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Types of voltage-dependent calcium channels involved in high potassium depolarization-induced amylase secretion in the exocrine pancreatic tumour cell line AR4-2J

CUI ZONG JIE
Beijing Agricultural University Faculty of Biological Sciences, Beijing 100094, China

ABSTRACT

In the perifused fura-2 loaded exocrine pancreatic acinar cell line AR4-2J pulses of high potassium induced repetitive increases in intracellular calcium. Attached cells when stimulated with high potassium secreted large amount of amylase. High potassium-induced secretion was dependent both on the concentration of potassium and duration of stimulation. High potassium induced increases in intracellular calcium were inhibited by voltage-dependent calcium channel antagonists with an order of potency as follows: nifedipine > ω-agatoxin IVA > ω-conotoxin GVIA. In contrast, the L-type calcium channel antagonist nifedipine almost completely inhibited potassium-induced amylase secretion, whereas the N-type channel antagonist ω-conotoxin GVIA was without effect. The P-type channel antagonist ω-agatoxin IVA had a small inhibitory effect, but this inhibition was not significant at the level of amylase secretion. In conclusion, the AR4-2J cell line possesses different voltage-dependent calcium channels (L, P, N) with the L-type predominantly involved in depolarization induced amylase secretion.

Key words: *AR4-2J, pancreatic acinar cells, amylase, secretion, calcium channels.*

Address all correpondence to: Beijing Agricultural University Faculty of Biological Sciences, Beijing 100094, Tel: 010-6289-1830, Fax: 010-6289-1055, E-mail: bauic@public.bta.net.cn

AR4-2J is a cell line originally derived from a transplantable, azaserine-induced murine tumour[1, 2], which contains significant amount of amylase and other digestive enzymes. This cell line contains a number of receptor systems: substance P[3], cholecystokinin[4], somatostatin[5, 6], gastrin[7], bombesin [8], muscarinic acetylcholine[8], vasoactive intestinal peptide[9], and amylin receptors[10], which duplicate the major receptor systems in normal pancreatic acinar cells. Therefore AR4-2J is a model widely used for studying mechanisms of exocrine pancreatic secretion.

In contrast to normal pancreatic acinar cells which do not generate voltage-activated ionic currents and therefore are electrically non-excitable[11, 12], AR4-2J cells have been shown to possess voltage-activated K^+, Na^+, Ca^{2+} channels[13], therefore this cell line also offers a convenient model for the study of excitable cells. Studies by Wiedenmann and coworkers found that this cell line contains small secretory vesicles (40-80 nm) that store classical neurotransmitters glycine and GABA. Immunocytochemical studies confirmed the existence of specific markers for these small vesicles: synaptophysin and S.V.2[14, 15]. In addiction, high potassium depolarization stimulated GABA release in this cell line[16].

Pharmacological and molecular biological studies identified the following voltage-dependent calcium channels: L, N, T, P, Q and R[17, 18]. The present study aimed to identify which of the above types of voltage-dependent calcium channels exist in AR4-2J cells by pharmacological means and found that the L-type predominated and it was solely responsible for depolarization induced amylase secretion.

MATERIALS AND METHODS

Cell culture

AR4-2J was purchased from American Type Culture Collection (Rockville, Maryland, USA). The cells were maintained in Leibovitz L-15/CO_2 medium (Gibco) supplemented with vitamins, glutamine (2 m*M*), glucose (44 m*M*), penicillin/ streptomycin (50 U/ml), and foetal calf serum (20%, Gibco) at 37℃ in a humidified 95% O_2/5% CO_2 atmosphere[19]. Cells were fed daily.

Amylase secretion

AR4-2J cells were subcultured in 24-well plate for 3 days. On day 3, wells were rinsed with modified Ringer's buffer (composition in m*M*: NaCl 118, KCl 4.7, $MgCl_2$ 1.16, $CaCl_2$ 2.0, NaH_2PO_4 1.16, glucose 14, bovine serum albumin 2 $mg.ml^{-1}$, soybean trypsin inhibitor 0.1 $mg.ml^{-1}$, HEPES 25, BME amino acid mixture, glutamine 2, pH adjusted to 7.3 with NaOH 1N, and oxygenated with 100% O_2 before use). Cells were stimulated in this buffer for 30 min, and secreted amylase was expressed as percentage of total. Amylase was assayed with the method of Ceska et al.[20]. High K^+ solution was made by molar replacement of Na^+.

