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The Role of K^+ Channels on Spontaneous Action Potential in Rat Clonal Pituitary GH_3 Cell Line

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The types of K^+ channel which determine the pattern of spontaneous action potential (SAP) were investigated using whole-cell variation of patch clamp techniques under current- and voltage-clamp recording conditions in rat clonal pituitary GH_3 cells. Heterogeneous pattern of SAP activities was changed into more regular mode with elongation of activity duration and afterhyperpolarization by treatment of TEA (10 mM). Under this condition, exposure of the class III antiarrhythmic agent E-4031 (5 μM) to GH_3 cells hardly affected SAP activities. On the other hand, the main GH_3 stimulator thyrotropin-releasing hormone (TRH) still produced its dual effects (transient hyperpolarization and later increase in SAP frequency) in the presence of TEA. However, addition of $BaCl_2$ (2 mM) in the presence of TEA completely blocked SAP repolarization process and produced membrane depolarization in all tested cells. This effect was observed even in TEA-untreated cells and was not mimicked by higher concentration of TEA (30 mM). Also this barium-induced membrane depolarization effect was still observed after L-type Ca^{2+} channel was blocked by nicardipine (10 μM). These results suggest that barium-sensitive current is important in SAP repolarization process and barium itself may have some depolarizing effect in GH_3 cells.

Key Words: Spontaneous action potential, Pacemaker, Pituitary gland, Barium, Repolarization, Inwardly rectifying K^+ current

INTRODUCTION

The change of spontaneous action potential (SAP) in its duration and frequency is important for various physiological functions such as cardiac pacemaker activity or transmitter- or hormone-induced activity. For past decades, therefore, the ionic currents underlying SAP have been examined extensively in various tissues. One of well-characterized systems is sino-atrial node of the heart. Hallmarks of these nodal cells include the presence of the characteristic pacemaker current (I_f), lack of the inward rectifier K^+ current ($I_{K,\ IR}$) and action potential that are relatively insensitive to the fast sodium channel blocker tetrodotoxin (See review: Irisawa et al, 1993).

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The rat clonal pituitary cell line, GH_3, has been also considerably focused by its intrinsic firing property. SAP of GH_3 cells are known to be stimulated by thyrotropin-releasing hormone (TRH), and the modulation of SAP is considered to be related to the secretion of growth hormone and prolactin (Taraskevich & Douglas, 1977; Gershengorn, 1986). It has been suggested that the depolarizing drive to action potential threshold is mediated by dihydropyridine-sensitive steady state Ca^{2+} channel currents in GH_3 cells (Scherbl & Hescheler, 1991). On the other hand, other investigators have suggested that background sodium conductance is responsible for driving cells to threshold for firing action potentials (Simasko, 1994). Compared to these studies for depolarizing drive to SAP, the ionic currents for repolarizing phase of SAP have not been extensively studied even though many voltage clamp studies have examined the characteristics of different types of K^+ currents in GH_3 cells (Dubinsky & Oxford, 1984; Ritchie, 1987; Oxford &

Wagoner, 1989; Simasko, 1991a). We, therefore, examined the effects of several K^+ channel blockers on the pattern of SAP using the whole-cell patch clamp technique under current- and voltage-clamp recording conditions in GH_3 cells.

METHODS

Cell culture

The clonal GH_3 pituitary cell line was purchased from the American Type Culture Collection (ATCC: Rockville, MD) and maintained in Ham's F-10 nutrient mixture supplemented with 15% horse serum, 2.5% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin under a humidified atmosphere of 95% air and 5% CO_2 at 36.5°C. Growth medium was replaced twice a week, and cells were split into subcultures once a week. During passages of the cells, aliquots of cells were plated on poly-D-lysine (0.05 mg/ml) coated glass cover slips and grown in 35 mm culture dishes for electrophysiological measurements. Cells used in this study were from passes 18 to 30, and a new stock cell line was prepared from cells frozen in liquid N_2 after 10 to 12 passage use. All cell culture reagents were purchased from GIBCO (Grand Island, NY).

Electrophysiological recordings and data analysis

Recordings were made from cells 2~7 days after plating, using the standard whole-cell patch-clamp method (Hamill et al, 1981) or the nystatin perforated patch-clamp method (Horn & Marty, 1988). Patch electrodes with resistance of 2~3 MΩ were filled with the pipette solution containing (mM); 30 KCl, 110 KOH, 90 aspartic acid, 10 HEPES, 1 $MgCl_2$, 5 MgATP, 2.5 di-tris phosphocreatine, 2.5 di-sodium phosphocreatine, 0.1 EGTA. Standard bath solution consisted of (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose. Isotonic K^+ bath solution was made by replacing an equivalent amount of NaCl. Nystatin was prepared as a stock solution (25 mg/ml) in DMSO and diluted to a concentration of 250 μg/ml using pipette solution and back-filled into the pipette after the tip of the pipette was initially filled with the nystatin-free solution. Bath solutions and drugs were applied into the recording chamber by gravity-fed perfusion system. All chemicals for solutions were obtained from Sigma Chemical (St. Louis, MO).

