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INHIBITORY EFFECTS OF LOADING WITH THE CALCIUM-CHELATOR 1,2-BIS(*o*-AMINOPHENOXY)ETHANE-*N*,*N*,*N*′,*N*′-TETRAACETIC ACID (BAPTA) ON AMYLASE RELEASE AND CELLULAR ATP LEVEL IN RAT PAROTID CELLS

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Abstract—Rat parotid cells were loaded with the Ca^{2+}-chelator 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), by incubation with the acetoxymethyl ester of BAPTA (BAPTA-AM). The BAPTA loading inhibited amylase release induced by β-adrenergic receptor stimulation without affecting the basal release, and the IC_{50} value was 25 μM. Incubation of cells with BAPTA-AM also suppressed cellular ATP levels considerably. At 100 μM BAPTA-AM, the ATP level fell to about 50% of the control. A decrease in ATP levels by incubation with oligomycin, a mitochondrial inhibitor, correlated well with the inhibition of amylase release. Although these results do not exclude the possibility that cytosolic free Ca^{2+} is involved in the regulation of cyclic AMP-mediated amylase release, they suggest that the amylase release inhibition with BAPTA loading may be due, in part, to a decrease in cellular ATP levels. Therefore, the exact mode of the BAPTA action must be interpreted with caution.

Amylase release from rat parotid glands is induced primarily by the activation of β-adrenergic receptor which leads to an increase in cellular cyclic AMP (cAMP) levels. The cAMP-mediated amylase release is thought to be independent of extracellular Ca^{2+}, but depletion of intracellular Ca^{2+} by incubation with high concentrations (1 mM or more) of the Ca^{2+}-chelator EGTA† considerably reduces the amylase release from parotid slices and cells [1–4]. As a result, a possible role for intracellular Ca^{2+} in amylase exocytosis through β-adrenergic receptor stimulation has been considered [5, 6].

BAPTA, a double aromatic analogue of EGTA, also has high selectivity for Ca^{2+} over Mg^{2+} [7]. Although BAPTA, as well as EGTA, is a membrane-impermeant compound, its acetoxymethyl ester (BAPTA-AM) can cross plasma membranes and is hydrolyzed by cellular esterases to BAPTA. The BAPTA trapped in the cells is believed to buffer intracellular free Ca^{2+} and to affect cell functions. Incubation with BAPTA-AM has been found to strongly inhibit a variety of Ca^{2+}-dependent cellular functions at concentrations below 100 μM [8–11].

In this study, we used the membrane-permeable Ca^{2+}-chelator BAPTA-AM to evaluate the role of cytosolic free Ca^{2+} in amylase release from rat parotid cells elicited by β-adrenergic receptor stimulation. The results show that the BAPTA loading suppresses not only the amylase release but also cellular ATP levels.

MATERIALS AND METHODS

Materials. Isoproterenol (ISO), dibutyryl cyclic AMP (DBcAMP), oligomycin, bovine testis hyaluronidase (Type 1-S), and bovine serum albumin were obtained from Sigma (St. Louis, MO). Collagenase (CLS II) was obtained from Cooper Biomedical (Malvern, PA), and Hanks' balanced salt solution from Gibco (Chagrin Falls, OH). BAPTA-AM, BAPTA, and EGTA were purchased from Dojin Laboratories (Kumamoto, Japan). The BAPTA-AM was stored at 4° in dimethyl sulfoxide as a 20 mM stock solution. All other reagents were from Wako Pure Chemical (Osaka, Japan).

Cell preparation. Male Wistar rats (about 300 g) were anesthetized with ether and killed by cardiac puncture. Dispersed parotid acinar cells were prepared as described previously [12]. Briefly, parotid glands were minced finely and incubated for 60 min at 37° in Hanks' balanced salt solution buffered with 20 mM Hepes–NaOH, pH 7.4 (HBSS-H), containing collagenase (100–150 units/mL) and hyaluronidase (0.25 mg/mL) under 95% O_2–5% CO_2. At 20-min intervals, the minced tissue was gently pipetted and gassed. Thereafter, the cells were filtered through two layers of gauze, washed twice with HBSS-H containing 0.1% bovine serum albumin, and suspended in the same medium.

