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High magnesium concentration inhibits ligand-stimulated calcium influx and hormone secretion in rat pituitary lactotropes with involvement of intracellular free magnesium

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The effects of extracellular magnesium concentration ($[Mg^{2+}]_{ex}$) on thyrotropin-releasing hormone (TRH)-stimulated intracellular free calcium mobilization and prolactin secretion were investigated concomitantly with measurement of the intracellular free magnesium concentration ($[Mg^{2+}]_i$). TRH-stimulated intracellular free calcium mobilization was significantly inhibited when the medium was replaced by high Mg^{2+} medium ($[Mg^{2+}]_{ex}$=10mM) in normal Ca^{2+} medium. The inhibitory effects of high Mg^{2+} became apparent concomitantly with an increase in $[Mg^{2+}]_i$ from 0.7 to 1.3 mM. High Mg^{2+} significantly inhibited TRH-induced PRL secretion in a dose-dependent manner in normal Ca^{2+} medium. TRH-stimulated inositol triphosphate (IP_3) production was rather augmented by the replacement with high Mg^{2+} medium. In summary, high Mg^{2+} inhibits Ca^{2+} influx stimulated by TRH in the rat pituitary lactotropes, possibly with the involvement of $[Mg^{2+}]_i$ increase. These results have general importance in relation to high Mg^{2+}-induced suppression of the biological functions of cells.

The roles of intracellular free calcium ($[Ca^{2+}]_i$) in relation to various cell functions have been extensively elucidated following the introduction of Ca^{2+}-sensitive fluorescent dyes, fura-2 or indo-1 (1,2). Numerous reports have indicated that $[Ca^{2+}]_i$ play an role in general cell functions, in various kinds of cells, i.e. proliferation of cells (3), contraction of muscles (4), synthesis and secretion of hormones (5,6). However, the role of intracellular free magnesium ($[Mg^{2+}]_i$) ,a similar bivalent cation , has not yet been clarified despite the fact that magnesium ion is well known as a co-factor of various enzymatic reactions and membrane transportations *in vitro* (7). The requirement of magnesium in these fundamental biochemical reactions surmised to relate to $[Mg^{2+}]_i$ in the functions of living cells.

On the other hand, it is well known that a high magnesium solution inhibits the cellular functions of various cells, i.e. myometrium (8), cardiac myocyte (9), or pancreatic acinar cells (10) stimulated by ligands. However, the mechanisms of these inhibitory effects have not been fully determined. Recently two Mg^{2+}-sensitive fluorescent dyes, mag-fura-2 and mag-indo-1, have been developed (11), which enable us to study intracellular free magnesium concentration.

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These dyes proved not to be influenced by the change in $[Ca^{2+}]_i$ (9,12). Recent advance in a digital imaging microscopic system has allowed us to measure $[Mg^{2+}]_i$ directly in single cells (13). We have previously reported that a high Mg^{2+} concentration inhibits $[Ca^{2+}]_i$ mobilization stimulated by oxytocin in human puerperal myometrial cells (14).

In this study, we tried to determine the effects of high Mg^{2+} concentration on TRH-stimulated $[Ca^{2+}]_i$ mobilization and hormone secretion in rat pituitary lactotropes. We also measured $[Mg^{2+}]_i$ and IP_3 production to elucidate the mechanisms of their inhibitory effects.

MATERIALS AND METHODS

Materials- Fura-2, fura-2-AM, mag-fura-2, mag-fura-2-AM were obtained from Molecular Probes, Inc. (Eugene, OR). TRH was obtained from Peninsula Laboratories, Inc. (Belmont, CA). Fetal bovine serum was obtained from Cell Culture Laboratories (Cleveland, OH). Trypsin, pancreatin, $MgSO_4$ and $CaCl_2$ were obtained from Sigma (St. Louis, MO). Hanks' balanced salt solution and Medium 199 were obtained from Gibco BRL, Inc. (Gaithburg, MD). Anti-rat PRL rabbit antibody was obtained from UCB-Bioproducts S.A (Brussels, Belgem). Biotynylated ant-rabbit goat IgG and fluorescein-labeled avidin were obtained from Vector Laboratories, Inc. (Burlingame, CA).

