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Role of Gap Junction in the Regulation of Renin Release and Intracellular Calcium in As 4.1 Cell Line

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Gap junction protein, connexin, is expressed in endothelial cells of vessels, glomerulus, and renin secreting cells of the kidney. The purpose of this study was to investigate the role of gap junction in renin secretion and its underlying mechanisms using As 4.1 cell line, a renin-expressing clonal cell line. Renin release was increased proportionately to incubation time. The specific gap junction inhibitor, 18-beta glycyrrhetinic acid (GA) increased renin release in dose-dependent and time-dependent manners. Heptanol and octanol, gap junction blockers, also increased renin release, which were less potent than GA. GA-stimulated renin release was attenuated by pretreatment of the cells with amiloride, nifedipine, ryanodine, and thapsigargin. GA dose-dependently increased intracellular Ca^{2+} concentration, which was attenuated by nifedipine, nimodipine, ryanodine, and thapsigargin. However, RP-cAMP, chelerythrine, tyrphostin A23, or phenylarsine oxide did not induced any significant change in GA-stimulated increase of intracellular Ca^{2+} concentration. These results suggest that gap junction plays an important role on the regulation of renin release and intracellular Ca^{2+} concentration in As 4.1 cells.

Key Words: As 4.1 cell line, Ca^{2+}, Gap junction, Hormone, Renin

INTRODUCTION

Gap junction channels are expressed in almost all mammalian tissues, where they facilitate chemical and electrical communication between adjacent cells (Loewenstein, 1981; Kummar & Gilular, 1992; Meda & Spray, 2000). Ions, second messengers, small metabolites, and other signaling molecules are able to pass through gap junction channels from one cell to another, thus enabling the coordinated cellular response, including secretion (Paul, 1986; Loewenstein & Rose, 1992; Vazquez et al, 2001; Takeda et al, 2005; Haefliger et al, 2006). Intercellular gap junction channels are formed by two hemichannels, named connexons, which are separately contributed by each of adjacent cells (White & Bruzzone, 1996). Connexons are formed by the hexameric assembly of membrane-spanning proteins, known as connexins (Cx), which belong to a family of at least 20 members in mammals (Beyer & Paul, 1990; Willecke et al, 1991; Kummar & Gilular, 1992).

The role of gap junctions in modulating intercellular communication under physiological and patholological conditions has been well documented (Yamasaki, 1990; Bennett et al, 1991; Klaus et al, 1992; Loewenstein & Rose, 1992). Especially, the kidney serves as an important model to study connexin diversity for the function of different cell types. Morphologically identifiable gap junctions have been described in juxtaglomerular (JG) apparatus, glomerulus, proximal and distal convoluted tubules, and collecting ducts. mRNAs for nine Cx species have been found in the kidney (Taugner et al, 1978; Barajas et al, 1994; Seul & Beyer, 2000). Among them, Cx43 and Cx40, which appear to be the prominent connexins in the adult kidney, are differentially regulated within the kidneys of renovascular hypertensive rats (Haefliger et al, 2001).

It has been reported that gap junctions promote the communication and coordinated activity between coupled neurons, and may help facilitate the synchronous release and pulsatile secretion of neurohormones (Hu, 1999; Vazquez et al, 2001). Especially, Cx36 controls insulin secretion and synchronization of Ca^{2+} oscillations in MIN6 cells (Calabrese et al, 2003). Recently, it has been reported that a vessel-specific Cx43 gene expression is increased in renin-dependent hypertension model (Haefliger et al, 1997), and that the replacement of Cx43 by Cx32 prevents the renin-dependent hypertension by decreasing expression and secretion of renin (Haefliger et al, 2006). Therefore, gap junctions may play an important role in the regulation of hormone secretions. There exist only a few reports on the regulation of renin release by gap junction. The aim of this study was to investigate the role of gap junction in renin release and its underlying mechanisms using As 4.1 clonal cell line, which contains Cx40 and 45 (Ryan et al, 2003).