Assay of amylase release. Aliquots (1 mL) of cell suspension were incubated at 37°, and the medium was collected by filtration through filter paper. To determine total amylase in the cells, a portion of the

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† Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid: BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; ISO, isoproterenol; and DBcAMP, dibutyryl cyclic AMP.

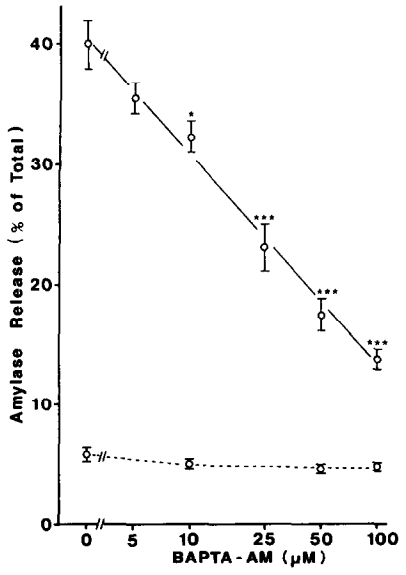

Fig. 1. Effect of incubation with BAPTA-AM on isoproterenol-stimulated amylase release. The parotid cells were preincubated with various concentrations of BAPTA-AM for 30 min and then stimulated for the following 30 min by 1 μM isoproterenol. Key: (O—O) isoproterenol-stimulated release, and (O····O) unstimulated basal release. The released amylase was expressed as a percent of the total amylase of the cells. Values are shown as means ± SE of four experiments. Key: * $P < 0.05$ and *** $P < 0.001$, significantly different from the stimulated control.

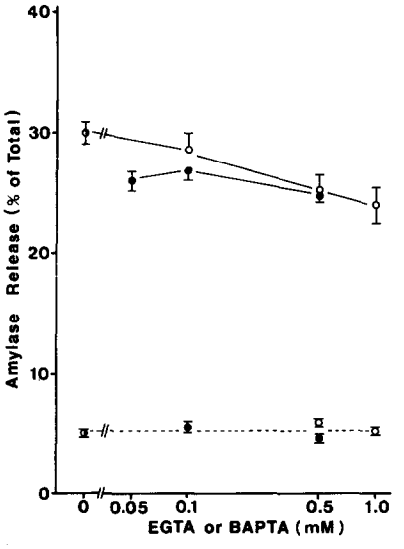

Fig. 2. Effects of incubation with EGTA and BAPTA on isoproterenol-stimulated amylase release. The parotid cells were preincubated in a Ca^{2+}-free medium containing various concentrations of EGTA (○) or BAPTA (●) for 30 min and then stimulated for the following 30 min by 1 μM isoproterenol. Key: (——) isoproterenol-stimulated release, and (· · · ·) unstimulated basal release. The released amylase was expressed as a percent of the total amylase of the cells. Values are shown as means ± SE of four experiments.

cell suspension was homogenized with a glass–Teflon homogenizer. Amylase activity in medium and homogenates was measured by the method of Bernfeld [13]. Parotid cells prepared here contained a total amylase activity of 50–80 units/mg of cell protein (one unit = milligrams of maltose formed per minute at 37°). The released amylase activity was expressed as percent of total amylase activity.

cAMP assay. Aliquots (1 mL) of cell suspension were incubated at 37° for various intervals and heated in a boiling water bath for 2 min to terminate the reaction and to extract intracellular cAMP. After centrifugation, cAMP in the supernatant fluid was measured by radioimmunoassay using a commercial assay kit (Yamasa Shoyu Co., Chiba, Japan).