Electrophysiological experiments were performed at 32~35°C with the use of Axopatch-1D amplifier and pCLAMP software package from Axon Instruments (Foster City, CA). Signals from the patch amplifier were filtered at 5 kHz. For current-clamp data, the signal was recorded on a chart record (Gould, Cleveland, OH) and videocassette recorder tape with the use of a VR-10 Instrutech digital data recorder (Elmont, NY). For voltage-clamp data, the signal was digitalized by a computer driven analog-to-digital converter (Indec Systems, Sunnyvale, CA) and stored on hard disk for subsequent analysis.

RESULTS

In standard whole-cell patch clamp mode, SAP activities were disappeared within 5~10 min after the membrane was ruptured by gentle suction, which suggested the importance of cytoplasmic factors in this automaticity. We therefore recorded SAP activities in the perforated patch clamp method using nystatin at concentration of 250 μg/ml in GH3 cells. Under this condition, 90.6% of GH3 cells (n=202/223) showed SAP activities, much higher percentage than earlier reports (Hedlund et al, 1988; Simasko, 1991b). Fig. 1 represents some typical examples of SAP activities in GH3 cells at high time resolution. The pattern and activity parameters of SAP are very heterogeneous from cell to cell. Some cells showed relatively regular shape and frequency of SAP, as shown in Fig. 1A. Range of duration and frequency of SAP in this type of cells were 63.2~121.9 ms (88.3±1.9 ms) and 0.51~0.79 Hz (0.60±0.01), respectively. Fig. 1E also represents an extreme example of very regular pattern of SAP with 2.7~23.1 ms range of duration (9.9±0.5 ms) and 3.2~12.1 Hz range of frequency (6.2±0.4 Hz). The other extreme was a very irregular pattern of SAP, as shown in Fig. C. However, the most frequently observed pattern of SAP in GH3 cells was a kind of mixed form like Fig. 1C & 1D. The amplitude of action potentials did not differ significantly among different patterns of spontaneous activity: the maximum negative potential was −68.8~−44.0 mV and the upstroke reached −2.4~+21.6 mV.

Tetraethylammonium (TEA) has been known as a blocker of delayed rectifying K^+ channels and Ca^{2+}-

