Chinese Journal of Physiology 44(4): 161-167, 2001

Functional Coupling of Voltage-Dependent L-Type Ca^{2+} Current to Ca^{2+}-Activated K^+ Current in Pituitary GH_3 Cells

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Abstract

Ca^{2+}-activated K^+ currents ($I_{K(Ca)}$) can contribute to action potential repolarization and after-hyperpolarization in GH_3 cells. In this study, we examined how the activation of $I_{K(Ca)}$ at the cellular level could be functionally coupled to Ca^{2+} influx through L-type Ca^{2+} channels. A 30-msec Ca^{2+} influx step to 0 mV was found to exhibit substantial contribution of Ca^{2+} influx through the activation of $I_{Ca,L}$ to the activation of $I_{K(Ca)}$. A bell-shaped relationship between the conditioning potentials and the integrated $I_{K(Ca)}$ was observed, suggesting that the magnitude of integrated $I_{Ca,L}$ correlates well with that of integrated $I_{K(Ca)}$ in the same cell. A linear relationship of integrated $I_{Ca,L}$ and integrated $I_{K(Ca)}$ was found with a coupling ratio of 69 ± 7. The value of the coupling ratio was unaffected by the presence of Bay K 8644 or nimodipine, although these compounds could effectively affect the amplitudes of both $I_{K(Ca)}$ and $I_{Ca,L}$. However, tetrandrine could decrease the coupling ratio. Paxilline or intracellular Ca^{2+} buffer with EGTA decreased the coupling ratio, while apamin had no effect on it. Interestingly, phorbol 12-myristate 13-acetate also reduced the coupling ratio significantly, whereas thapsigargin increased this value. Thus, the present study indicates that the activation of $I_{K(Ca)}$ during brief Ca^{2+} influx, which is inhibited by paxilline, is coupled to Ca^{2+} influx primarily through the L-type channels. The selective modulation of $I_{K(Ca)}$ by second messengers or Ca^{2+} release from internal stores may affect the coupling efficiency and hence cellular excitability.

Key Words: L-type Ca^{2+} current, Ca^{2+}-activated K^+ current, GH_3 cells

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Received June 1, 2001; Revised July 5, 2001; Accepted July 6, 2001.

Introduction

Elevation of intracellular Ca^{2+} mediated by influx through voltage-dependent Ca^{2+} channels participates in a wide variety of cellular functions, including exocytosis, excitation-contraction coupling, synaptic plasticity, ion channel gating, gene expression, and the growth and death of neurons (1). Ca^{2+} ions that enter through Ca^{2+} channels can regulate different cell processes, including the generation of Ca^{2+}-activated K^+ current ($I_{K(Ca)}$) and afterhyperpolarizing potentials (1, 14, 16). The magnitude of $I_{K(Ca)}$ present in neurons or neuroendocrine cells can control the action potential waveform and regulate cell excitability.

Lactotrophs are known to secrete prolactin at elevated basal rates in the absence of normal hypothalamic or hypophysiotropic factors (8). Spontaneous secretion of prolactin was believed to require the presence of spontaneous Ca^{2+}-dependent action potentials. The activity of voltage-dependent L-type Ca^{2+} channels plays an important role in controlling spontaneous action potentials (11). Repetitive action potentials, as occurs in neurons, were thus thought to be of importance in controlling changes in intracellular Ca^{2+} and basal and stimulated secretion from lactotrophs (11, 17).