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METHODS

Cell culture for renin release in As 4.1 cell

As 4.1 cells (American Type Culture Collection No. CRL-2193) are a main renin-expressing clonal cell line derived from the kidney neoplasm of a transgenic mice. Cell cultures were maintained at 37°C in humidified room air containing 5% CO_2. As 4.1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS). Media were changed every other day, and cell cultures were split when reached confluence. To measure renin release, As 4.1 cells were cultured onto 12 well dish in DMEM with 10% FBS for 72 hr before experiments. Before experiment, medium was changed with fresh DMEM supplemented with 10% FBS. To measure renin release as a function of incubation time, cells were incubated for 1, 4, and 8 hrs at 37°C and 65 μl of aliquots was taken each time. To define the role of gap junction in renin release, 18-beta glycyrrhetinic acid (a specific gap junction inhibitor; GA, Sigma, St Louis) at 1, 3, 10, or 30 μM concentration was added after 1 hr of incubation and 65 μl of aliquots was taken at 1, 4 and 8 hrs. Heptanol or octanol (a gap junction blocker) at 10, 30 or 100 μM concentration was also added. To investigate the effect of Na^+/Ca^{2+} channel or L-type Ca^{2+} channel on GA-induced renin release, cells were preincubated with amiloride (300 μM) or nifedipine (30 μM) for 1 hr, and 65 (l of aliquots was taken. Then, 10 μM GA was added and incubated for 3 more hrs. Ryanodine (10 μM) or thapsigargin (1 μM), a sarcoplasmic reticulum Ca^{2+} blocker, was also used for pretreatment.

Measurement of renin concentration

After centrifugation of the medium for 5 min at 10,000 $\times$g at 4°C, 50 μl aliquots were added to 450 μl of renin substrate obtained from bilateral nephrectomized rats, containing 50 μl maleate buffer (pH 5.88, 1.5 M), 10 μl each of 8-hydroxyquinoline (3.4 mM), neomycin (0.4%) and phenylmethyl- sulfonylfluoride (7.5 mM) (Cho et al, 1989). The mixture was then incubated at 37°C for 60 min in a shaking incubator. For stopping the reaction, the mixture was kept on ice. Angiotensin I (ANG I) generated was measured by radioimmunoassay and expressed as nanograms of ANG I per hr per well.

Fluorimetric determination of intracellular Ca^{2+} concentration

To measure intracellular Ca^{2+} concentration, As 4.1 cells were loaded with 6 μM fura-2 AM and then incubated for 30 min at 37°C (Lee et al, 2000). Fura-2 loaded cells were washed twice with Ca^{2+} free-loading solution and were transferred into the measuring chamber on the stage of an inverted microscope. The extracellular solution contained (in mM) 140 NaCl, 5 KCl, 0 or 1 $CaCl_2$, 11 glucose, and 10 HEPES (pH 7.35 with NaOH). Fluorescence was measured at a determined site (1 cell bodies) through pinhole with alternating dual-excitation wavelengths of 340 nm and 380 nm and emission wavelength of 510 nm. Using a Ca^{2+} microspectroflurometric system (PTi system; Suite F South Brunswick, NJ, USA), the curve was obrained from the following equation:

$$[Ca^{2+}]_i = K_d\ (R - R_{min})/(R_{max} - R) \times \beta$$

where R is the ratio of fluorescence at 340 nm and 380 nm, R_{max} and R_{min} are the ratios for fura-2 at 340 nm and 380 nm in the presence of saturating Ca^{2+} and zero Ca^{2+}, respectively, and β is the ratio of fluorescence of fura-2 at 380 nm in zero and saturating Ca^{2+}. K_d is the dissociation constant of fura-2 for Ca^{2+} and Ca^{2+} to be 229 nM.

