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Growth Hormone-Releasing Factor Reduces Voltage-Gated Ca^{2+} Channel Current in Rat GH_3 Cells

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Summary. The action of GRF on GH_3 cell membrane was examined by patch electrode techniques. Under current clamp with patch electrode, spontaneous action potentials were partially to totally eliminated by application of GRF. In the case of partial elimination, the duration of remaining spontaneous action potentials was prolonged and the amplitude of afterhyperpolarization was decreased. The evoked action potential in the cells which did not show spontaneous action potentials was also eliminated by GRF. In order to examine what channels were affected by GRF, voltage-clamp analysis was performed. It was revealed that voltage-gated Ca^{2+} channel current and Ca^{2+}-induced K^+ channel current were decreased by GRF, while voltage-gated Na^+ channel and delayed K^+ channel were not affected. The decrease of Ca^{2+}-induced K^+ channel current was considered to be a consequence of the decrease of voltage-gated Ca^{2+} channel current. Therefore it is likely that the effect of GRF on GH_3 cells was due to the block of voltage-gated Ca^{2+} channels. The elimination of action potential under current clamp corresponded to the block of voltage-gated Ca^{2+} channels and the prolongation of action potential could be explained by the decrease of Ca^{2+}-induced K^+ channel current. The amplitude decrease of afterhyperpolarization could also be explained by the reduction of Ca^{2+}-induced K^+ channel current. Thus the results under current clamp well coincide with the results under voltage clamp. Hormone secretion from GH_3 cells was not stimulated by GRF. However, the finding that GRF solely blocked voltage-gated Ca^{2+} channel suggested the specific action of GRF on GH_3 cell membranes.

Key Words GH_3 cells · GRF · action potential · Ca^{2+} channel · Ca^{2+}-induced K^+ channel

Introduction

The secretion and synthesis of anterior pituitary hormones are controlled by neurohypophysiotrophic hormones which act at their specific receptors on the cell membrane. Electrophysiological analyses of various anterior pituitary cell lines have revealed that these cell lines can generate action potential, which is dependent on calcium and/or sodium ions [1, 16, 17, 22, 25, 29]. The electrophysiological method has an advantage that very early changes of ion fluxes through plasma membrane can be detected, which allow a good approach to investigate the mechanism of stimulus-secretion coupling. In rat GH_3 cells, membrane potential changes caused by various agents have been reported [7, 8, 14, 16, 20, 29]. Especially the action of thyrotropin-releasing factor (TRH) has been intensively explored (7, 16, 19, 20, 29). TRH causes initial hyperpolarization of the membrane potential due to the increased conductance of potassium ions and then depolarization due to the decreased conductance of potassium ions. The frequency of spontaneous action potential is increased during the depolarizing phase, which enhances the influx of calcium ions. These electrical phenomena have been reported to correlate with hormone secretion [26–28].

Recently hypothalamic growth hormone-releasing factor (GRF) has been identified [11, 23], which stimulates growth hormone (GH) secretion [3, 4, 30, 31] and GH mRNA production [2, 10]. Although GH_3 cells secrete GH in response to TRH or high concentration of potassium ions in the medium, it has been reported that GRF has no stimulatory effects on GH secretion nor mRNA production in GH_3 cells [32]. The unresponsiveness of GRF may be due to the lack of GRF receptors on the plasma membrane and/or to the defects of post-receptor mechanism. In the present study, we have investigated the membrane potential changes of GH_3 cells caused by GRF in an effort to determine whether the unresponsiveness to GRF is due to the lack of GRF action on the cell membrane.

Materials and Method

Cell Culture

The experiments were carried out on rat clonal GH_3 cells. GH_3 cells were a gift from Professor S. Ozawa (Gunma University, Japan). GH_3 cells were grown in Ham F-10 medium supple-

Table 1. Ionic composition of external media (mM)[a]

	Na	Choline	K	Ca	Ba	Mg	La	TEA[b]	TMA[c]	Cl
Normal	148.75	0	6.0	2.5	0	1.0	0	0	0	141.75
2.5 Ba Na-free TEA	0	0	6.0	0	2.5	1.0	0	128.75	20	141.75
2.5 Ca Na-free	0	128.75	6.0	2.5	0	1.0	0	0	20	141.75
0.5 La	148.75	0	6.0	2.5	0	1.0	0.5	0	0	143.25

[a] All media were buffered with 20 mM HEPES at pH 7.4 and contained 5.56 mM glucose.
[b] TEA: tetraethylammonium.
[c] TMA: tetramethylammonium.

mented with 15% horse serum and 2.5% fetal calf serum. The medium was exchanged twice a week and subculture was carried out once a week. At the subculture, the cells were transferred to Falcon dishes (35 × 10 mm). After 4 to 7 days of culture, electrophysiological studies were performed.