There are several lines of evidence to suggest a tight coupling between Ca^{2+} current (I_{Ca}) and $I_{K(Ca)}$. For example, it has been reported that there appeared to be a subtle interaction between large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels, ryanodine receptors, and L-type I_{Ca} ($I_{Ca,L}$) in cerebellar granule cells (2). It was found that a part of K^+ outward current in neostriatal projection neurons is sensitive to Ca^{2+} entry by the activation of I_{Ca} (20). A coupling between L-type Ca^{2+} channels and small-conductance Ca^{2+}-activated K^+ channels was also reported (10). However, quantitative data concerning the co-regulation of these two currents at the cellular level have not been clarified. Because the different types of Ca^{2+} channels may differentially participate in the activation of $I_{K(Ca)}$, it remains unclear whether large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels can functionally be coupled to L-type Ca^{2+} channels. Therefore, the purpose of this study was to study whether the activation of $I_{Ca,L}$ and $I_{K(Ca)}$ can be functionally coupled in GH_3 lactotrophs, and to determine the coupling efficiency of integrated $I_{Ca,L}$ and $I_{K(Ca)}$ in the same cell.

Materials and Methods

Cell Preparation

GH_3 cells were obtained from the Culture Collection and Research Center (CCRC-60015, Hsinchu, Taiwan) (22). Cells were cultured in 50-ml Ham's F-12 medium (Life Technologies, Grand Island, NY, USA) that was supplemented with 15% horse serum (v/v), 2.5% fetal calf serum (v/v), and 2 mM L-glutamine (Life Technologies) in a 5% CO_2 atmosphere. Cells were subcultured once a week, and a new stock line was generated from frozen cells (frozen in 10% glycol in medium plus serum) every 3 months. The experiments were performed after 5 or 6 days of subcultivation (60 to 80% confluence).

Electrophysiological Measurements

Immediately before each experiment, GH_3 cells were dissociated and an aliquot of cell suspension was transferred to a recording chamber positioned on the stage of an inverted phase-contrast microscope (Diaphot-200; Nikon, Tokyo, Japan). Cells were bathed at room temperature (20-25 °C) in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Patch pipettes were prepared from Kimax capillary tubes (Vineland, NJ) using a vertical two-step pipette puller (model PB-7; Narishige, Tokyo, Japan) and polished on a Narishige microforge (model MF-83). The resistance of the patch pipette was 3-5 MΩ when immersed in normal Tyrode's solution. Ionic currents were recorded with glass pipettes in the whole-cell configuration of patch-clamp technique, using an RK-400 patch amplifier (Biologic, Claix, France) (5, 22). All potentials were corrected for liquid junction potential that would develop at the tip of the pipette when the composition of pipette solution was different from that of bath.

Data Recording and Analysis

The signals consisting of voltage and current tracings were monitored with a digital storage oscilloscope (model 1602; Gould, Valley View, OH, USA) and recorded on-line using a digital audio tape recorder (model 1204, Biologic). After the experiments, the stored data were then fed back and digitally acquired at 5-10 kHz (Digidata 1320A acquisition system; Axon Instruments, Union City, CA,

USA) at the sampling rate of 10 kHz with a 1 kHz 4-pole Bessel filter. The data were processed on a Pentium III-grade computer (Slimnote VX_3; Twinhead, Taipei, Taiwan) and pClamp 8.02 software package (Axon Instruments).

To quantify Ca^{2+} influx or K^+ efflux during the voltage pulses, ion currents were integrated over the depolarizing pulses. Least-squares fits were performed with the aid of a Marquardt-Levenberg algorithm routine that was included in Microcal Origin 6.0 (Microcal Software Inc., Northampton, MA, USA). To measure the coupling ratio of integrated $I_{Ca,L}$ and $I_{K(Ca)}$, each cell was held at the level of –50 mV, and a 30-msec step to 0 mV followed by a 1-sec depolarizing pulse to +50 mV was applied. Then, the difference in outward currents obtained in the presence of 1.8 and 3.6 mM $CaCl_2$ was taken in each cell. When net inward ($I_{Ca,L}$) and outward ($I_{K(Ca)}$) currents were integrated, the coupling ratio would be obtained.

All values are reported as means ± standard error of the mean (SEM). The paired or unpaired Student's *t* test and one-way analysis of variance with least-significance-difference method for multiple comparisons were used for the statistical evaluation of differences among the means. Differences between the values were considered significant when $p < 0.05$.