At the beginning of each experiment, cells were washed in saline solution, and GA was added then in the presence or absence of Ca^{2+} channel modulators, such as nifedipine (30 μM) or nimodipine (10 μM). To define the intracellular calcium source induced by GA, As 4.1 cells were also pretreated for 20 min with ryanodine (10 μM), ruthenium red (100 μM), U73122 (1 μM), thapsigargin (1 μM), RP-cAMP (100 μM), chelerythrine (1 μM), tyrphostin A23 (3 μM), or phenylarsine oxide (300 nM) in Ca^{2+}-free extracellular solution and then GA was added.

Statistical analysis

The results are given as mean$\pm$SEM. The statistical significance of the differences was assessed using Student's t test. The critical level of significance was set at $p<0.05$.

RESULTS

Effects of gap junction blockers on renin release

To measure renin release as a function of incubation time, cells were incubated for 1, 4, and 8 hrs at 37°C. As shown in Fig. 1A, renin release was increased from 22.3$\pm$4.1 to 61.1$\pm$17.8, and 90.2$\pm$21.5 ng ANG I/hr/well (n=8) at 4 and 8 hrs, respectively. The increase of renin release was time-dependent. To define the role of gap junction in renin release, GA was added after 1 hr of incubation and aliquots was taken at 4 and 8 hrs; renin release was increased by 3 μM GA from 19.7$\pm$4.3 to 122.3$\pm$20.7, and 168.6$\pm$32.1 ng ANGI/hr/well (n=8), respectively (Fig. 1A). Renin releases in response to 1, 3, 10, and 30 μM GA were dose-dependent, showing 67.3$\pm$16.1, 122.3$\pm$20.7, 230.2$\pm$25.1, and 294.1$\pm$35.8 ng ANGI/hr/well at 4 hrs, respectively (Fig. 1B). Increases of renin release by 1, 3, 10, and 30 μM GA were 3.2$\pm$0.3, 6.6$\pm$0.5, 12.1$\pm$1.2, and 16.4$\pm$2.0 fold, respectively which were significantly higher than the control value (2.6$\pm$0.3 fold). Heptanol (n=9) and octanol (n=7) also increased renin release, however, were less potent than GA (Fig. 2).

GA-stimulated renin release was significantly attenuated by pretreatment of the cells with amiloride (300 μM, n=5), a Na^+/Ca^{2+} exchanger blocker (Fig. 3A). GA-stimulated renin release was also attenuated by pretreatment with nifedipine (L-type Ca^{2+} cannel blocker, 30 μM, n=6), ryanodine (sarcoplasmic reticulum Ca^{2+} release inhibitor, 10 μM, n=6), or thapsigargin (sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor, 1 μM, n=6) (Fig. 3B). However, they did not influence basal renin release at a given concentration.

Effects of gap junction blocker on the regulation of intracellular Ca^{2+}

To elucidate whether GA-stimulated renin release was

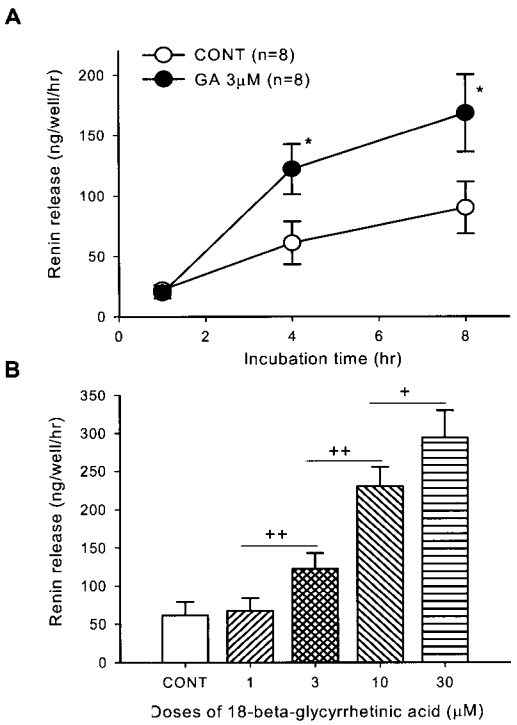

Fig. 1. Effect of 18-beta glycyrrhetinic acid (GA) on renin release in As4.1 cell line. (A) Increase of renin release by 3 μM GA as a function of incubation time. (B) Dose-response curve of renin release by GA. *compared to control group, $p< 0.05$, $^{+}$: compared to corresponding value, $^{+}p<0.05$, $^{++}p<0.01$.