Culture of rat anterior pituitary cells-Female Wistar rats (180-200 g) from Nihon Dohbutsu Co.(Osaka Japan) were decapitated, and an anterior cell suspension was prepared by gentle trypsinization as described previously (15). For measurement of $[Ca^{2+}]_i$ or $[Mg^{2+}]_i$, cells were cultured on 0.5-mm glass slips that were sealed under a 10-mm hole in the bottom of 35-mm culture dishes. For the study of PRL secretion, cells, at a density of $0.5X10^6$ per ml were cultured in 24-well Falcon culture plates in an atmosphere of 5% CO_2 and 95% air at 37°C. In both studies, the cells were used after 3 days of culture.

Purification of lactotropes-A suspension of rat pituitary cells was used for labeling of PRL by a fluorescence double-antibody method. Anti-rat PRL rabbit antibodies were applied at 37°C for 20 min. The cells were washed twice with Hanks' balanced salt solution (HBSS). Biotinylated anti-rabbit goat IgG was applied for 40 min at room temperature, and cells were washed twice with HBSS. Fluorescein-labeled avidin was applied for the next 10 min at room temperature, and the cells were washed twice again with HBSS. Then lactotrope-enrich cells were separated by fluorescence-activated cell sorting (EPICS ELITE, Coulter Electronics, Inc. Miami, FL) as described previously (17).

Hormone assay-Prolactin levels in the medium were determined by a double-antibody radioimmunoassay using a kit provided by NIDDK, Natinal Hormone and Pituitary Program. The results are expresses as μg rPRL-RP-3 standard/ml.

Microscopic fluorometry-$[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ were monitored by a digital imaging fluorescence microscope using Ca^{2+} or Mg^{2+}-sensitive fluorescent dyes, fura-2 or mag-fura-2. The cells were loaded with one of the dyes by modification of the method of Grynkiewicz et al.(1) as previously described (13,16). Briefly, cells were loaded with fura-2 or mag-fura-2 by incubation at 37°C for 60 min in Medium 199 containing 5μM fura-2-AM or mag-fura-2-AM. The cells were then rinsed with HBSS and placed on the microscope stage. The $[Mg^{2+}]_i$ of cells was measured at 100-msecond intervals using a digital imaging microscope system (Model M1000, Interdec Corp.,Osaka, Japan). The fluorescent excitation beam was directed onto the cells, and the emission images were recorded and analyze with a computer. The ratio of the intensities of fluorescent emission at 355 nm and 370 nm was calculated. Calibration of $[Mg^{2+}]_i$ was performed from a standard curve of various Mg^{2+} concentrations prepared just before each experiments. A thermostatic device maintained the temperature of the bathing medium at 37±1°C for the duration of the experiments.

IP_3 production-Dispersed rat pituitary cells were incubated on ice for 30 min in HBSS without Ca^{2+} and Mg^{2+}, decanted into thin wall tubes and centrifuged. After aspirating supernatants, 1 ml of HBSS with the various concentrations of Ca^{2+} and Mg^{2+} was added to each tube. After incubation at 37°C for 30 min to achieve equilibrium, 10 μl of 10^{-5} M TRH was added, and the mixture was promptly vortexed. After 10 seconds of incubation at 37°C, 0.2 ml of 20% ice cold perchloric acid was quickly added. The tubes were transferred into in a ice bath. Then incubated on ice for 20 min. The precipitated protein was removed by centrifugation. The supernatants were adjusted to pH 7.5 with KOH, and the insoluble precipitates ($KClO_4$) were removed by

centrifugation. The amount of IP_3 in the supernatant was measured by competitive binding assay using Amersham TRK 1000 kit.