Drugs and Solutions

Thapsigargin and apamin were obtained from Research Biochemicals (Natick, MA, USA). Bay K 8644 was obtained from Biomol (Plymouth Meeting, PA, USA) and tetrandrine was from Aldrich (Milwaukee, WI, USA). Phorbol 12-myristate 13-acetate (PMA) and nimodipine were purchased from Tocris Cookson Ltd. (Bristol, UK), and paxilline was from Alomone (Jerusalem, Israel). All other chemicals were commercially available and of reagent grade. The composition of normal Tyrode's solution was as follows (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5 and HEPES-NaOH buffer 5.5 (pH 7.4). To record membrane currents, the patch pipette was filled with a solution (in mM): KCl 140, $MgCl_2$ 1, Na_2ATP 3, Na_2GTP 0.1, EGTA 0.1 and HEPES-KOH buffer 5 (pH 7.2). In some experiments, 10 mM EGTA was included inside the pipette to chelate intracellular Ca^{2+} strongly.

Results

With the use of a two-step voltage protocol, ion currents in GH_3 cells were recorded in the whole cell configuration of the patch clamp technique (22). In these experiments, the activation of $I_{K(Ca)}$ was studied by stepping the voltage to various potentials that can activate Ca^{2+} current followed by a test pulse to +50 mV in which the activation of $I_{K(Ca)}$ was evaluated. As shown in Fig. 1, the effect of stepping the pulse on membrane currents was obtained in the presence of 1.8 and 3.6 mM $CaCl_2$. Notably, there is a small inward current that is evident during the prepulse and the magnitude of this inward current is greater in the presence of 3.6 mM $CaCl_2$ than in the presence of 1.8 mM $CaCl_2$. Similar results were obtained in 12 different cells. Current amplitudes measured at the end of the long-lasting depolarizing pulses in the presence of 1.8 and 3.6 mM $CaCl_2$ were 487 ± 23 and 825 ± 58 pA (n = 12), respectively. This small inward current was believed to be due to Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels, because nimodipine (1 mM) blocked this current and Bay K 8644 enhanced it. Furthermore, the 1-sec long-lasting depolarizing pulse to +50 mV after conditioning pulse was noted to induce $I_{K(Ca)}$ that was sensitive to inhibition by paxilline (1 mM). Paxilline was reported to be a potent inhibitor of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels (7, 9). When the difference between these two currents was taken, a significant inward current followed by a gradual outward current was observed (Fig. 1B). The time course of rise of this outward current was well described by an exponential function with a time constant of 238 msec (Fig. 1B). Thus, when extracellular Ca^{2+} was increased from 1.8 to 3.6 mM, the major component of outward currents obtained during the long-lasting depolarizing pulses to +50 mV was $I_{K(Ca)}$ that was mediated through BK_{Ca} channels. More interestingly, these results suggested that in GH_3 cells, a 30-msec step to 0 mV before the +50 mV test pulse could produce the increased Ca^{2+} influx through voltage-dependent Ca^{2+} channels and result in a substantial activation of $I_{K(Ca)}$ observed at the level of +50 mV.

To further test the possibility that the modulation of $I_{K(Ca)}$ results from Ca^{2+} influx through L-type Ca^{2+} channel, various conditioning pulses were designed and delivered before a 1-sec long-lasting pulse voltage was applied to evoke $I_{K(Ca)}$. As shown in Fig. 2A, the cell was depolarized from –50 mV to various potentials that ranged from –40 to +40 mV in 20-mV increments with a duration of 30 msec. After these conditioning pulse, a 1-sec depolarizing pulse to +50 mV was then used to

measure a family of $I_{K(Ca)}$. $I_{K(Ca)}$ elicited by these depolarizing pulses was significantly enhanced when extracellular Ca^{2+} was increased to 3.6 mM. The presence of 3.6 mM extracellular Ca^{2+} increased outward currents at potentials between –40 and +50 mV, with a maximal effect near 0 mV.