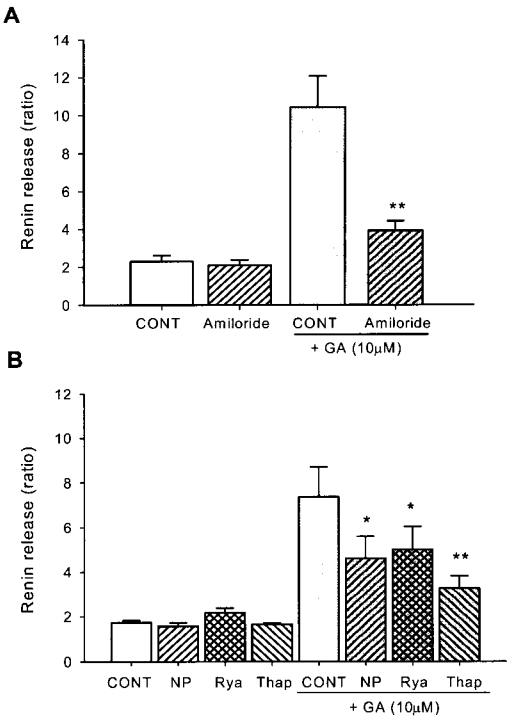

Fig. 3. Effects of Ca^{2+} channel modulator on GA (10 μM)-induced renin release. Amiloride, nifedipine, ryanodine and thapsigargin attenuated GA-induced renin release. CONT: vehicle-treated group, NP: nifedipine, Rya: ryanodine, Thap: thapsigargin. *compared to GA only treated control group, $*p<0.05$, $**p<0.01$.

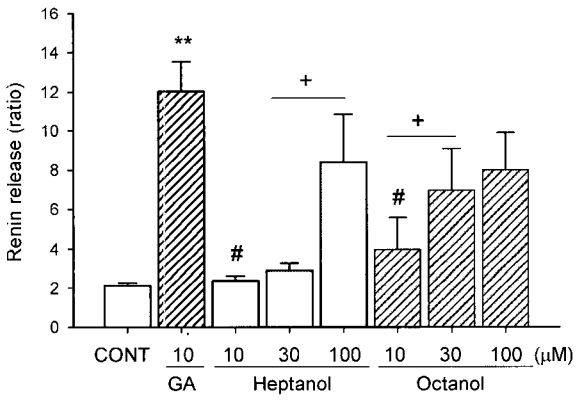

Fig. 2. Comparison of renin release in response to 18-beta glycyrrhetinic acid (GA), heptanol and octanol on renin release in As 4.1 cell line. **compared to control group, $p<0.01$, $^{+}$compared to corresponding value, $p<0.05$, $^{\#}$compared to GA group, $p<0.05$.

related to Ca^{2+} concentration, intracellular Ca^{2+} concentration was determined using fluorometry. Fig. 4A illustrates representative tracings (a, b) and averaged values (c) of fluorescence signals showing the effect of GA on intracellular Ca^{2+} concentration in the presence or absence of 1 mM $CaCl_2$ extracellular solution. GA concentration dependently increased intracellular Ca^{2+} concentration in manner with an EC50 of 15.28 ± 1.89 μM and 11.32 ± 4.04 μM with (n=6) or without Ca^{2+} extracellular solution (n=6). GA-induced increase in intracellular Ca^{2+} concentration was significantly attenuated in the absence of 1 mM $CaCl_2$ extracellular solution (Fig. 4B). Fig. 3C shows the effect of L-type Ca^{2+} channel blocker nifedipine and nimodipine on GA-induced increase of $[Ca^{2+}]_i$ in the presence of 1 mM $CaCl_2$ extracellular solution. GA-induced increase of intracellular Ca^{2+} concentration was significantly attenuated by nifedipine (30 μM, n=6) or nimodipine (10 μM, n=5).