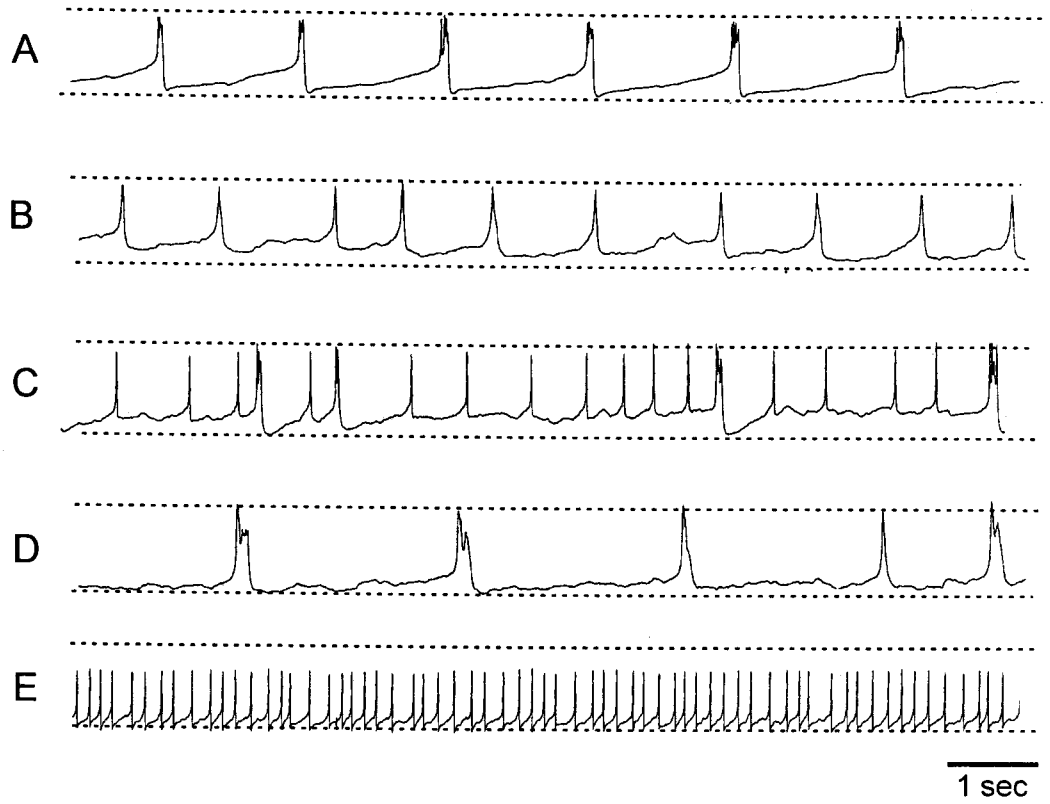

Fig. 1. Heterogeneous pattern of spontaneous action potentials in rat clonal pituitary GH_3 cells. Representative examples of heterogeneous cell showing spontaneous action potentials (SAP) in GH_3 cells using the nystatin (250 μg/ml) perforated patch-clamp method. The pattern and activity parameters of SAP are very heterogeneous from cell to cell (A-E). Upper and lower dotted lines represent 0 mV and −60 mV, respectively, in this and following figures.

activated K^+ channels ($I_{K,Ca}$) depending on TEA concentration used (Ritchie, 1987; Simasko, 1991a). Application of TEA to GH_3 cells prolonged the duration of SAP and reduced the frequency of SAP in a dose-dependent manner (Fig. 2A). The amplitude of action potential was increased by TEA and the increase was in both directions: more depolarization of upstroke potentials and more hyperpolarization of maximum negative potentials. In addition, slow depolarization phase after repolarization phase became prominent in 10 mM TEA, resulting in a pattern of SAP which looks similar to that of sino-atrial node cells of the heart. Furthermore, various patterns of SAP which were shown in Fig. 1 were changed into a more regular pattern in the presence of 10 mM TEA, as was shown in Fig. 2A. Under voltage-clamp condition, currents were evoked every 15 sec by a 500 msec depolarizing voltage step from −80 mV to various testing potential (−60 to +20 mV). Sustained outward currents were inhibited at all testing voltage step and inhibited 59.4% and 88.4% at +20 mV testing potential by 1 and 10 mM TEA application, respectively (Fig. 2B). On the other hand, it was interesting that the main GH_3 stimulator, TRH, still produced its dual effects (transient hyperpolarization and later increase in SAP frequency) under this condition (Fig. 2C).

The facts that GH_3 cells were still producing SAP activities and repolarization reached more negative potential in the presence of 10 mM TEA suggest the presence of TEA-insensitive outward current contributing to the repolarization. Under the condition of blocking most delayed rectifying and Ca^{2+}-activated K^+ currents by TEA (10 mM), we examined the type (s) of ionic current responsible for SAP repolarization period because repolarization process still occurred in sino-atrial node cells of the heart where Ca^{2+}- activated K^+ currents were absent (Sanguinetti & Jurkiewicz, 1990). $I_{K,\ IR}$ is known to be blocked by class III antiarrhythmic drugs, such as E-4031, and