When the difference between the two currents recorded at the same level of the conditioning pulse was taken, $I_{Ca,L}$ and $I_{K(Ca)}$ could be integrated over the depolarizing pulses. Fig. 2B displays the digitally subtracted current plotted as a function of conditioning pulse. Integrated $I_{K(Ca)}$ was evident between –40 and +50 mV, with a maximal effect near +0 mV. Likewise, when the plot of integrated $I_{Ca,L}$ as a function of membrane potential (i.e., the prepulse potential) was taken, a bell-shaped relationship was obtained (Fig. 2C). Indeed, when the plot of integrated $I_{Ca,L}$ as a function of integrated $I_{K(Ca)}$ was constructed, a linear relationship with a coupling ratio (i.e., slope) of 69 ± 7 (n = 10) could be found (Fig. 3). These results reveal that the voltage dependency of this Ca^{2+}-dependent bell-shaped relationship of integrated $I_{K(Ca)}$ coincides with the voltage dependency of Ca^{2+} entry through L-type Ca^{2+} channels expressed in GH_3 cells.

Various agents that can modify $I_{Ca,L}$, $I_{K(Ca)}$, or both, were also used to examine their effects on the coupling ratio. Table 1 provides a summary of the coupling ratios obtained under control conditions and after the exposure of various agents. Nimodipine, Bay K 8644 and apamin caused no effect on the values of the coupling ratio. Apamin is known to be a blocker of small-conductance Ca^{2+}-activated K^+ channel. Nimodipine and Bay K 8644 are the blocker and opener of $I_{Ca,L}$, respectively. Thus, changes in the magnitude of $I_{K(Ca)}$ could be concomitantly associated with the alteration of $I_{Ca,L}$, because there was no change in coupling ratio in the presence of nimodipine or Bay K 8644. However, the presence of tetrandrine was noted to reduce the value of coupling ratio. It was previously reported that tetrandrine can suppress the amplitude of $I_{Ca,L}$ and the activity of BK_{Ca} channels (21, 23). Paxilline, an inhibitor of BK_{Ca} channels, also caused a reduction in the value of coupling ratio. Therefore, it is possible that BK_{Ca} channels are predominantly activated in response to an increase in intracellular Ca^{2+} induced by the activation of $I_{Ca,L}$. When the cells were dialyzed with a high concentration (10 mM) of EGTA, the coupling ratio of integrated $I_{Ca,L}$ and $I_{K(Ca)}$ was reduced. These results could be due to the fact that changes in the level of intracellular Ca^{2+} can significantly influence the magnitude of $I_{K(Ca)}$. Indeed, thapsigargin, an agent