Electrophysiological Study

For electrophysiological analysis, the gigaohm seal patch-clamp techniques [13] were employed. All cells were settled in a whole cell clamp condition with patch electrode, where current-clamp recording as well as voltage-clamp recording were performed. The amplifier of LMT 5 (List Electronics) was used in the present experiment. Low-pass filter (cut-off frequency = 1 KHz) was employed. In some experiments membrane potential changes were recorded by intracellular glass microelectrode containing 3 M KCl with a resistance of 20 to 30 MΩ. The results were essentially the same between the two techniques, except for higher membrane resistance in the case of patch electrode. The patch electrodes were filled with a solution containing 151.28 mM KCl, 1 mM $MgCl_2$, and 20 mM HEPES. The pH was adjusted at 7.4. In some experiments 5 mM EGTA was added in the intracellular solution for calcium buffering, which did not change the results. The composition of the extracellular solutions are listed in Table 1. Temperature was kept at 32 to 35°C.

The resistance of patch electrodes was ranged from 2 to 8 MΩ. The seal resistance of patch electrodes over 10 GΩ could be obtained after application of negative pressure less than 50 cm H_2O. After formation of giga-seal, negative pressure over 100 cm H_2O was applied in order to obtain whole cell clamp condition, which was assumed by a sudden increase of the capacitive surge and the membrane noise. The current due to capacity of the cell membrane lasted about a few msec, which did not disturb the analysis of the present experiments. Sometimes the series resistance increased during recordings, which was recognized by the prolongation of the time constant in capacitive current. This phenomenon was considered to be the occlusion of the pipet opening by the membrane fragment [5]. If this resistance could not be effectively removed by the additional suction, the cell was discarded. This phenomenon was occasionally observed when the positive pressure was applied to the glass capillary containing GRF (*see below*). The data in such an experiment were also discarded.

The maximum inward current in the present experiment did not exceed 350 pA, thus the membrane potential error was almost negligible in the case of inward current. However, the amplitude of outward current was usually large, which caused underestimation of the true clamped potential. To reduce this potential error, the patch electrodes with relatively low resistance (less than 5 MΩ) were used for recording outward current.

Under whole cell clamp condition, diffusion of ions between the pipet and the cell interior occurs and the compensation of liquid junctional potential is needed. The compensation was carried out in a manner similar to that reported by Hagiwara and Ohmori [12]. The liquid junctional potential between the normal solution and the other solution used (internal and external) was directly measured, assuming the junctional potential at the tip of 3 M KCl in these solutions to be negligible. The value of liquid junctional potential was −2 to −4 mV, which was compensated in each experiment.

hpGRF (1-44) was purchased from Protein Research Foundation, Osaka, Japan. TRH was a gift from Takeda Chemical Industry, Japan. GRF was dissolved in the external solution and it was filled in a glass capillary with a tip diameter of a few microns. GRF concentration in the capillary was 5 μM. The tip of the glass capillary was positioned 20 to 30 μm apart from the recording cell. The pressure of 0.2 to 0.3 kg/cm² was applied to administer GRF. In order to examine the artefact caused by the pressure, the control glass capillary containing only the external solution was positioned at the same distance apart from the recording cell and the same pressure was applied. With the control capillary, no obvious membrane potential change was observed.

Assay of Growth Hormone

For hormone assay, the cells grown in Falcon dishes were washed two times with serum-free Ham F-10 medium and then the cells were incubated with 2 ml of serum-free Ham F-10 medium containing GRF or TRH. After incubation for 30 min the medium was aspirated, which was centrifuged by 1000 rpm for 5 min. GH in the supernate was assayed by double-antibody RIA provided by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases Rat Pituitary Hormone Distribution Program.

Data are expressed as mean ± SD. Statistical analysis was done with Student's *t*-test.