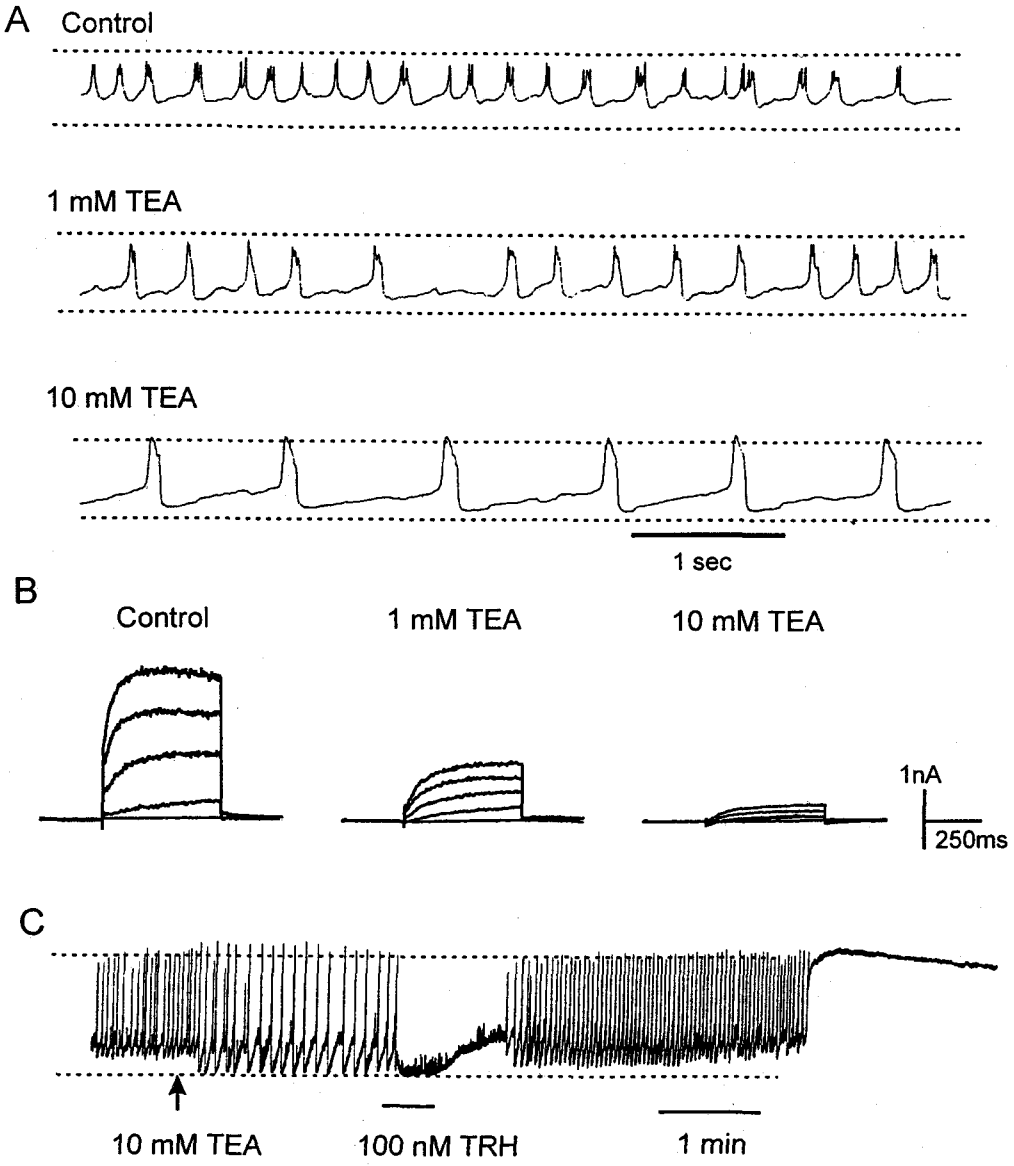

Fig. 2. Modulatory effects of tetraethylammoium (TEA) on SAP activities in GH_3 cells. (A) Under current-clamp conditions using the perforated patch clamp method, application of TEA prolonged the duration and reduced the frequency of SAP in a dose-dependent manner (1, 10 mM). (B) Under voltage-clamp condition using low concentration Ca^{2+} chelator EGTA (0.1 mM), application of 1 and 10 mM TEA inhibited the sustained outward currents by 59.4% and 88.4% at +20 mV, respectively. Cells were held at −80 mV and given 20 mV voltage steps for 500 msec to various testing potentials. (C) The effects of thyrotropin-releasing hormone (TRH) in the presence of 10 mM TEA. Application of TRH (100 nM) into GH_3 cells still produced an initial phase of transient hyperpolarization and a sustained increase in the rate of action potential production in the presence of 10 mM TEA. Application of drug is indicated by black bar under the traces in this and following figures.