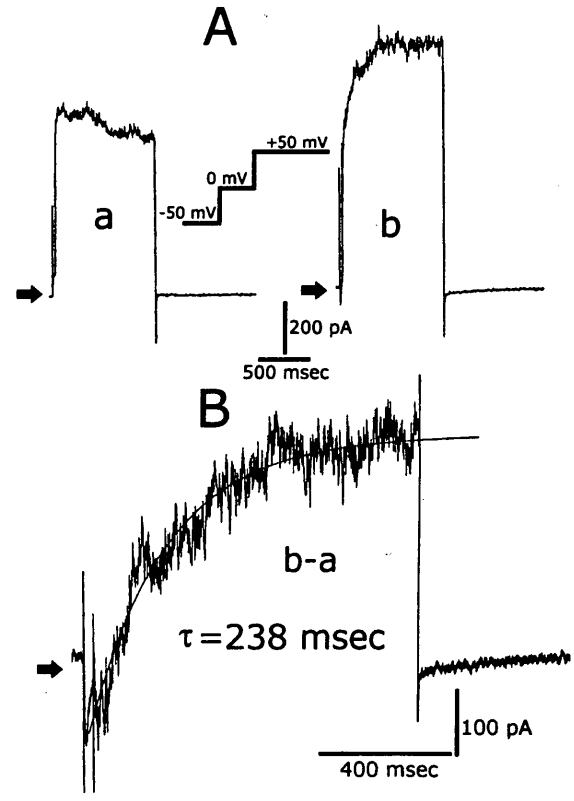

Fig. 1. Depolarizing stimuli-induced Ca^{2+}-activated K^+ current ($I_{K(Ca)}$) in GH_3 cells. Cells were bathed in normal Tyrode's solution. A two-step voltage pulse was used in this experiment. The cell was held at the level of –50 mV, and a 30-msec step to 0 mV before the +50 mV voltage pulse produces Ca^{2+} influx through voltage-dependent Ca^{2+} channels and subsequently activates $I_{K(Ca)}$. In panel A, labeled a and b were obtained in the presence of 1.8 and 3.6 mM $CaCl_2$, respectively. Inset shown in panel A indicates the voltage protocol. The time course of current rise shown in panel (B) was well fitted by a single exponential with a value (τ) of 238 msec. Arrows indicate the zero current level. Of note, there is a small inward current that is considered as a Ca^{2+} current.

that induces Ca^{2+} release from inositol triphosphate-sensitive stores, was found to increase the value of coupling ratio. Interestingly, phorbol 12-myristate 13-acetate (PMA), which is an activator of protein kinase C, also caused a reduction of this value.

Discussion

The important finding of this study is that $I_{K(Ca)}$ is large and can be activated by depolarizing stimuli which activate voltage-dependent L-type Ca^{2+} currents ($I_{Ca,L}$) and hence induce Ca^{2+} influx in pituitary GH_3 cells. $I_{Ca,L}$ and $I_{K(Ca)}$ were found to be tightly co-regulated in the same cell. Elevation of extracellular Ca^{2+} from 1.8 to 3.