Results

Current-Clamp Recording

The Elimination and Prolongation of Spontaneous Action Potential

Figure 1 shows typical records of membrane potential changes of GH_3 cells caused by GRF. The rest-

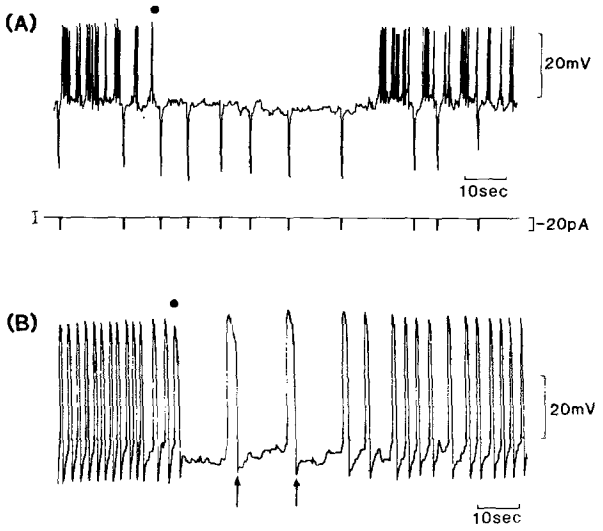

Fig. 1. Membrane potential changes of GH_3 cells under current clamp using patch electrode. (*A*) The record in the normal medium. Constant current pulses of −20 pA were applied in the inter-spike intervals. The current pulses are shown in the lower part of the figure. The dot indicates the timing of GRF administration of 1 sec. Following GRF, spontaneous action potentials were inhibited and were recovered after 54 sec. (*B*) The record in the normal medium. The application of GRF is indicated by the dot. Following GRF, the frequency of spontaneous action potential was decreased and the duration of action potentials was prolonged. In the prolonged action potential, the amplitude of afterhyperpolarization was decreased, which is indicated by arrows in the figure. Resting potential, −50 mV

ing potential of the record in Fig. 1*A* was −51 mV and the repetitive spontaneous action potentials were observed before application of GRF. When GRF was applied for a short period (1 sec in this case), spontaneous action potentials disappeared without changes in the resting potential. The membrane conductance, which is indicated by the potential deflections by the constant current pulses, remained unchanged as well. After cessation for 54 sec, spontaneous action potentials were resumed. Figure 1*B* represents another record, where the frequency of action potential was decreased and the duration of action potential was prolonged by application of GRF. In addition, the amplitude of afterhyperpolarization was decreased in the prolonged action potentials (arrows in Fig. 1*B*). The resting potential change was not observed. The similar phenomena to Fig. 1*B* were also observed in the cell of Fig. 1*A*, when the glass capillary containing GRF was located at a different position from that in Fig. 1*A*.

The same effects of GRF were observed by microelectrode techniques. These effects of GRF on GH_3 cells were consistent, observed in 45 of 50 cells (90%).

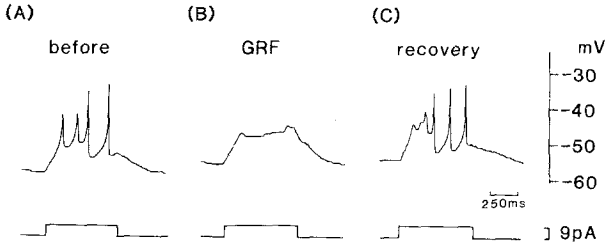

Fig. 2. Evoked action potentials by depolarizing current pulse in the cell which did not show spontaneous action potentials. The amplitude of applied current was 9 pA. Resting potential was −54 mV. In the normal medium: (*A*) is the record before application of GRF, (*B*) during GRF administration, (*C*) recovery of action potential at 60 sec after termination of GRF administration

The Elimination of Evoked Action Potential

The effect of GRF was examined in the cells which did not show spontaneous action potentials (silent cells). GRF caused no change in membrane potential and conductance, similar to the cells showing spontaneous action potentials. In silent cells action potential could be evoked by the positive current pulse (Fig. 2*A*). The evoked action potentials were eliminated by GRF and they were completely recovered at 60 sec after termination of GRF administration (Fig. 2*B* and 2*C*).

From the above results, it was concluded that GRF eliminated action potential and prolonged its duration, coupled with the amplitude decrease of afterhyperpolarization in GH_2 cells. Since the action potential of GH_3 cells is composed of voltage-gated Ca^{2+} and Na^+ channels [5, 7, 16, 18, 22], the elimination of action potential was considered to be ascribable to the block of either of the two channels or both. The prolongation of action potential and the decreased amplitude of afterhyperpolarization indicated the block of delayed K^+ channels and/or Ca^{2+}-induced K^+ channels existed in GH_3 cells [5, 6].