Intracellular calcium ($[Ca^{2+}]_c$) measurement

Sub-confluent AR4-2J cells were harvested, loaded with fura-2 AM (5 μM, 37℃ for 30 min). Fura-2 loaded AR4-2J cells were sandwich positioned between layers of Biogel P_2 beads (Bio Rad) and perifused with modified Ringer's solution (see above) in a quartz flow cuvette, and $[Ca^{2+}]c$ measured in a Spex DM3000CM spectrofluorometer, the ratios of F340/F380 were shown as indication of $[Ca^{2+}]c$ changes (Fig 1). In separate experiments, fura-2 loaded AR4-2J cells were resuspended and the suspension constantly stirred magnetically, F340 and F380 was measured in

Spex DM3000CM. Since these cells were in suspension, at the end of the experiments cells were permeabilized for $[Ca^{2+}]_c$ calibration, and the fluorescence traces converted to$[Ca^{2+}]_c$ in n*M* (Fig 3).

Materials

Bay K 8644 was from CalBiochem (La Jolla, CA, USA). ω-Conotoxin GVIA, ω-agatoxin IVA were from Peptide Institute Inc. (Osaka, Japan). Fura-2 AM was from Molecular Probes (Portland, OR, USA).

RESULTS

High potassium induced increases in $[Ca^{2+}]_c$

The fact that high potassium induced depolarization increased $[Ca^{2+}]_c$ in perifused AR4-2J cells (Fig 1) indicates that AR4-2J cells are excitable cells. In these experiments, AR4-2J cells were positioned into the light path of the Spex DM3000CM spectrofluorometer and continuously perifused with modified Ringer's solution at a flow rate of 1 ml.min^{-1}.. High potassium pulse (100 m*M*) was given at indicated time, pulse duration ranged from 2 sec to 1 min. From these experiments, it is obvious that potassium dose dependently increased $[Ca^{2+}]_c$. What is significant is that an 1 min pulse did not necessarily caused a larger increase in $[Ca^{2+}]_c$ than a 30 sec pulse, indicating that large and fast increase in $[Ca^{2+}]_c$ may desensitize the

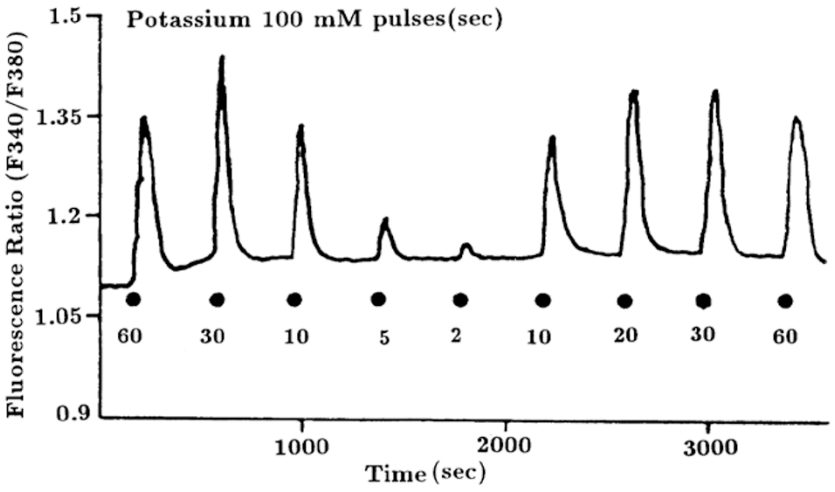

Fig 1. High potassium (100 m*M*) pulses induced increases in $[Ca^{2+}]_c$ in perifused AR4-2J cells. Potassium pulses (100 m*M*) of indicated duration (in sec) were given and increases in $[Ca^{2+}]_c$ were recorded as ratios of fura-2 fluorescence F340/F380. Trace is representative of 4 similar experiments.

voltage-dependent calcium channels, even on this macroscopical scale. This dose-response relationship conforms to the general bell-shaped dose-response curve for amylase secretion in the exocrine pancreatic acinar cells[21].