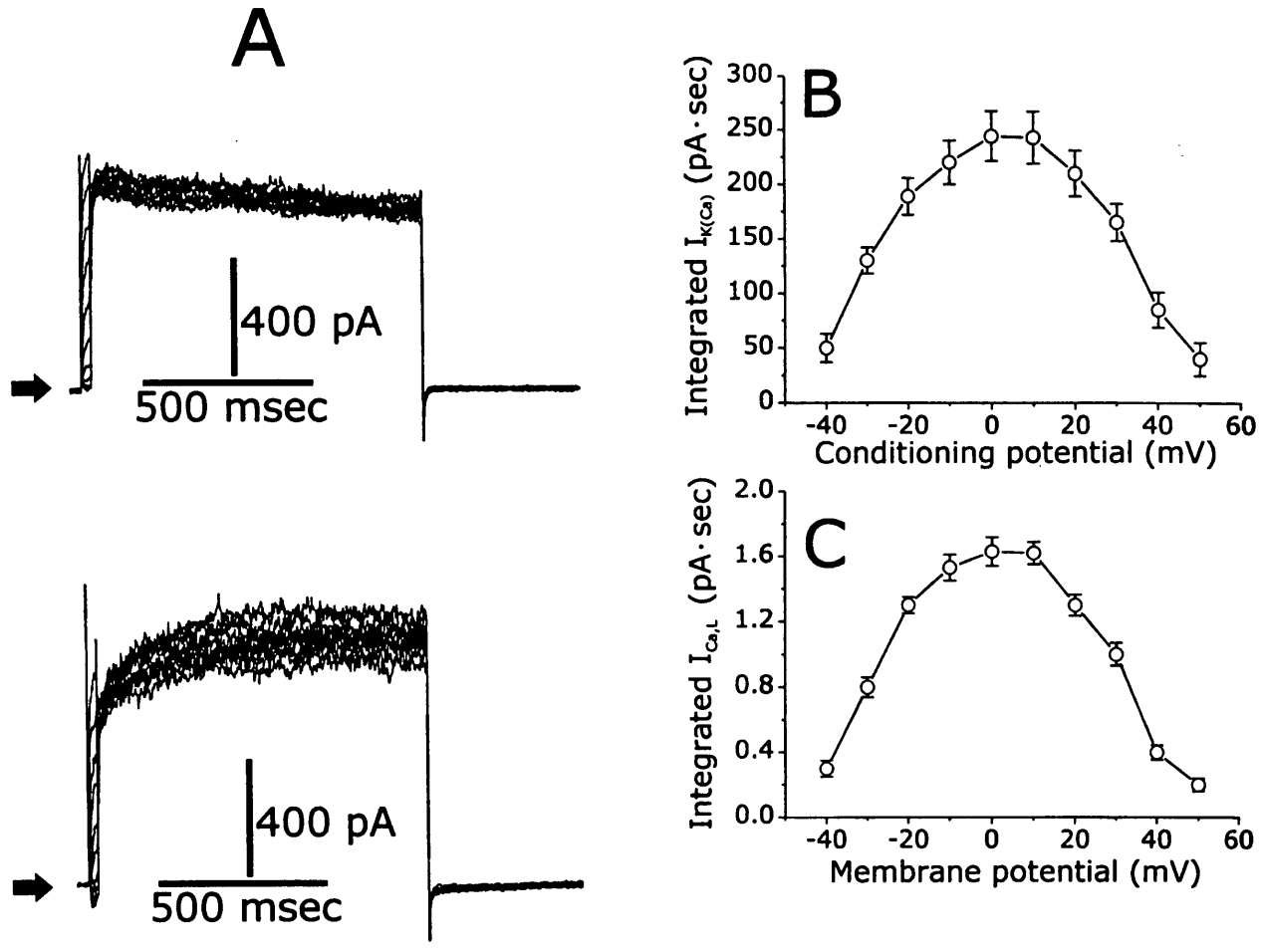

Fig. 2. Relationship of voltage-dependent L-type Ca^{2+} currents and Ca^{2+}-activated K^+ currents in GH_3 cells. The cell was held at the level of –50 mV. Various voltage pulses ranging from –40 to +50 mV in 10-mV increments was applied with a duration of 100 msec, before being stepped to +50 mV for 1 sec. Panel A shows superimposed current traces obtained in the presence of 1.8 (upper part) and 3.6 (lower part) mM $CaCl_2$. Panel (B) shows the relationship of conditioning pulses versus integrated $I_{K(Ca)}$. Panel (C) shows the relationship of membrane voltage versus integrated $I_{Ca,L}$. Each bar represents the mean ± SEM (n = 5-8).

6 mM could modulate both amplitude and kinetics of $I_{K(Ca)}$. The present study suggests that the intracellular Ca^{2+} may not be distributed homogeneously, because GH_3 cells always exhibited spontaneous action potentials (11, 17). Because firing pattern is voltage dependent, increase in $I_{K(Ca)}$ may have a protective effect by forcing the membrane potential towards hyperpolarized levels. As a result, prolactin secretion in these cells would be attenuated (8).

The evidence that the amplitude of $I_{K(Ca)}$ induced by an increase in intracellular Ca^{2+} concentration due to the activation of $I_{Ca,L}$ is supported by the following findings: a) elevation of extracellular Ca^{2+} produced enough intracellular Ca^{2+} to cause significant increase in the amplitude of $I_{K(Ca)}$, and b) the bell-shaped curves for integrated $I_{K(Ca)}$ versus conditioning potential and $I_{Ca,L}$ versus membrane potential were superimposable. However, the amplitude of $I_{K(Ca)}$ increased by the activation of $I_{Ca,L}$ was unaffected by apamin, but by paxilline, suggesting that the activity of BK_{Ca} channels was primarily responsible for the activation of $I_{K(Ca)}$ caused by the depolarizing stimuli that induced Ca^{2+} influx through Ca^{2+} channels. Indeed, the coupling ratio of integrated $I_{Ca,L}$ and $I_{K(Ca)}$ was not modified in the presence of apamin, a blocker of small-conductance Ca^{2+}-activated K^+ channels.