Analysis of data-Data are shown as mean values plus or minus the standard error of multiple determinations in at least three replicate experiments. Homoscedasticity of data was analyzed by Bartlett's test. The significance of differences was assessed of variance or Kruskal Wallis test, followed by multiple comparisons by the method of Dunnett or Scheffe and a P value of less than 0.05 were considered significant. For tests of two independent groups, equal variance was analyzed by the F test. The significance of differences was assessed by the Mann-Whitney test, and a P value of less than 0.05 was considered significant.

RESULTS

Effect of high magnesium concentration on TRH-stimulated $[Ca^{2+}]_i$ increase-The changes in $[Ca^{2+}]_i$ of the pituitary cells stimulated by TRH in normal HBSS ($[Ca^{2+}]_{ex}$=1.2mM, $[Mg^{2+}]_{ex}$=0.8mM) are shown in Fig. 1a. TRH increased $[Ca^{2+}]_i$ from 121nM (basal level) to 456nM (peak level) within a few seconds. The increases in $[Ca^{2+}]_i$ induced by TRH under the following four conditions are compared in Fig.1a-d, in the normal medium (a), 30 min after replacement of the normal medium with Ca^{2+}-depleted medium (b), 30 min after replacement of the normal medium with a high Mg^{2+} medium ($[Mg^{2+}]_{ex}$=10mM)(c), 30 min after replacement of