Since GA caused an increase of intracellular Ca^{2+} concentration without Ca^{2+} extracellular solution, we next tried to find out the source of intracellular calcium induced by GA. Therefore, As 4.1 cells were incubated for 20 min with ryanodine (10 μM, n=6), ruthenium red (non-selective Ca^{2+} channel blocker, 100 μM, n=5), U73122 (phospholipase C inhibitor, 1 μM, n=6), thapsigargin (1 μM, n=6), RP- cAMP (protein kinase A inhibitor, 100 μM, n=6), chelerythrine (protein kinase C inhibitor, 1 μM, n=5), tyrphostin A23, or phenylarsine oxide before perfusion of GA in Ca^{2+}-free extracellular solution (Fig. 5A). GA-induced increase in intracellular Ca^{2+} concentration was attenuated by ruthenium red, ryanodine, or thapsigargin but not by U73122 in Ca^{2+}-free extracellular solution (Fig. 5B). However, RP-cAMP, chelerythrine, tyrphostin A23, phenylarsine oxide (300 nM, n=6) did not cause any significant changes of GA-induced increase of Ca^{2+} (Fig. 5B).

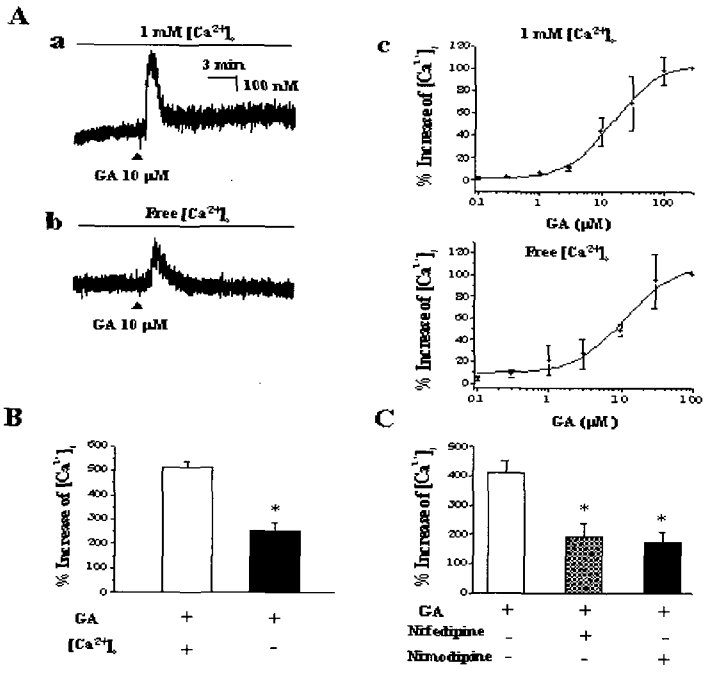

Fig. 4. Effects of extracellular Ca^{2+} and L-type Ca^{2+} channel blockers on GA-induced increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated As 4.1 cells loaded with fura-2/AM. The arrows indicate the starting point of perfusion of GA. (A) Representative tracings (a, b) and averaged values (c) of fluorescence signals showing the effects of different dose of GA on $[Ca^{2+}]_i$ in the presence or absence of 1 mM $CaCl_2$ extracellular solution. GA increased $[Ca^{2+}]_i$ *in a dose-dependent manner*. (B) Changes in $[Ca^{2+}]_i$ by GA under Ca^{2+}-free or Ca^{2+} containing extracellular solutions. GA-induced increase in $[Ca^{2+}]_i$ was attenuated in Ca^{2+}-free solution. (C) Changes in $[Ca^{2+}]_i$ by GA in the presence of nifedipine and nimodipine, and in GA-induced increase of $[Ca^{2+}]_i$. Both nifedipine and nimodipine attenuated GA-induced increase of $[Ca^{2+}]_i$. **compared to control* group, $p<0.05$.