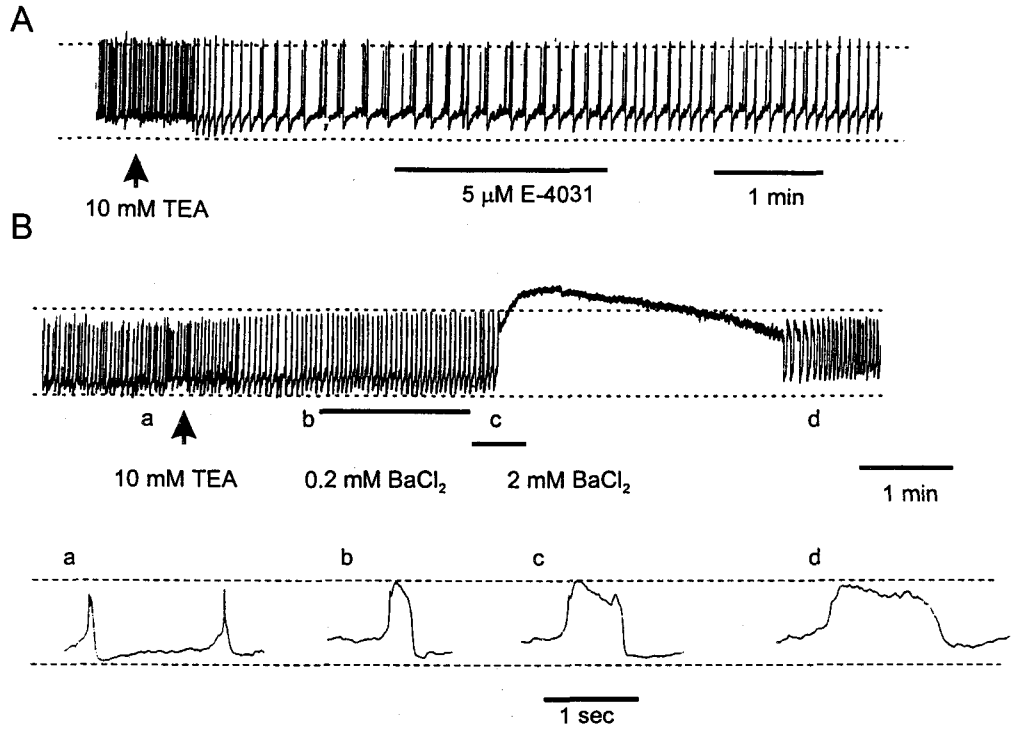

Fig. 3. The effects of variable K^+ channel blockers on SAP activities in GH_3 cells. (A) In the presence of 10 mM TEA, exposure of E-4031 (5 μM), class III antiarrhythmic drug to GH_3 cells did not cause significant changes of SAP activities. (B) Dose-dependent effect of barium ion in the presence of TEA (10 mM). a-d are high resolution voltage traces.

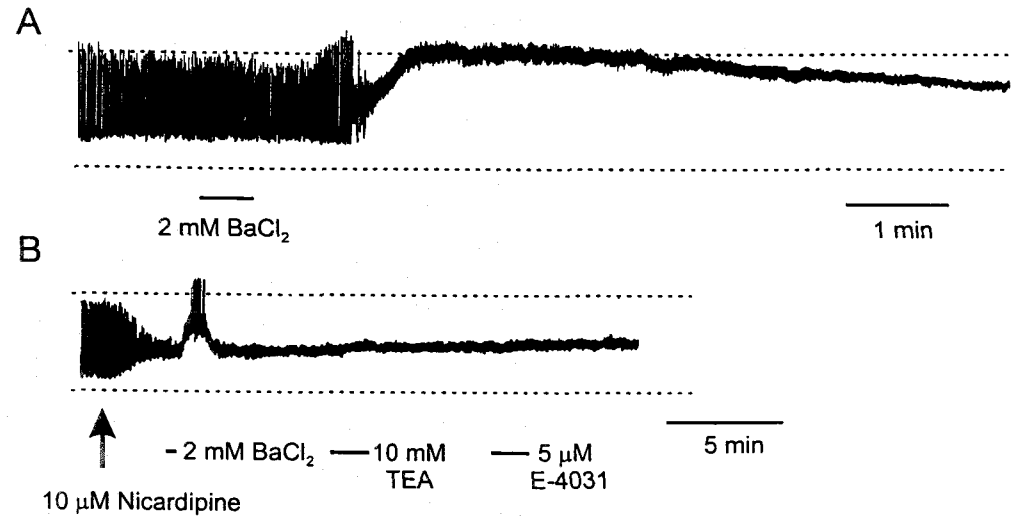

Fig. 4. Characteristics of barium-mediated modulation of SAP activities in GH_3 cells. (A) Addition of $BaCl_2$ (2 mM) in GH_3 cells was completely blocked SAP repolarization process and produced membrane depolarization in normal extracellular recording solution. (B) In the presence of L-type Ca^{2+} channel blocker, nicardipine (10 μM), this barium-induced membrane depolarization effect was still observed. However, there is no change of membrane potential by application of TEA (10 mM) or E-4031 (5 μM).

the presence of E-4031-sensitive component of K^+ currents has been reported in GH_3 cells (Weinsberg et al, 1997). The effect of E-4031 was examined in the presence of 10 mM TEA. Exposure of E-4031 (5 μM) did not cause significant changes in SAP of GH_3 cells (n=7, Fig. 3A). However, addition of 0.2 mM $BaCl_2$ prolonged the repolarization profoundly, resulting in a very long duration of SAP in the presence of 10 mM TEA. Addition of higher concentration of barium (2 mM) completely blocked repolarization process and produced membrane depolarization up to +20 mV in all tested cells (n=4, Fig. 3B). Membrane depolari-zation effect of high concentration of barium also occurred when SAP was recorded in normal extracellular recording solution without TEA treatment (n=3, Fig. 4A). In some population of GH_3 cells, even low concentration (0.2~0.3 mM) of barium also blocked SAP activities and produced membrane depolarization (n=3/8, data not shown). To exclude the possibility that this effect was caused by the permeability of barium through Ca^{2+} channels, we examined the effect of barium after L-type Ca^{2+} channel was blocked by the treatment with L-type blocker, nicardipine (10 μM). After SAP activity was stopped by nicardipine, barium (2 mM) still produced membrane depolarization effect (n=5/6, Fig. 4B).