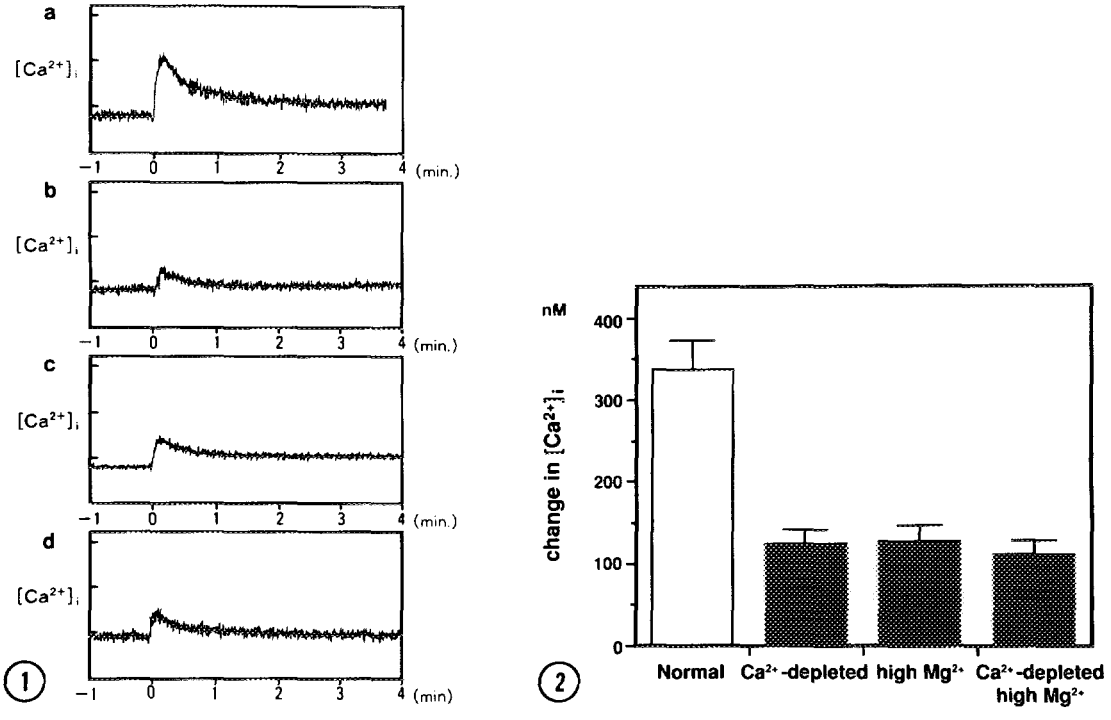

Fig. 1. **Changes in $[Ca^{2+}]_i$ stimulated by TRH under various conditions.** (a) in normal medium, (b) in Ca^{2+}-depleted medium, (c) in normal medium with a high Mg^{2+} concentration (10mM),and (d) in Ca^{2+}-depleted medium with a high Mg^{2+} concentration. TRH 10^{-7}M was added from a stock solution of 10^{-5} M.

Fig. 2. **Comparison of increase in $[Ca^{2+}]_i$ stimulated by TRH in various conditions.** The increase in $[Ca^{2+}]_i$ is expressed as the net increase in $[Ca^{2+}]_i$ from basal levels ranging from 80 - 120 mM.

the normal medium with a high Mg^{2+} medium in the absence of Ca^{2+} (d). As shown in Fig. 1b, in Ca^{2+}-depleted medium, the increase in $[Ca^{2+}]_i$ stimulated by TRH was 37% of that when Ca^{2+} was present. As shown in Fig.1c, in the high Mg^{2+} medium, the increase in $[Ca^{2+}]_i$ stimulated by TRH was reduced to 38% of that in the normal mediums. As shown in Fig. 1d, in Ca^{2+}-depleted medium, the high Mg^{2+} medium did not suppressed the $[Ca^{2+}]_i$ increase stimulated by TRH in comparison with in the Ca^{2+}-depleted medium. The increases of $[Ca^{2+}]_i$ stimulated by TRH in a various conditions are summarized in Fig. 2.

Effect of high magnesium concentration on basal $[Mg^{2+}]_i$ and $[Ca^{2+}]_i$-The effect of high Mg^{2+} medium on $[Mg^{2+}]_i$ is shown in Fig. 3a. The $[Mg^{2+}]_i$ in the rat pituitary cells in the resting condition was 0.7±0.13mM (n=40). When the extracellular Mg^{2+} concentration ($[Mg^{2+}]_{ex}$) increased from 0.8 mM to 10 mM, $[Mg^{2+}]_i$ was not changed immediately, but after 15 min, $[Mg^{2+}]_i$ increased gradually from 0.7 to 1.3 mM . However, the change in $[Ca^{2+}]_i$ was not observed in the presence of a high concentration of external Mg^{2+} ($[Mg^{2+}]_{ex}$=10mM) even for more 30 min (Fig. 3b). TRH did not have any effect on $[Mg^{2+}]_i$ in normal and high Mg^{2+} medium.

Effect of high magnesium concentration on basal and TRH-stimulated PRL secretion-The basal secretion of PRL from the anterior pituitary cells under various conditions of $[Mg^{2+}]_{ex}$ is shown in Fig. 4. The mean (±S.E.M.) secretion of PRL from the anterior pituitary cells in the normal medium (Mg^{2+}=0.8mM) was 7.70(±0.31) μg/ml. When the $[Mg^{2+}]_{ex}$ was elevated from 0.8 mM to 2.5, 5.0, 7.5, 10.0 mM, the basal level of PRL secretion was suppressed in a dose-dependent manner. The effects of high Mg^{2+} concentration on TRH-stimulated PRL secretion in the