It has been shown that T-type Ca^{2+} channel may be functionally coupled to BK_{Ca} channel (10). However, in our study, $I_{K(Ca)}$ in response to depolarizing stimuli was not affected in the presence of $NiCl_2$ (100 μM) (data not shown), although Bay K 8644 effectively increased the amplitude of $I_{K(Ca)}$. In addition, changes in membrane

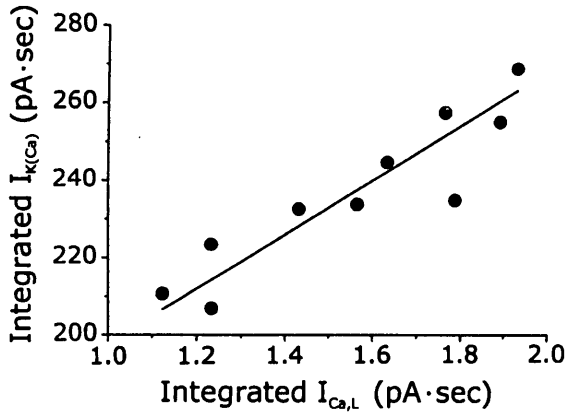

Fig. 3 The linear relationship of integrated $I_{Ca,L}$ versus integrated $I_{K(Ca)}$ for 10 cells with r = 0.91 and slope = 69. Notably, integrated $I_{Ca,L}$ was each correlated with integrated $I_{K(Ca)}$.

Table 1. Summary of Data Showing Influence of Various Agents on the Coupling Ratio of Integrated ICa,L and IK(Ca) in GH3 Cells

	n	Coupling Ratio
Control	10	69 ± 7
Nifedipine (1μM)	6	68 ± 8
Bay K 8644 (1μM)	5	69 ± 9
Apamin (200 nM)	5	68 ± 9
Intracellular dialysis with EGTA (10 mM)	6	16 ± 5*
Thapsigargin (1μM)	5	75 ± 7*
Paxilline (1 μM)	6	21 ± 8*
PMA (5 μM)	6	25 ± 7*

All values are mean ± SEM. PMA: phorbol 12-myristate 13-acetate. n: the number of cells examined. *Values significantly different ($P<0.05$) from control.

potential used in this study were known to fall within a range in which T-type Ca^{2+} current would have been inactivated. It is thus unlikely that T-type Ca^{2+} channel predominantly contributes to the activation of $I_{K(Ca)}$ observed in this study.

In the present study, a coupling ratio of integrated $I_{K(Ca)}$ and $I_{Ca,L}$ with a value of about 69 was obtained, suggesting that a feedback modulation of these currents, i.e., $I_{K(Ca)}$ and $I_{Ca,L}$, might exist. This result is compatible with the fact that the regulation of $I_{K(Ca)}$ plays a role in maintaining the hyperpolarizing state and thus determining firing properties of GH_3 cells in response to depolarizing stimuli (16). The input resistance in these cells was high in the potential range examined in this study (22). It is thus of importance to note that if very small $I_{Ca,L}$ can have such influence on $I_{K(Ca)}$ and there are more BK_{Ca} channels than $I_{Ca,L}$ channels in GH_3 cells, it is possible that the much larger $I_{K(Ca)}$ can summate to have greater effect on membrane potential and thereby strongly influence surrounding channels. On the other hand, no change in the value of coupling ratio was found in the presence of nimodipine or Bay K 8644, although the amplitude of $I_{Ca,L}$ can be affected by these compounds. These results can be interpreted to mean that there appears to be a tight coupling of these currents (i.e., $I_{Ca,L}$ and $I_{K(Ca)}$). However, tetrandrine was found to reduce the value. This result could be due to the possibility that tetrandrine not only blocks $I_{Ca,L}$, but also suppresses the BK_{Ca} channel activity (21, 23). The finding that paxilline decreased the value of coupling ratio is in agreement with the earlier reports that these compounds are a potent blocker of BK_{Ca} channels (7, 9).