ATP assay. After incubation for 30 min or 60 min at 37°, an equal volume of trichloroacetic acid (10%) was added to the cell suspension. The solution was neutralized with sodium acetate (1.5 M), and the ATP level was determined by the luciferin–luciferase method using a commercial assay kit (Boehringer-Mannheim, F.R.G.).

RESULTS

Effect of incubation with BAPTA-AM on amylase release. Parotid cells were preincubated with various concentrations of BAPTA-AM for 30 min in the presence of extracellular Ca^{2+} (1.26 mM) and then stimulated by 1 μM ISO for the following 30 min (Fig. 1). The treatment with 5–100 μM BAPTA-AM inhibited the ISO-stimulated amylase release in a dose-dependent manner. The apparent IC_{50} value (concentration producing a 50% inhibition) was estimated to be about 25 μM. Basal amylase release was unaffected by incubation with BAPTA-AM. The BAPTA loading also inhibited DBcAMP-stimulated amylase release at rates similar to those observed with ISO-stimulated release (data not shown). When the experiment was carried out with Ca^{2+}-free HBSS-H, the inhibitory effect of BAPTA-AM incubation was essentially similar to that obtained in the presence of Ca^{+2}. Since the hydrolysis of BAPTA-AM in cells produces formaldehyde and acetate which may have toxic effects on secretory responses, amylase release induced by ISO was examined in the presence of 400 μM formaldehyde or acetate (as would be produced by complete hydrolysis of 100 μM BAPTA-AM). Incubation with the reagents had no effect on the ISO-induced amylase release (data not shown).

Figure 2 shows the effects of incubation with the membrane-impermeant BAPTA and EGTA on the amylase release. Cells were preincubated for 30 min in Ca^{2+}-free HBSS-H containing various concentrations of BAPTA or EGTA and then stimulated by 1 μM ISO for the following 30 min. The inhibitory effects were much weaker than those observed for the cells incubated with BAPTA-AM. Amylase release induced by 1 μM ISO was about 80% of the control (no chelator) even in the presence of 500 μM BAPTA or 1 mM EGTA.

Effect of incubation with BAPTA-AM on cAMP accumulation. Cells were incubated in the presence and absence of 100 μM BAPTA-AM for 30 min and then stimulated by 1 μM ISO for various periods

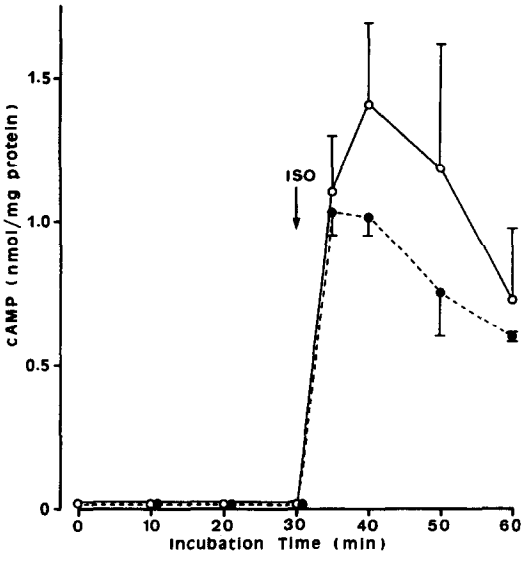

Fig. 3. Effect of incubation with BAPTA-AM on cAMP formation. The parotid cells were preincubated in the presence (●····●) or absence (○—○) of 100 μM BAPTA-AM for 30 min and then stimulated for the following 30 min by 1 μM isoproterenol. Values are shown as means ± SE of three experiments.