Whole Cell Clamp Recording

The Reduction of Voltage-Gated Ca^{2+} Channel Current

In order to clarify what channels were influenced by GRF, voltage-clamp analyses were performed. Figure 3*A* shows voltage-gated Ca^{2+} channel current recorded in 2.5 mM Ca Na-free medium. The membrane potential was fixed at −55 mV, where the outward current was not prominent. Holding potential was −80 mV. By application of GRF, the amplitude of Ca^{2+} inward current was decreased to about

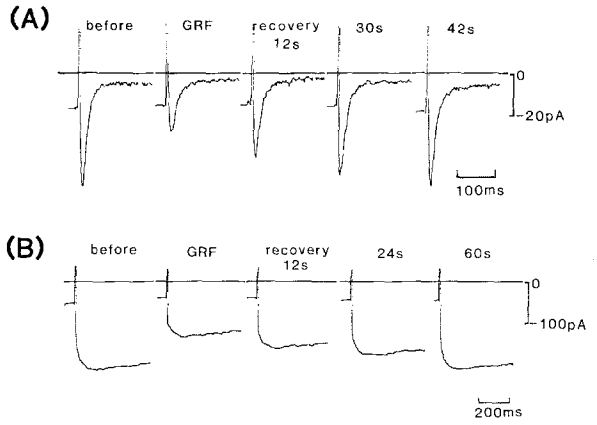

Fig. 3. Inhibition of voltage-gated Ca^{2+} channel current caused by GRF. (*A*) Records in 2.5 mM Ca Na-free medium. Holding potential was −80 mV and the membrane potential was fixed at −55 mV. (*B*) Records in 2.5 mM Ba Na-free TEA medium. Holding potential was −100 mV. The membrane potential, −8 mV

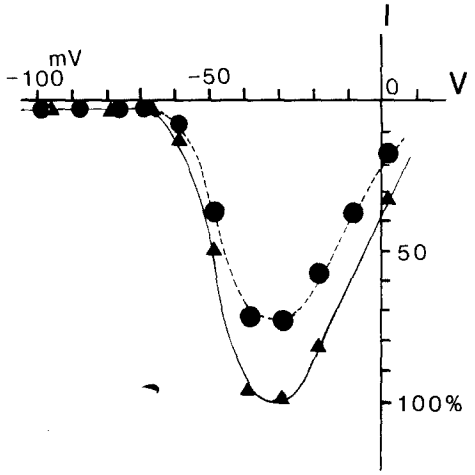

Fig. 4. *I-V* relation of Ba^{2+} current through voltage-gated Ca^{2+} channels, in 2.5 mM Ba Na-free TEA medium. Filled triangles connected with the solid line indicate the control record. Filled circles with broken line, the record during GRF administration. The peak of maximum inward current was −340 pA, which is represented as 100% in the figure. Holding potential was −100 mV

50% of the original level and almost completely recovered at 42 sec after termination of GRF application. There appeared to be no change in kinetics. Figure 3*B* shows the changes of Ba^{2+} current through Ca^{2+} channels, recorded in 2.5 mM Ba Na-free TEA medium. The membrane potential was −8 mV. Ba^{2+} current, which does not show distinct inactivation process, was reversibly reduced by GRF.

I-V relation of peak inward current through Ca^{2+} channels is shown in Fig. 4. These data were obtained in 2.5 mM Ba Na-free TEA medium, where contribution of outward current was negligible. The amplitude of Ca^{2+} channel current was inevitably decreased when the recording exceeded about 10 min (rundown of Ca^{2+} channel current), which was also observed during the experiment of Fig. 4. In this experiment, the control *I-V* curve was obtained first and then GRF was applied at each potential step, obtaining the ratio of maximally decreased current to the control. After recovery from the reduction at one potential step, the recording at another potential step was performed. During this procedure, the amplitude of the control Ca^{2+} channel current was decreased due to the rundown of Ca^{2+} channel current so that the effect of the rundown was superimposed on the reduction of Ca^{2+} channel current caused by GRF, which would be proportional to the effect on the control. Provided that the rundown did not occur, the amplitude of the control current should be the same as that recorded first and the really reduced current by GRF could be estimated by the ratio of maximally reduced current to the control, which are plotted in Fig. 4. Filled triangles in Fig. 4 indicate the amplitude of peak inward current without GRF and filled circles, those with GRF. The peak of maximum inward current is represented as 100%. The amplitude of inward current at each potential step was decreased to about 70% of the control level. The threshold and configuration of *I-V* curve was not changed.