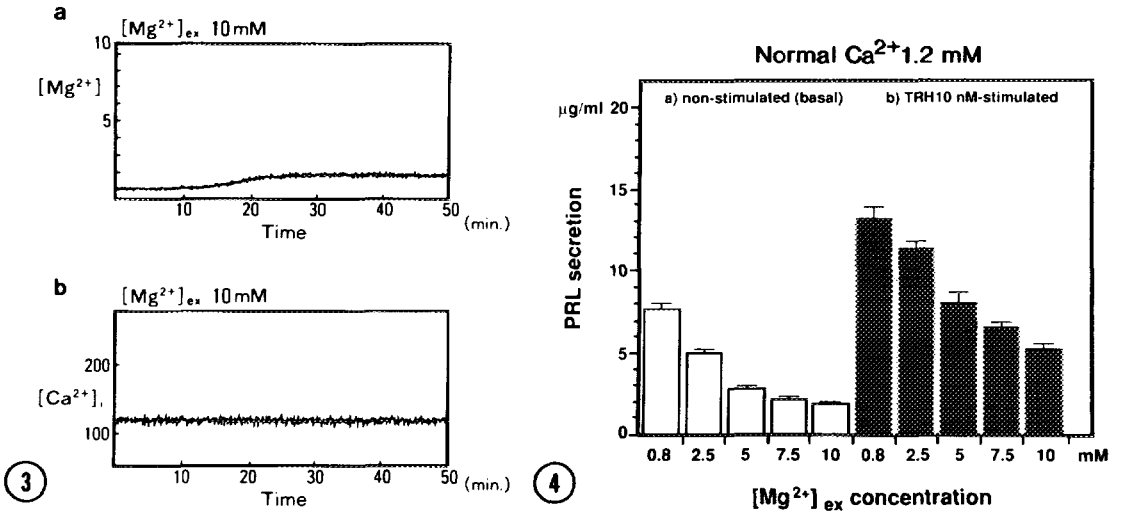

Fig. 3. **Changes in $[Mg^{2+}]_i$ and $[Ca^{2+}]_i$ by replacement of the normal HBSS with a high concentration Mg^{2+} medium.**

Fig. 4. **Effect of various concentrations of Mg^{2+} on PRL secretion.** (a) basal secretion, (b) TRH-stimulated PRL secretion. Mg^{2+} concentration in medium were changed by adding them from a high Mg^{2+} stock solution . The change in osmolality of medium was minimum, and the pH was adjusted to 7.40±0.05.

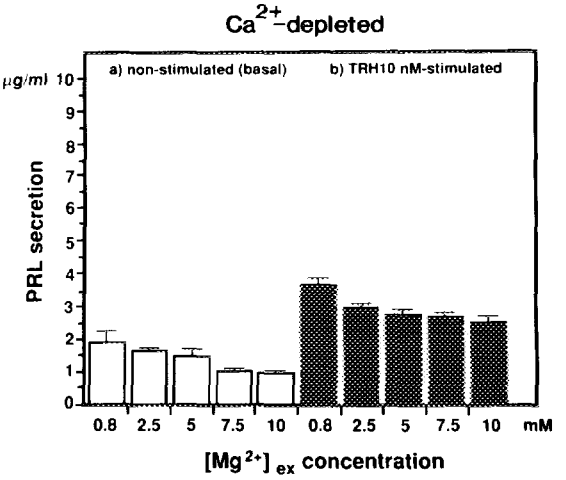

Fig. 5. **Effects of various concentrations of Mg^{2+} on PRL secretion in Ca^{2+}-depleted medium.** (a) basal, (b) TRH-stimulated PRL secretion.

normal Ca^{2+} medium are shown in Fig. 4. The mean(±S.E.M.) secretion of PRL from the anterior pituitary cells stimulated by TRH(10nM) for 2 hours was 13.30(±0.76) μg/ml. When $[Mg^{2+}]_{ex}$ was elevated from 0.8mM to 2.5, 5.0, 7.5, 10.0 mM, the secretion of PRL was suppressed in a dose dependent manner.