When the cells were dialyzed with a high concentration (10 mM) of EGTA, it was found that the coupling ratio of integrated $I_{Ca,L}$ and $I_{K(Ca)}$ was significantly reduced. Thus, it is reasonable to expect that change in intracellular Ca^{2+} concentration can influence the coupling efficiency of integrated $I_{Ca,L}$ and $I_{K(Ca)}$. However, thapsigargin, an inhibitor of endoplasmic Ca^{2+}-ATPase, could increase the value of coupling ratio. This result raises the possibility that the depletion of internal stores may be implicated in the regulation of functional coupling of $I_{Ca,L}$ and $I_{K(Ca)}$. Indeed, a previous report showed that activation of sarcolemmal Ca^{2+}-activated K^+ channels was induced by a rise in intracellular Ca^{2+} elicited by Ca^{2+} release from sarcoplasmic reticulum (6). Furthermore, PMA, an activator of protein kinase C, significantly reduced the value of coupling ratio. The activation of protein kinase C in GH_4C_1 cells was previously reported to suppress the BK_{Ca} channel activity (18). Taken together, the results lead us to propose that the coupling efficiency of $I_{Ca,L}$ and $I_{K(Ca)}$ be dynamically regulated by second messengers or phosphorylation process inside the cell.

Both the BK_{Ca} and Ca^{2+} channels have been found to be located along the secretory zones, as suggested by immunofluorescent staining (15). In a previous report, Ca^{2+} transients were measured using EFP18 during membrane depolarization-induced Ca^{2+} influx (3). The results revealed that intracellular Ca^{2+} beneath the surface membrane rose quickly and reached millimolar levels at early times (3). It has also been demonstrated that BK_{Ca} channels can be activated by the plume, or domain, of ions entering through closely associated Ca^{2+} channels (2, 4, 10, 12, 13, 19). It is possible that the L-type Ca^{2+}

channels and the BK_{Ca} channels are physically co-localized in GH_3 cells. In other words, the proximity of L-type and BK_{Ca} channels allows BK_{Ca} channels to be activated rapidly by Ca^{2+} influx through neighboring L-type Ca^{2+} channel. Therefore, feedback control of Ca^{2+} channels by BK_{Ca} channels could occur within a few milliseconds by deflecting membrane potential toward the equilibrium potential of K^+ ions, resulting in the closure of the Ca^{2+} channels. This is also in agreement with the physiological role of BK_{Ca} channels, allowing them to be activated during the falling phase of the action potential to cause repolarization of action potential and generation of the fast after-hyperpolarization (20, 24).

In summary, our study provides the evidence of a functional interaction between $I_{Ca,L}$ and $I_{K(Ca)}$ in GH_3 lactotrophs. This interaction is also likely to be the case in the other neuroendocrine cells. The co-regulation of these two currents may shape the duration of action potential in which they could influence Ca^{2+} influx and hormonal release. The localized effect between these ion channels may reflect a more global feedback mechanism that potently controls Ca^{2+} entry and ultimately controls Ca^{2+} homeostasis in the cell.

Acknowledgments

This work was supported by grants from National Science Council (NSC-89-2320-B075B-016) and Kaohsiung Veterans General Hospital (VGHKS-90-06 and VGHKS-90-73) and VTY Joint Research Program, Tsou's Foundation (VTY-89-P3-23), Taiwan.

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