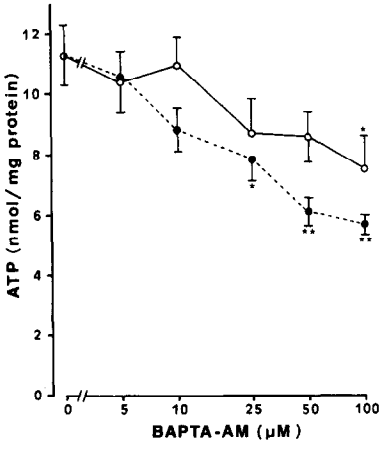

Fig. 4. Effect of incubation with BAPTA-AM on cellular ATP levels. The parotid cells were incubated with various concentrations of BAPTA-AM for 30 min (○—○) or 60 min (●····●). Values are shown as means ± SE of four experiments. Key: (*) $P < 0.05$ and (**) $P < 0.01$, significantly different from the control.

(Fig. 3). Incubation with BAPTA-AM had no significant effect on cAMP accumulation induced by ISO, although the mean value of the cAMP level was somewhat lower than that in the absence of BAPTA-AM.

Cellular ATP levels. The ATP levels of control cells incubated without BAPTA-AM were 11.3 ± 1.0 nmol/mg protein (mean ± SE, N = 8), with no change in the value up to 60 min. Figure 4 shows the changes in ATP levels of cells incubated with various concentrations of BAPTA-AM for 30 or 60 min. Incubation for 30 min at concentrations above 25 μM tended to reduce the ATP levels, and the difference was statistically significant ($P < 0.05$) at 100 μM BAPTA-AM. When cells were incubated with BAPTA-AM for 60 min, the cellular ATP levels were considerably suppressed in a dose-dependent manner. At 100 μM BAPTA-AM, the ATP level fell to about 50% of the control.

To assess the correlation between amylase release and cellular ATP levels, the effects of oligomycin, a mitochondrial inhibitor, were examined (Fig. 5). When parotid cells were preincubated with various concentrations of oligomycin for 30 min and then stimulated by 1 μM ISO for the following 30 min, the striking inhibition of amylase release was observed with increasing concentrations of oligomycin (Fig. 5A). The IC_{50} value was about 14 ng/mL. Oligomycin had no effect on the basal amylase release. Incubation with oligomycin also caused a marked depression of cellular ATP levels (Fig. 5B) which correlated closely with the inhibitory effect on ISO-stimulated amylase release. The IC_{50} values for cellular ATP levels were 18 ng/mL with a 30-min incubation and 11 ng/mL with a 60-min incubation.

DISCUSSION

This study demonstrates that incubation with BAPTA-AM strongly inhibited amylase release from rat parotid cells induced by β-adrenergic receptor stimulation. The inhibition was statistically significant at BAPTA-AM concentrations above 10 μM and the IC_{50} value was very low (25 μM). Although the membrane-impermeant EGTA has been used extensively to assess the involvement of cytosolic free Ca^{2+} in the amylase secretory process, lengthy preincubation (1 hr or more) in a Ca^{2+}-free medium containing a high concentration (1 mM or more) of the chelator is required for a dramatic inhibition of amylase release [1–4]. Compared with EGTA, the treatment with BAPTA-AM was found to be very effective in inhibiting amylase release. With BAPTA-AM, the DBcAMP-stimulated amylase release was also reduced, and cAMP formation was not inhibited significantly by incubation with the chelator, indicating that the inhibitory effect of BAPTA loading on amylase release is not due to receptor blockade or decreased cAMP formation.

However, we cannot easily conclude that the inhibition of the secretory response by incubation with BAPTA-AM is a result of a blocking of the role of intracellular free Ca^{2+} in the transduction of β-receptor stimulation. Recently, Hincke [14] reported that lengthy incubation of rat parotid cells with EGTA causes both inhibition of amylase release and depression of cellular ATP levels, suggesting that treatment with EGTA inhibits the amylase release via energy depletion in the cells. In the present study, incubation with BAPTA-AM reduced cellular ATP levels considerably. Treatment with oligomycin caused a marked decrease in ATP levels and a concomitant decline in ISO-stimulated amylase release, and it is assumed that the changes in ATP levels affect the secretory activity strongly. The importance of cellular ATP in exocytotic secretion has also been