Under voltage clamp experiment using 0.1 mM internal EGTA concentration, effects of TEA and Ba^{2+} on outward rectifying K^+ currents were examined. Currents were evoked by a 200 msec test pulse to various potentials (from −120 to +20 mV) after

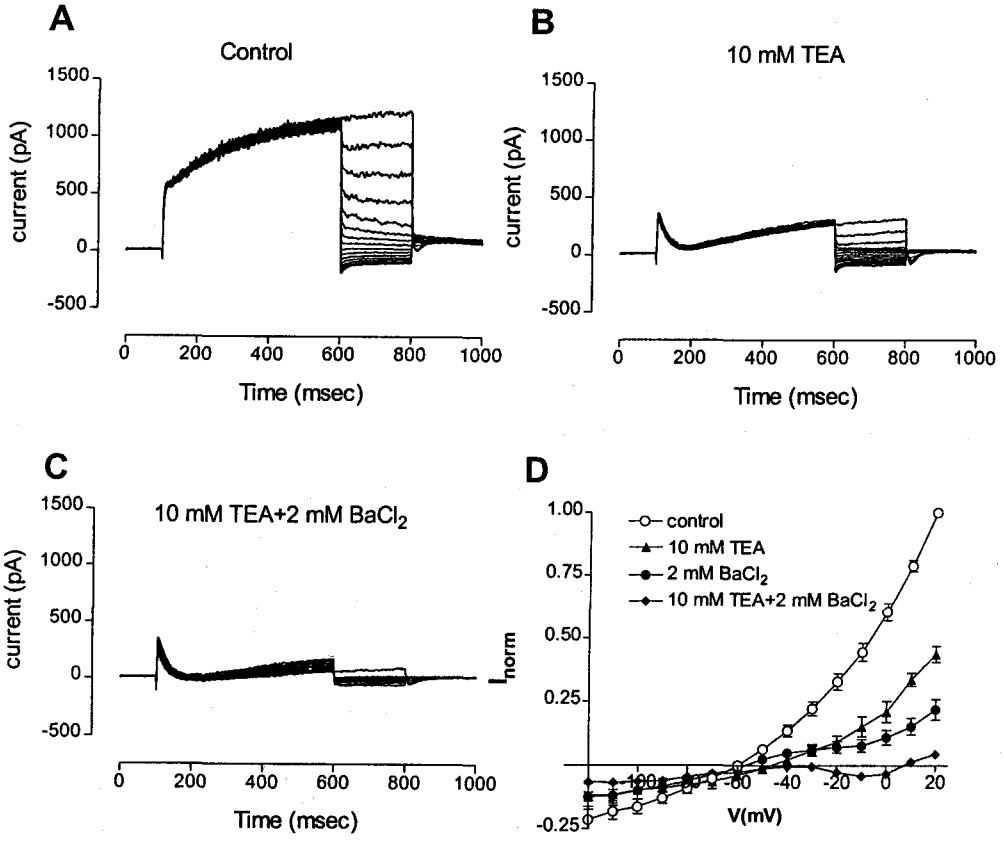

Fig. 5. Comparison between barium-mediated and TEA-mediated inhibition on outward rectifying K^+ currents in GH_3 cells. (A-C) Under voltage clamp experiment using 0.1 mM internal EGTA concentration, outward currents were evoked by a 20 mV voltage step for 200 msec to various testing potentials (from −120 to +20 mV) after holding cells at +20 mV for 500 ms. (A) for control, (B) for treatment of 10 mM TEA, and (C) for co-treatment of $BaCl_2$ (2 mM) and TEA (10 mM). (D) Current-voltage (I-V) relationship of normalized sustained current before (open circles) and during drug application (TEA for closed triangles, n=4; $BaCl_2$ for closed circles, n=4; TEA+$BaCl_2$ for closed diamonds, n=4).

holding cells at +20 mV for 500 msec to check drug's effect on repolarization period in SAP activity (Fig. 5). From pooled data (n=4 for each treatment), the relationship between the normalized sustained current and voltage before and during drug application is shown in Fig. 5D. The amplitude of outward currents measured at +20 mV was reduced by 56.7± 3.3% (n=4) in 2 mM $BaCl_2$, showing that the percentage of inhibition by barium ion is not larger than that by 10 mM TEA (78.1±4.0%, n=4). Co-treatment of $BaCl_2$ (2 mM) and TEA (10 mM) produced 95.7± 0.9% inhibition of outward currents in 4 tested cells. Because barium-mediated effect was observed even in TEA-untreated cells and was not mimicked by higher concentration of TEA (30 mM), it is unlikely that complete failure of repolarization process by barium is explained by the effect of barium on outward rectifying K^+ currents. In isotonic K^+ external and 10 mM EGTA containing internal solutions to block $I_{K,Ca}$, barium blocked both peak and sustained currents more effectively than TEA over all tested voltage range (data not shown). These results suggest that this barium-sensitive current is important in SAP repolarization process and barium itself may have some depolarizing effect in GH_3 cells.