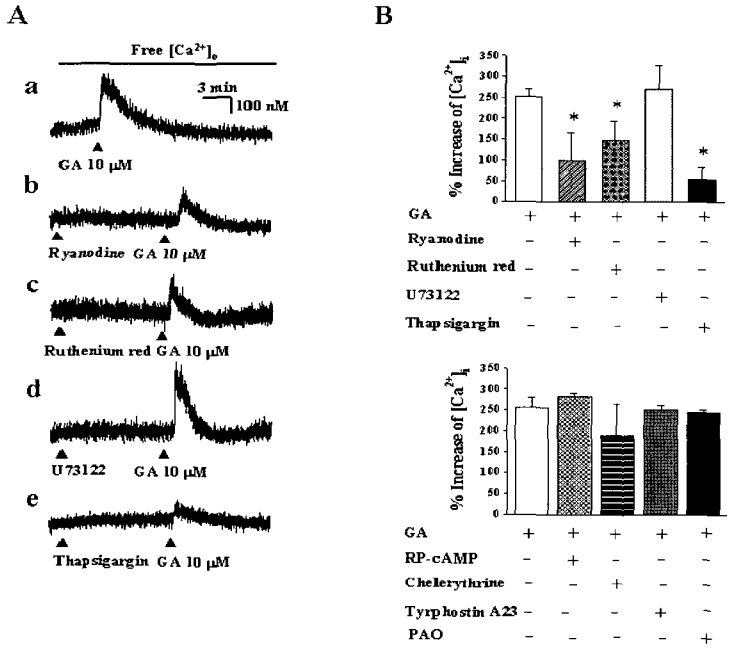

Fig. 5. Effects of inhibitor for ryanodine receptor, phospholipase C, sarcoplasmic reticulum Ca^{2+} channel, PKA, PKC, tyrosine kinase, tyrosine phosphatase on GA-induced increase of $[Ca^{2+}]_i$ in Ca^{2+}-free extracellular solution. (A) Representative tracings of fluorescence signals showing the effect of GA on $[Ca^{2+}]_i$ in the absence of 1 mM $CaCl_2$ extracellular solution. The fura-2-loaded As 4.1 cells were incubated for 20 min with ryanodine (b), ruthenium red (c), U73122 (d), thapsigargin (e), before perfusion of GA. (B) Averaged values of fluorescence signals showing the effect of GA on $[Ca^{2+}]_i$ in the presence of various inhibitors. Ryanodine, ruthenium red and thapsigargin attenuated an increase of $[Ca^{2+}]_i$ by GA in Ca^{2+}-free extracellular solution, but other inhibitors did not. $^*p<0.05$ compared to the control.

DISCUSSION

Intercellular communication via gap junction is thought to play an important role in control of various cellular functions, including cell growth, migration, differentiation, and electric coupling (Bennett et al, 1991; Loewenstein & Rose, 1992; Haefliger et al, 1997; Takeda et al, 2005). Especially, several studies have shown a close relationship between gap junctions and hormone secretions (Hu, 1999; Calabrese et al, 2003; Ryan et al, 2003; Michon et al, 2005). However, the role of gap junction in renin release has not yet been well defined. The present study shows that GA inceased renin release and intracellular Ca^{2+} concentration in As 4.1 cells, an in vitro model for JG cells, and both responses were markedly attenuated by treatment of the cells with nifedipine, ryanodine, or thapsigargin. Therefore, we suggest that gap junction may play an important role in the regulation of renin release and intracellular Ca^{2+} concentration in As 4.1 cell line.