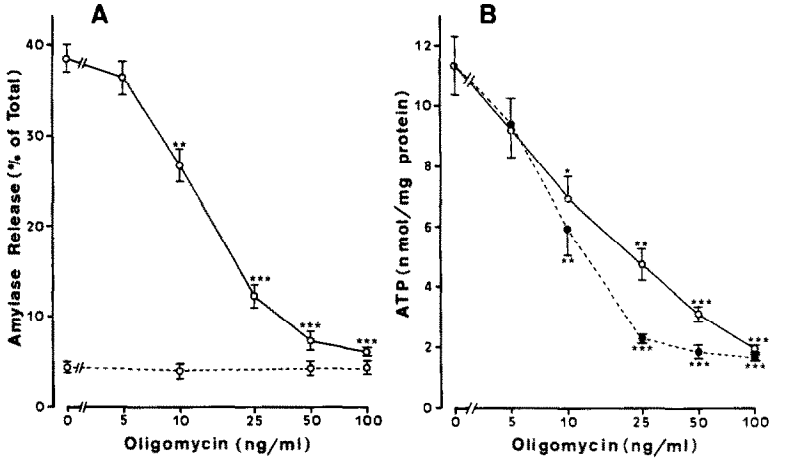

Fig. 5. Effect of incubation with oligomycin on amylase release (A) and cellular ATP levels (B). In panel (A), the parotid cells were preincubated with various concentrations of oligomycin for 30 min and then stimulated for the following 30 min by 1 μM isoproterenol. Key: (○—○) isoproterenol-stimulated amylase release, and (○····○) unstimulated basal release. The released amylase was expressed as a percent of the total amylase of the cells. In panel (B), the cells were incubated with oligomycin for 30 min (○—○) or 60 min (●····●). Values are shown as means ± SE of four experiments. Key: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$, significantly different from the control.

suggested for other secretory cells [15, 16]. These results lead to the conclusion that the inhibition of parotid amylase release with BAPTA loading may be due, in part, to a decrease in cellular ATP levels. Although it is not clear by what mechanism the treatment with BAPTA-AM causes the decline of cellular ATP, several key dehydrogenases in mitochondria are thought to be Ca^{2+}-sensitive [17]. Therefore, it seems likely that the depletion of intracellular free Ca^{2+} by the Ca^{2+}-chelator would result in the inhibition of mitochondrial functions.

The loading of cells with BAPTA has been employed in various cell systems, such as mast cells [8], pancreatic acini [9], enterocytes [10] and parotid acini [11], to prevent increases in cytosolic free Ca^{2+} induced by cellular activation. In these studies, the cells were incubated with 20–100 μM BAPTA-AM, which may have caused a considerable decrease in cellular ATP levels. Therefore, the exact mode of action of BAPTA loading on the Ca^{2+}-dependent cellular functions must be interpreted with caution.

Thus, although BAPTA loading may be inappropriate for an evaluation of the role of cytosolic Ca^{2+}, our data do not exclude completely the possibility that Ca^{2+} is involved in the regulation of amylase release in response to β-adrenergic receptor stimulation. In this study, the IC_{50} value (14 ng/mL) of oligomycin for amylase release coincided approximately with its value (11 ng/mL) for cellular ATP levels. With BAPTA-AM, however, concentrations above 100 μM were required for a 50% depression of ATP levels, while the IC_{50} value for amylase release was 25 μM. This suggests that the decrease in ATP levels was not the only cause of the inhibition of amylase release by BAPTA loading.

On the basis of the measurements using quin 2 or fura 2, some investigators [18–20] have reported that ISO can induce an increase in cytosolic free Ca^{2+} in rat parotid cells, although the magnitude of the response is smaller than that seen after stimulation with carbachol and substance P. The concentrations of ISO that are required for the Ca^{2+}-mobilization appear to be higher than the concentrations for stimulating amylase release. Accordingly, further studies are necessary to determine whether the mobilized Ca^{2+} is linked to the regulation of amylase release.

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