The amplitude of the decreased Ca^{2+} channel current was dependent of GRF concentration in the glass capillary, although the final concentration of GRF at the cell surface could not be accurately determined. When GRF concentration in the capillary was lowered to less than 100 nM, the reduction of Ca^{2+} channel current was not prominent. On the other hand, GRF of 10 μM in the capillary reduced Ca^{2+} channel current more prominently but complete elimination was not observed. In the case of 5 μM GRF in the capillary, the Ca^{2+} channel currents were decreased to 67 ± 15.7% of the control level (n = 18).

The Reduction of Outward Current

Figure 5*A* shows the changes of outward current caused by GRF, recorded in the normal medium. The membrane potential was +10 mV. The outward current was reversibly reduced by GRF. The outward current of GH_3 cells is composed of delayed K^+ channel current and Ca^{2+}-induced K^+ channel current. It was obvious that Ca^{2+}-induced K^+ channel current was reduced by GRF through the decrease of voltage-gated Ca^{2+} channel current.

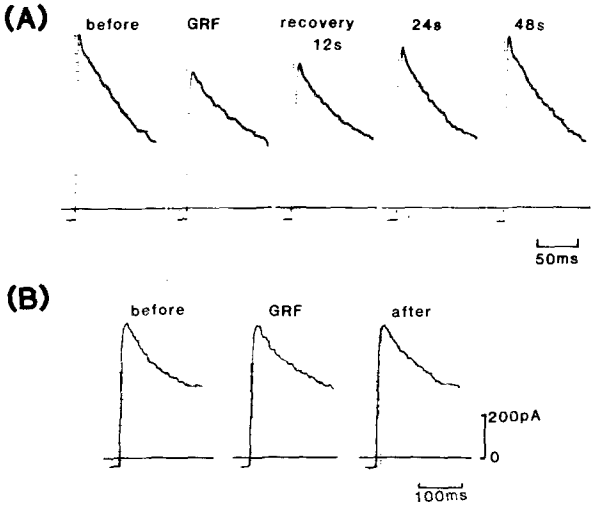

Fig. 5. The changes of outward current caused by GRF. (*A*) Records in the normal medium. Holding potential was −80 mV. The membrane potential, +10 mV. (*B*) Records in 0.5 mM La medium. Holding potential, −80 mV. The membrane potential, 0 mV

Therefore it was necessary to determine whether delayed K^+ channel current was reduced. Since Ca^{2+} channels are completely blocked in 0.5 mM La medium, it was considered that the changes of outward current in this medium could be attributed to delayed K^+ channel current, assuming that GRF did not change intracellular Ca^{2+} concentration[1]. The outward current in 0.5 mM La medium showed no change by GRF (Fig. 5*B*). The reduction of outward current in the normal medium was, therefore, considered to be due to the decrease of Ca^{2+}-induced K^+ channel current.

We also examined the effect of GRF on voltage-gated Na^+ channel current but GRF showed no effects (*data not shown*). From the above results, it was concluded that the elimination of action potential under current clamp was due to the decrease of voltage-gated Ca^{2+} channel current and the prolongation of action potential and that the decreased amplitude of afterhyperpolarization were ascribable to the decrease of Ca^{2+}-induced K^+ channel current. Since the decrease of Ca^{2+}-induced K^+ channel current was thought to be a consequence of decreased voltage-gated Ca^{2+} channel current, the direct effect of GRF on GH_3 cells was considered to be the block of voltage-gated Ca^{2+} channels.

[1] This assumption would be valid because GRF did not change the resting potential nor the conductance in the normal medium as well as in 0.5 mM La medium. If the significant change of intracellular Ca^{2+} concentration occurs, it should be detected by the changes of the resting potential and input resistance.

Table 2. Hormone release from GH_3 cell

Additions	*n*	GH release ng/10^6 cells/30 min[a]	Significance
Control	4	5.48 ± 0.34	
TRH 10^{-6} M	4	7.67 ± 0.27	$P < 0.001$
GRF 10^{-8} M	4	5.18 ± 0.39	*NS*
GRF 10^{-7} M	4	5.79 ± 0.80	*NS*

[a] Mean ± SD.

We have examined the effects of transferrin, vasoactive intestinal peptide and lutenizing hormone-releasing factor on Ca^{2+} channel current. Each protein in a concentration of 10 μM showed no effects, which indicated that the action of GRF was specific to the plasma membrane of GH_3 cells.

Hormone Secretion

Hormone secretion from GH_3 cells is shown in Table 2. GH release was increased 1.7 times by TRH, whereas it was not altered by GRF.