It has been reported that various types of connexin such

as 37, 40, 43 and 45 are found in human kidney (Paul, 1986; Beyer & Paul, 1987; Zang & Nicholson, 1989; Klaus et al, 1992; Seul & Beyer, 2000) as well as in As 4.1 cell line (Cho et al, 1989). Especially, the most prominent location of gap junctions in the kidney is blood vessels and mostly Cx40 is present. Haefliger et al (18) found that Cx40 mRNA expression and its protein level are increased in both kidneys of two kidney one-clip (2K1C) animals, but Cx43 mRNA expression is increased only in unclipped right kidney. Therefore, they suggest that cell-to-cell communication mediated by Cx40 may be implicated in the function of renin-secreting cells. However, the replacement of Cx43 by Cx32 decreases basal renin gene expression and attenuates the response of renin secretion to low salt diet and hypoperfusion (Ryan et al, 2003), suggesting that Cx43 and Cx40 may play an important role in the regulation of renin release. In the present study, GA-stimulated renin release was significantly attenuated by pretreatment of the cells with amiloride, nifedipine, ryanodine, or thapsigargin, strongly suggesting that gap junction participates partly in the regulation of renin release by changing intracellular Ca^{2+} concentration. At present, however, we do not know which type of connexin in As 4.1 cell line is more important for the regulation of renin release.

The mechanisms of the involvement of gap junctions in various tissue functions are not fully understood. However, there is an evidence to indicate that second messengers such as Ca^{2+} and inositol triphosphate can transverse gap junctions and cause a physiological response in neighboring cells. To define whether GA-stimulated renin release is related to Ca^{2+} concentration, intracellular Ca^{2+} concentration was determined using fluorometry. We found that GA increased intracellular Ca^{2+} concentration in a concentration-dependent manner and this response was attenuated by omission of extracellular Ca^{2+} and the presence of L-type Ca^{2+} channel blocker. GA-induced increase of intracellular Ca^{2+} concentration was also attenuated by ruthenium red, ryanodine, or thapsigargin in Ca^{2+}-free buffer, but not by U73122. However, RP-cAMP, chelerythrine, tyrphostin A23, and phenylarsine oxide did not cause any significant changes of GA-induced increase of intracellular Ca^{2+} concentration. These results show that an increase of intracellular Ca^{2+} by GA may have originated from L-type Ca^{2+} channel and sarcoplasmic reticulum. Based on these data, we speculate that the blocking of gap junction by GA may directly inhibit the movement of Ca^{2+} ions through gap junction or may indirectly activate other Ca^{2+} channel. It has been reported that connexin 36 controls synchronization of Ca^{2+} oscillations and insulin secretion in MIN6 cells (Calabrese et al, 2003). In patch clamp experiments of isolated jejunal myocytes, GA decreases L-type Ca^{2+} current (Takeda et al, 2005). Our data, therefore, are not consistent with the above reports. We could not offer any reason of why gap junction regulates intracellular Ca^{2+} concentration differently. One possible explanation could be differences in cell types and experimental methods. Taken the above results together, we suggest that gap junction plays an important role in the regulation of intracellular Ca^{2+} concentration.

Intracellular Ca^{2+} is the common final pathway through which renin secretion from JG cells is governed. In contrast to other secretory cells in which an increase of intracellular Ca^{2+} usually promotes hormone secretion, renin secretion in JG cells is well known to inversely relate to Ca^{2+} concentration (Fray et al, 1987; Park et al, 1992). In the present in vivo and in vitro experimental conditions, an inverse relationship between renin release and Ca^{2+} concentration was well-defined: TMB-8 (100 μM, Ca^{2+}-calmodulin inhibitor) and the omission of extracellular Ca^{2+} caused an increase of renin release in As 4.1 cell line from 1.8-fold to 21.5-fold and 6.7-fold, respectively (n=4, data not shown). Nevertheless, exact mechanisms involved in GA-induced renin release, which is related to intracellular Ca^{2+} concentration in As 4.1 cell line remain to be elucidated.

In conclusion, we have shown that GA-induced increases of renin release and intracellular Ca^{2+} concentration were attenuated by treatment of As 4.1 cells with nifedipine, ryanodine, or thapsigargin. Therefore, we suggest that gap junction may play an important role in the regulation of renin release and intracellular Ca^{2+} concentration in As 4.1 cell line.

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