DISCUSSION

In the present investigation, we examined the effect of several K^+ channel blockers on SAP activity in a rat clonal pituitary cell line GH_3 and found the important role of barium ion on SAP repolarization process. As a useful model for investigations into the regulation of hormone, a clonal pituitary cell line GH_3 has been widely used for its similarity with comparable cells in the normal animal. GH_3 has been shown to secrete continuously both growth hormone and prolactin, which strongly related with its intrinsic firing property. The ionic basis of action potentials in these cells is of functional importance when considering possible mechanism by which various agents such as thyrotropin-releasing hormone (TRH), somatostatin, or other hypothalamic peptides acting on these cells to modulate hormone release and to alter the firing frequency. Since TRH has been known as a main stimulator of hormone release in GH_3 cells (Taraskevich & Douglas, 1977; Tashjian, 1979), many studies have focused on what electrical change is accompanied with increased hormone release by TRH to understand the ionic mechanisms of SAP in GH_3 cells.

Stimulation of prolactin release by TRH in GH_3 cells is biphasic and linked to a similar biphasic changes in electrical activity, consisting of an initial phase of transient hyperpolarization and a sustained increase in the rate of action potential production (Gershengorn, 1986; Bjoro et al, 1990). It has been well established that the initial phase is caused by the release of Ca^{2+} ions from intracellular pools and resulted in a transient hyperpolarization of the cell membrane via the opening of Ca^{2+}-activated K^+ channels (Dubinsky & Oxford, 1985; Lang & Ritchie, 1987). However, the conductance pathway(s) responsible for the increased production of action potentials has been difficult to identify. Initial studies suggested that TRH-induced increase in action potential production is at least partly caused by a decrease in the membrane K^+ conductance and resulting in a depolarization of the cell membrane and Ca^{2+} entry through voltage-sensitive channels, and increased prolactin secretion (Dubinsky & Oxford, 1985; Ozawa & Sand, 1986). Recent evidences further suggest that an inwardly rectifying K current ($I_{K,\ IR}$), present at the resting potential (Bauer et al, 1990) and reduced by TRH, could play a major role in determining the firing rate of GH_3 cells and its enhancement by TRH. (Barros et al, 1992; Barros et al, 1994) The importance of $I_{K,\ IR}$ in determining the firing rate of SAP in GH_3 cells was also suggested by Weinsberg et al. (1997). They showed the class III antiarrhythmic agent E-4031 potently blocked $I_{K,\ IR}$ and induced an increase in the frequency of SAP, suggesting an important role of $I_{K,\ IR}$ in controlling cell excitability. The ionic currents underlying the action potentials in GH_3 cells were also investigated using channel blockers as well as TRH. Along with Ca^{2+} and background sodium currents proposed for the depolarizing drive to action potential threshold (Scherbl & Hescheler, 1991; Simasko, 1994), two classes of K^+ currents were described: one voltage dependent, one Ca^{2+} dependent. As was reported by Lang & Ritchie (1987) and Ritchie (1987), at least two pharmacologically distinct Ca^{2+}-activated K^+ currents (TEA-sensitive B_K currents & TEA-insensitive but apamin-blockable S_K currents) and a 4-aminopyridine (4-AP) sensitive voltage-dependent K^+ currents exist in GH_3 cells. In addition, more recently Simasko (1991a) reported the existence of a TEA-sensitive delayed rectifier-like current that has a slow rate of inactivation

after removal of bath Ca^{2+} to isolate voltage- dependent K^+ currents from Ca^{2+}-activated K^+ currents. While these previous studies have been intensively focused on the characteristics of several K^+ currents using voltage clamp experiments, not much study has been attempted for examining the effect of several K^+ channel blockers on SAP activity and the nature of the currents responsible for repolarization of SAP.

Taraskevich & Douglas (1980) reported that TEA prolongs the duration of both evoked and spontaneous action potentials. As was reported by Simasko (1991a), TEA also blocks a delayed rectifier-like current in GH_3 cells in addition to Ca^{2+}-activated K^+ current. Thus the observations with TEA do not establish which of these K^+ currents is involved in the repolarization, but indicates that activation of Ca^{2+}-dependent K^+ current is at least involved. Involvement of Ca^{2+}-dependent step in the pattern of SAP was also suggested by Simasko (1991b) in GH_3 cells. Increasing intracellular Ca^{2+} buffering capacity with Ca^{2+}-chelator caused the duration of SAP to increase without altering other parameters of membrane potential activity. This result suggests that the duration of SAP in GH_3 cells is regulated by the accumulation of free intracellular Ca^{2+} although the exact nature of this Ca^{2+}-dependent step remains to be determined. More systematic approach to determine the role of specific K^+ currents in the repolarization process was recently attempted by Sankaranarayanan & Simasko (1998) using K^+ channel blockers. According to their study, TEA, 4-AP, charybdotoxin, and apamin all caused a significant increase in duration, while TEA and charybdotoxin also caused an increase in peak amplitude, suggesting the importance of Ca^{2+}-dependent and voltage-dependent K^+ currents.