Effect of high magnesium concentration on basal and TRH-stimulated prolactin secretion in Ca^{2+}-depleted medium-The effect of high Mg^{2+} on basal PRL secretion is shown in Fig. 5. The mean(±S.E.M.) basal PRL secretion from the anterior pituitary cells in Ca^{2+}-depleted medium ($[Mg^{2+}]_{ex}$=0.8mM) was 1.95(±0.18) μg/ml. In the absence of extracellular calcium, the basal secretion of PRL was significantly suppressed as compared with in its presence (7.70 vs1.95)

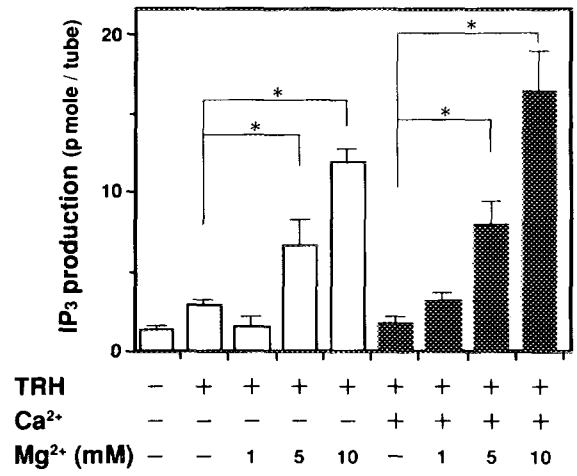

Fig. 6. **Effects of various concentrations of Mg^{2+} on IP_3 production stimulated by TRH.** 10^{-7}M TRH was added from a stock solutions of 10^{-5}M.

compared with Fig. 4. When $[Mg^{2+}]_i$ were elevated from 0.8 mM to 2.5, 5.0, 7.5, 10.0 mM, the basal secretion of PRL was slightly suppressed in Ca^{2+}-depleted medium (Fig. 5). The effects of high Mg^{2+} concentration on TRH-stimulated PRL secretion in Ca^{2+}-depleted medium are shown in Fig. 5. High Mg^{2+} medium also inhibited the TRH-stimulated secretion of PRL in a dose dependent manner.

Effects of high magnesium concentration on TRH-stimulated IP_3 production-IP_3 production stimulated by TRH in various conditions are shown in Fig. 6. TRH-stimulated IP_3 production was not inhibited in high Mg^{2+} medium in normal Ca^{2+} or in Ca^{2+}-depleted medium. TRH-stimulated IP_3 production was rather augmented significantly when $[Mg^{2+}]_i$ was increased to 5, or 10 mM in normal Ca^{2+} or Ca^{2+}-depleted medium.

DISCUSSIONS

In this study, we demonstrated that high Mg^{2+} medium inhibited TRH-stimulated Ca^{2+} influx and prolactin secretion and that these inhibitory effects were paralleled with the increase in $[Mg^{2+}]_i$.

TRH are well known to stimulate PRL and TSH secretions in the pituitary gland (18). The $[Ca^{2+}]_i$ mobilization are closely related to PRL secretion stimulated by TRH. In our study, in fact, TRH stimulated $[Ca^{2+}]_i$ increse, which was partially inhibited by omission of Ca^{2+} in medium. The high Mg^{2+} medium also inhibited both of the $[Ca^{2+}]_i$ increase and PRL secretion. However it was unclear what mechanisms were involved in this inhibitory effect. The gradient in the Mg^{2+} concentration inside and outside of the cell membrane may possibly affect cell function. But in our study these inhibitory effect was not apparent just after the replacement of the medium. Latent effects of the high Mg^{2+} concentration on $[Mg^{2+}]i$ was mainly involved in suppressive effects on TRH-stimulated $[Ca^{2+}]_i$ mobilization and PRL secretion. In fact, it took about 30 min for the high magnesium solution to show its inhibitory effects. and those inhibitory effects became apparent concomitantly with the increase of $[Mg^{2+}]_i$.

Comparing the effects of the high Mg^{2+} solution on TRH-stimulated $[Ca^{2+}]_i$ mobilization and PRL secretion, in the normal and Ca^{2+}-depleted medium, we surmised that the impaired Ca^{2+} influx plays a key role in the inhibitory effects of the high Mg^{2+} concentration. In cardiac myocytes, an increase in $[Mg^{2+}]_i$ has been reported to inhibit L-type calcium channels (19). Moreover Ohya et al.(20) reported that Mg^{2+} inhibited the calcium channel current in single pregnant rat myometrial cells using whole-cell patch clamp methods. Similar mechanisms are thought to be involved also in the secretory cells.