High potassium stimulated amylase secretion in a dose-, and time-dependent manner

In AR4-2J cells, as in other secretory cells, the direct consequence of an increase in $[Ca^{2+}]_c$ is the stimulation of secretion, of amylase in this case. Indeed, high potassium (100 m*M*) caused significant increase in amylase secretion (Fig 2). The increase in amylase secretion was a gradual process, the fastest secretion occurred within the first min (Fig 2A), and the rate gradually sloped off with time. This profile bears comparison with secretion triggered by normal secretagogues such as cholecystokinin.

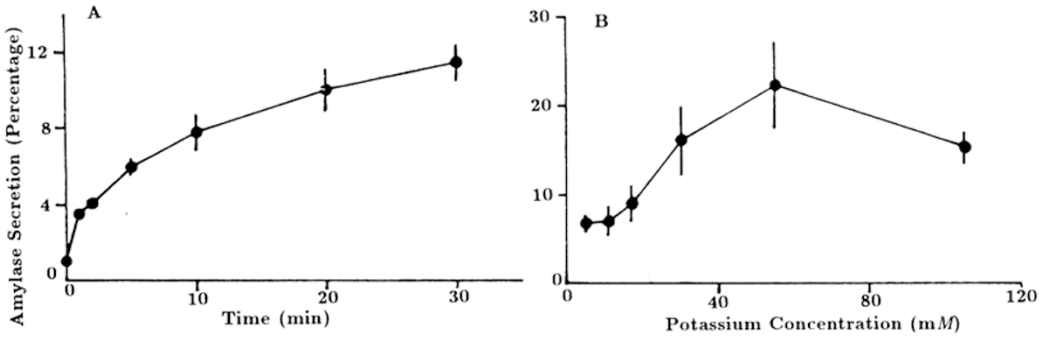

Fig 2. High potassium induced amylase secretion from adherent AR4-2J cells.
A. Time-dependence. Potassium 100 m*M* was added to each culture well, and amylase secreted over the indicated time period expressed as percentage of total content present in cells before stimulation. n=3.
B. Concentration-dependence. Potassium of indicated concentration was added to each culture well, and amylase secreted in 30 min expressed as percentage of total content present in cells before stimulation. n=3. Data are presented as m ± sem.

Amylase secretion induced by high potassium depolarization also depended on the concentration of potassium used (Fig 2B). At K^+ 11 m*M* (it was 5 m*M* in control), no obvious secretion was observed. Stimulation of secretion was seen only when K^+ had been raised to 17 m*M*. Significant stimulation was achieved with a concentration of 30 m*M*. Maximal stimulation was seen at 55 m*M*. As with physiological secretagogues, K^+ stimulation of amylase secretion also conforms to the bell-shaped dose-response curve. Supra-maximal K^+ concentration of 105 m*M* induced a supra-maximal inhibition. Therefore 55 m*M* was needed for maximal stimulation, higher

concentrations do not necessarily get higher amount of amylase secreted.

Voltage-dependent calcium channel types involved in high potassium-induced increases in $[Ca^{2+}]_c$

To test what types of calcium channels were activated by high potassium depolarization, the effects of selective calcium channel antagonists on high potassium depolarization induced increases in $[Ca^{2+}]_c$ were investigated. The involvement of the L-type channels was examined with the dihydropyridine antagonist nifedipine, whereas ω-conotoxin GVIA and ω-agatoxin IVA were used to selectively block the N- and the P-types respectively. Fig 3 shows that KC1 55 mM induced marked increase in $[Ca^{2+}]_c$. This increase was significantly inhibited by the L-type antagonist nifedipine, the N-type antagonist ω-conotoxin GVIA decreased $[Ca^{2+}]_c$ increase further (Fig 3A). Similarly, after inhibition by nifedipine, the P-type antagonist ω-agatoxin IVA was able to decrease $[Ca^{2+}]_c$ further also (Fig 3B). From Fig 3, it is obvious that nifedipine had the greatest inhibitory effect; ω-conotoxin GVIA although exhibited obvious inhibition, it was less effective than ω-agatoxin IVA.