In our experiments, TEA (10 mM) also prolonged the duration of SAP in all heterogeneous population of GH_3 cells and changed the behavior of SAP activities from irregular to more regular pattern. Since GH_3 cells were still represent spontaneous firing potentials even with elongated duration of SAP in the presence of TEA, our study mainly focused on the nature of the currents responsible for repolarization of SAP after blocking most TEA-sensitive currents (Ca^{2+}-dependent and voltage-dependent K^+ currents). For the role of A-like voltage-dependent K^+ currents in SAP repolarization process of GH_3 cells, the variable effects of 4-AP, A-like K^+ current blocker, itself on the duration of SAP were reported (Sand et al, 1980; Rogawski, 1988 & 1989). In this study, however, exposure of 4-AP (5 mM) to GH_3 cells produced only small membrane depolarization with no further change of duration and upstroke potentials in the presence of TEA (data not shown). Exposure of $I_{K,IR}$ blocker, E-4031, to GH_3 cells hardly affected SAP activities in TEA treated cells. Simasko & Sankaranarayanan (1997) first reported the presence and characterization of hyperpolarization-activated current in GH_3 cells. They demonstrated that the current was insensitive to bath application of TEA, 4-AP, and barium but was completely blocked by cesium. However, application of cesium to GH_3 cells did not exert any effect on SAP activity, suggesting the I_f component does not serve as a pacemaking current in these cells.

In various other cell types that possess Ca^{2+}-dependent action potential, barium ions commonly increase the amplitude and duration of the action potentials, presumably by blocking voltage-dependent K^+ current and can substitute for Ca^{2+} in action potential production. In TEA-treated GH_3 cells, the addition of barium in the presence of physiological Ca^{2+} level prolonged the duration of SAP further at low concentration (0.2~0.3 mM) and completely blocked SAP repolarization process with membrane depolarization at high concentration of barium (2 mM). In some tested cells, this effect was observed even with low concentration of barium and in TEA untreated cells. Barium has been known to block inward rectifying K^+ channel and a substitute for Ca^{2+} in voltage-dependent Ca^{2+} channel in GH_3 cells (Scherbl & Hescheler, 1991; Barros et al, 1992). It is unlikely that an inwardly rectifying K^+ current ($I_{K,IR}$) is involved in this barium-mediated effect because the blockage of $I_{K,IR}$ component by TRH or E-4031 produced an increase in the frequency of SAP in GH_3 cells. (Barros et al, 1992; Barros et al, 1994; Weinsberg et al, 1997)

One way to find the nature of the currents responsible for repolarization of SAP is to block the targeted currents completely using pharmacological tools. TEA has been known to inhibit the Ca^{2+}-activated K^+ current with IC_{50} of 1 mM and to inhibit the majority of the delayed-rectifier K^+ current at concentration of 10 mM (Ritchie, 1987) in GH_3 cells. In some, but not all, cells application of 30 mM TEA, for further complete inhibition of these K^+ currents, stopped SAP, but never produced the depolarization effect that mediated by barium. This is the first difference between barium- and TEA-mediated effects. Second

difference is the consistency of response and the number of responded cells by these agents. Barium (2 mM) always blocked SAP repolarization with membrane depolarization in all tested cells while TEA (30 mM) produced variable effects, either prolongation of the duration or stopping firing, suggesting the main role of barium in repolarization process of SAP. Compared to barium, less effectiveness of TEA in prolongation of action potentials was also reported by Taraskevich & Douglas (1980).

Because barium produced much less inhibition of outward K^+ current than TEA did as shown in Fig. 5, possible explanation needs for the role of barium in SAP repolarization of GH_3 cells. Taraskevich & Douglas (1980) also reported that replacement of all Ca^{2+} by barium (10 mM) resulted in enormous prolongation of SAP duration and increased tendency for depolarization. They suggested that barium might enhance hormone secretion by sustaining the action potential or membrane depolarizing effect of barium itself. This depolarization effect is unlikely by the activation of L-type Ca^{2+} channel, the main type of Ca^{2+} channel in these cell, because it was still observed after this channel was blocked by its selective blocker nicardipine. Our result also clearly suggests that it is neither mediated by inwardly rectifying K^+-currents, nor by hyperpolarization-activated currents. Background K^+ channels being Ba^{2+}-sensitive, but insensitive to all other K^+-channel blockers, might be the target channel of this barium-mediated depolarization in GH_3 cells as Prior et al (1998) reported in rabbit renal arcuate artery. In conclusion, this barium-mediated repolarization process is important in understanding the ionic basis of spontaneous action potentials in GH_3 cells as well as voltage-dependent Ca^{2+} and background sodium currents responsible for the depolarizing drive to action potential threshold.

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