The concentration of $[Mg^{2+}]_{ex}$ which we selected for $[Ca^{2+}]_i$ study was rather high in comparison with physiological conditions, but many researchers have adopted this concentration as a high Mg^{2+} solution in in vitro studies. Of course, we determined preliminarily that these high Mg^{2+} medium did not have irreversible effects on general cell function during each experiments. We also rechecked that these cells responded to TRH again after replacement of the high Mg^{2+} medium with normal medium.

We measured $[Mg^{2+}]_i$ for the first time in the pituitary cells. The basal level of $[Mg^{2+}]_i$ was around 0.7 mM which is consistent with the value report by Murphy et al (19). First, we tried to

determined whether TRH itself has any effects on $[Mg^{2+}]_i$ or not, because Lennard reported that $[Mg^{2+}]_i$ was decreased by acetylcholine addition to pancreatic acinar cells (20). For that purpose, pituitary lactotropes were purified using fluorescence- activated cell sorting, as described previously (17), and 90%- purified lactotropes were collected. TRH, however, did not affects the $[Mg^{2+}]_i$ of these cells. Next, we determined the time-resolved changes in $[Mg^{2+}]_i$ by replacement of the normal medium with the high Mg^{2+} medium. It took about 30 min for the cells to attain a stable levels of $[Mg^{2+}]_i$. In this condition, the high $[Mg^{2+}]_{ex}$ medium had a suppressive effects on TRH-induced $[Ca^{2+}]_i$ mobilization. But $[Mg^{2+}]$i did not increased farther despite the gradient of Mg^{2+} concentration between inside and outside of cell membrane. The cell membrane of living cells is supposed to possess a system which maintains $[Mg^{2+}]_i$ in a physiological range against a high Mg^{2+} medium.

In our study TRH-stimulated PRL secretion was inhibited in a dose dependent manner more precisely than the effects on $[Ca^{2+}]_i$ mobilization. Moreover the suppressive effect of a high concentration Mg^{2+} were also exerted on the basal secretion of PRL. The mechanism of this inhibitory effect on the basal secretions is obscure. The possibility of other factors than $[Ca^{2+}]_i$ mobilization can not be ruled out. The inhibitory effects on basal and TRH stimulated PRL secretion were also present in Ca^{2+}-depleted medium. However these effects were not as prominent as those in TRH-stimulated PRL secretion in the normal medium.

Next, we measured IP_3 production stimulated by TRH under various condition of $[Ca^{2+}]_{ex}$ and $[Mg^{2+}]_{ex}$ to determine the mechanism of the effect of a high Mg^{2+} concentration on Ca^{2+} influx. The results of the experiments revealed that high Mg^{2+} increased the response of IP_3 production stimulated by TRH both in normal and Ca^{2+}-depleted medium. Therefore, at least, the signal transduction to the extent of IP_3 production surmised not to be impaired. Therefore TRH-stimulated $[Ca^{2+}]_i$ increase is supposed not to be via its suppressive effects on binding to receptors nor on IP_3 production but to be via the inhibitory effects on Ca^{2+} influx through calcium channels. Precise studies are required to confirm these hypothesis.

In this study, we demonstrated that a high concentration Mg^{2+} medium inhibits TRH-stimulated $[Ca^{2+}]_i$ mobilization and PRL secretion in the pituitary lactotropes . The mechanism of the effect was via the inhibitory effects on calcium influx which was accompanied by the increase in $[Mg^{2+}]_i$. These results indicated one of the mechanisms in relation to high Mg^{2+} induced inhibitory effects on the biological function of cells.

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