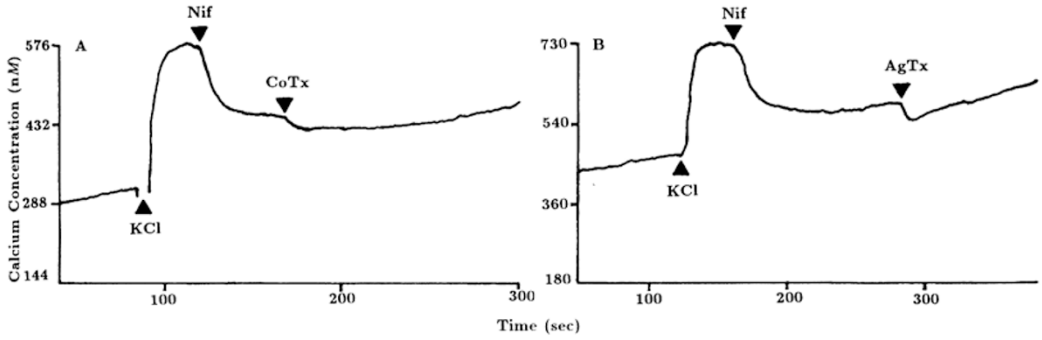

Fig 3. Inhibition of high potassium-induced increases in $[Ca^{2+}]_c$ by different calcium antagonists. F340 and F380 of Fura-2 loaded AR4-2J cells in suspension was monitored continuously and subsequently converted into $[Ca^{2+}]_c$ in n*M*. Cells were first depolarized with KC1 55 m*M*. This was followed by sequential additions of nifedipine 10 μ*M* and ω-conotoxin GVIA 1 μ*M* (A), or nifedipine 10 μ*M* and ω-agatoxin IVA 100 n*M* (B). These traces are representative of a number of experiments, with a total number of treatments as follows: nifedipine, 23; ω-conotoxin GVIA, 11; ω-agatoxin IVA, 10. Nif, nifedipine; CoTx, ω-conotoxin GVIA; AgTx, ω-agatoxin IVA.

Voltage-dependent calcium channel types involved in high potassium-induced amylase secretion

The involvement of three types of voltage-dependent calcium channels in high

potassium induced amylase secretion was examined. None of the type specific antagonists seemed to have any effect by itself ($P>0.05$, n=3-4). It is apparent that the L-type accounted for most of amylase secreted, nifedipine decreased 55 m*M* K^+ induced secretion completely back to control levels ($P<0.05$, n=3) (Fig 4A). Whereas ω-conotoxin GVIA was completely without effect ($P>0.05$, n=4) (Fig 4B), ω-agatoxin IVA had only neglegible effect ($P>0.05$, n=3) (Fig 4C).