Discussion

The results in the present experiment revealed the membrane potential changes of GH_3 cells caused by GRF. Under voltage clamp it was found that voltage-gated Ca^{2+} channel current and Ca^{2+}-induced K^+ channel current were reduced by GRF, while voltage-gated Na^+ channel and delayed K^+ channel currents were not affected. The reduction of Ca^{2+}-induced K^+ channel current was considered to be a consequence of the reduction of voltage-gated Ca^{2+} channel current. Therefore it was concluded that the effect of GRF on GH_3 cells was solely the blockade of voltage-gated Ca^{2+} channels. Under current clamp, GRF caused elimination and prolongation of action potential without changes in resting potential and input resistance. The prolonged action potential was coupled with the amplitude decrease of afterhyperpolarization. The elimination of action potential corresponds to the reduction of voltage-gated Ca^{2+} channel current. The prolongation of action potential can be explained by the reduction of Ca^{2+}-induced K^+ channel current which is attributable to the reduction of voltage-gated Ca^{2+} channel current. The decreased amplitude of afterhyperpolarization was explained by the reduction of Ca^{2+}-induced K^+ channel current as well. Thus the results under current clamp well coincide with the results under voltage clamp.

One may suspect that responses to hormone are altered by the employment of patch electrode, because it is known that solutes of patch electrode diffuse into the cytoplasm and the intracellular ionic environment changes [9]. However, this possibility was unlikely in the present experiment, because the same phenomena were observed by mirocelectrode techniques and the compositional change of patch electrode solution did not alter the results.

The final concentration of GRF at the cell surface could not be accurately determined. However, it should not be much lower than GRF concentration in the glass capillary, because 0.5 mM La medium in the capillary completely eliminated voltage-gated Ca^{2+} channel current. When GRF concentration in the capillary was lowered to less than 100 nM, the reduction of Ca^{2+} channel current was not prominent. This result suggested that GRF more than 100 nM was required to reduce voltage-gated Ca^{2+} channel current. This concentration was higher than that required to stimulate GH secretion in rat pituitary cells, where 1 nM of GRF maximally stimulates hormone release [11, 23]. Although the reduction of voltage-gated Ca^{2+} channel current by high concentration of GRF may be an unphysiological phenomenon, it might have a role in producing inhibition of secretion in normal cells that respond to GRF.

The findings that GRF did not affect voltage-gated Na^+ channel and delayed K^+ channel may support the notion that GRF action was specific to the plasma membrane, i.e., voltage-gated Ca^{2+} channel, which is different from the effects of Ca^{2+} channel blocking agents, such as verapamil. In GH_3 cells it has been reported that verapamil blocks voltage-gated Ca^{2+} channels in a concentration over 3×10^{-5} M [21]. At this concentration voltage-gated Na^+ channel and delayed K^+ channel are blocked as well. The evidence that GRF solely affected voltage-gated Ca^{2+} channel indicated specific binding of GRF molecule to the plasma membrane, through which the entry of ions through voltage-gated Ca^{2+} channels was blocked, which was further supported by the evidence that transferrin, vasoactive intestinal peptide and lutenizing hormone-releasing factor showed no effects.

Since GRF blocked voltage-gated Ca^{2+} channels, hormone release from GH_3 cells may be expected to decrease. However, basal hormone release in GH_3 cells is independent of extracellular Ca^{2+}, although extracellular Ca^{2+} ions are required in stimulated hormone release such as in the case of high K^+ ions in the medium [22]. Therefore it was reasonable that GRF did not decrease basal hormone release in GH_3 cells.

It has been reported that TRH blocks an early voltage-dependent K^+ conductance in GH_3 cells, which causes prolongation of Ca^{2+} action potential [15, 24]. The finding in the present experiments resembled previous observations. The mechanism for prolongation of action potential by TRH has been reported to be due to the block of delayed K^+ channels. However, it is found that TRH in a rather high concentration (over 100 nM) blocks voltage-gated Ca^{2+} channels under voltage clamp (Dr. Y. Fukushima, *personal communication*). Therefore it is probable that TRH prolongs Ca^{2+}-dependent action potential through the block of voltage-gated Ca^{2+} channels and the resulting blockade of Ca^{2+}-induced K^+ channel current. If this is the case, it follows that GRF possesses similar action to TRH on voltage-gated Ca^{2+} channels. TRH is a secretagogue in GH_3 cells, while GRF is not. It may be that GRF binds its receptor but the mechanism of membrane-signal transduction is impaired in GH_3 cells, which explains why GRF does not stimulate hormone release nor mRNA production.

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