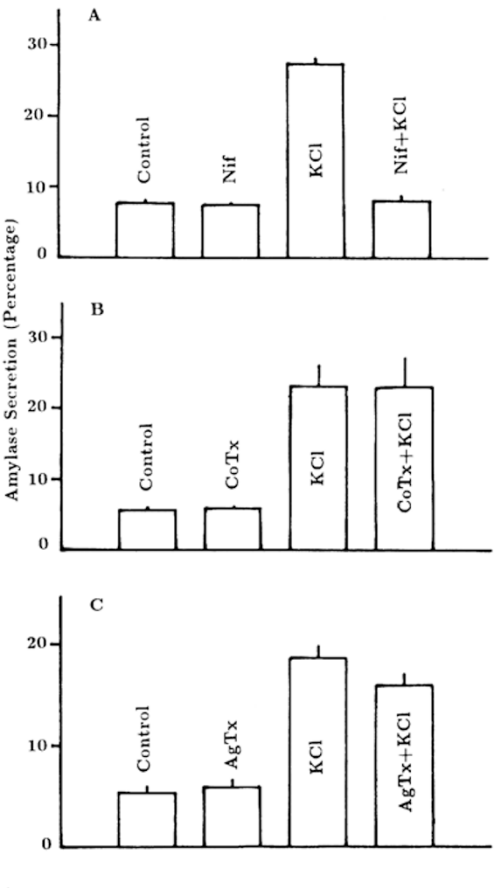

Fig 4. Inhibition of high potassium- induced amylase secretion by voltage-dependent calcium channel antagonists. For each set of experiments, vesicle (control), potassium 55 m*M*, antagonist (nifedipine 10 μ*M*, ω-conotoxin GVIA 1 μ*M*, ω-agatoxin IVA 100 n*M*), or potassium 55 m*M* plus antagonist was added to each culture well respectively, and amylase secreted in 30 min expressed as percentage of total present in cells before experiment. In A, n=3; in B, n-4; in C, n=3. Nif, nifedipine; CoTx, ω-conotoxin GVIA; AgTx, ω-agatoxin IVA. Data are presented as m ± sem.

DISCUSSION

In this study, it was found that high potassium depolarization could: 1) increase $[Ca^{2+}]_c$ in a dose-dependent manner; 2) stimulate amylase secretion in a time- and concentration-dependent fashion. High potassium by depolarizing the plasma membrane, activates the voltage-dependent calcium channels. Openning of the voltage-dependent calcium channels leads to Ca^{2+} influx resulting in a net increase in $[Ca^{2+}]_c$ which may in turn stimulate amylase secretion. High potassium induced increases in intracellular calcium were blocked by the voltage-dependent calcium channel an-

tagonists with an order of potency: nifedipine > ω-agatoxin IVA > ω-conotoxin GVIA. In contrast, high potassium depolarization induced amylase secretion was completely abolished by the L-type calcium channel antagonist nifedipine, the P-type antagonist ω-agatoxin IVA had little effect, whereas the N-type antagonist ω-conotoxin GVIA was completely without effect. These data indicate that there are at least three types (L, P, and N) of voltage-dependent calcium channels in the exocrine pancreatic acinar cell line AR4-2J, but the L type accounts for nearly all of the amylase secreted during high potassium depolarization. Therefore AR4-2J cell line is not only a model for studying the secretory mechanisms of non-excitable cells, but could also serve as a convenient model for excitable cells.

The fact that both high potassium induced increase in $[Ca^{2+}]_c$, and amylase secretion conforms to the bell-shaped dose-response curve is of much interest. This is different from stimulation by secretagogues[21, 22]. In the case of high potassium, supra-maximal inhibition of amylase secretion may be due to a supra-maximal inhibition of $[Ca^{2+}]_c$ increases. Whereas in the case of secretagogue induced amylase secretion, $[Ca^{2+}]_c$ increases generally do not conform to the bell-shaped dose-response curve, with supra-maximal stimulation resulting in an elevated plateau increase in $[Ca^{2+}]_c$[22, 23].

The difference in the dose-response curves in $[Ca^{2+}]_c$ increases induced by potassium depolarization and by secretagogues may provide a useful foothold for detailed investigation into the intriguing phenomenon of supra-maximal inhibition induced by secretagogues. Because in the former case supra-maximal inhibition in calcium increase may partly account for the supra-maximal inhibition in amylase secretion.

Judging from the experiments with calcium channel antagonists nifedipine, ω-conotoxin GVIA and ω-agatoxin IVA, it was mainly the L-type calcium channels that were involved in high potassium induced amylase secretion. Recently, it was found that in pancreatic B cells, L-type calcium channels co-localize with insulin-containing secretory granules[24]. Calcium-dependent exocytosis has been proposed to be regulated by a SNARE complex composed of secretory vesicle membrane proteins v-SNARE (synaptobrevin, synaptotagmin) and plasma membrane proteins t-SNARE (syntaxin, SNAP-25)[25]. There is evidence that both L- and N-type calcium channels can directly interact with the SNARE complex proteins syntaxin and SNAP-25[26, 27]. Whether in AR4-2J cells, L-type calcium channels would, like in other excitable cells, directly regulate the exocytotic SNARE complex will remain an open question at the present time. On the other hand, cytosolic calcium increase in AR4-2J cells by calcium influx through voltage-dependent calcium channels may, like in normal pancreatic acinar cells which are non-excitable, stimulate amylase secretion through the activation of calcium/calmodulin-dependent protein kinase II[28].

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