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Modulation of L-type Ca^{2+} Channel Currents by Various Protein Kinase Activators and Inhibitors in Rat Clonal Pituitary GH_3 Cell Line

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L-type Ca^{2+} channels play an important role in regulating cytosolic Ca^{2+} and thereby regulating hormone secretions in neuroendocrine cells. Since hormone secretions are also regulated by various kinds of protein kinases, we investigated the role of some kinase activators and inhibitors in the regulation of the L-type Ca^{2+} channel currents in rat pituitary GH_3 cells using the patch-clamp technique. Phorbol 12,13-dibutyrate (PDBu), a protein kinase C (PKC) activator, and vanadate, a protein tyrosine phosphatase (PTP) inhibitor, increased the Ba^{2+} current through the L-type Ca^{2+} channels. In contrast, bisindolylmaleimide I (BIM I), a PKC inhibitor, and genistein, a protein tyrosine kinase (PTK) inhibitor, suppressed the Ba^{2+} currents. Forskolin, an adenylate cyclase activator, and isobutyl methylxanthine (IBMX), a non-specific phosphodiesterase inhibitor, reduced Ba^{2+} currents. The above results show that the L-type Ca^{2+} channels are activated by PKC and PTK, and inhibited by elevation of cyclic nucleotides such as cAMP. From these results, it is suggested that the regulation of hormone secretion by various kinase activity in GH_3 cells may be attributable, at least in part, to their effect on L-type Ca^{2+} channels.

Key Words: GH_3 cell, L-type Ca^{2+} channels, Protein kinase

INTRODUCTION

The rat clonal pituitary cell line, GH_3, which was derived from a rat anterior pituitary tumor, has properties of somatomammotrophs (Tashjian et al, 1968), and GH_3 cells secrete both prolactin and growth hormone spontaneously and in response to thyrotropin-releasing hormone (TRH). An increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) due to influx through L-type Ca^{2+} channels, together with release from the intracellular Ca^{2+} store, has been regarded as an important mechanism for the secretion of the hormones (Albert et al, 1984; Aizawa et al, 1985; Gershengorn et al, 1986; Imai & Gershengorn, 1986; Cohen et al, 1988; Hescheler et al, 1988; Mollard et al, 1988; Rosenthal et al, 1988; Kuan et al, 1990; Mollard, 1990; Gollasch et al, 1991; Shorte & Schofield, 1991; Thorn & Petersen, 1991; Tornquist, 1991; Suzuki et al, 1992). Among the Ca^{2+} channels present in the GH_3 cells, L-type Ca^{2+} channels seem to contribute most largely to the influx of Ca^{2+} (Gershengorn, 1986; Gollasch et al, 1991, 1993).

It was suggested that various important physiological stimuli to GH_3 cells, such as TRH, vasopressin or angiotensin, exert their actions by the modulation of various protein kinase activities (Aizawa & Hinkle, 1985; Gershengorn, 1986; Bjoro et al, 1990; Gollasch et al, 1991, 1993). However, the modulation of the L-type Ca^{2+} channels by protein kinases has not been fully elucidated in GH_3 cells. In the present study, we have investigated the effect of phosphorylation by PKC, tyrosine kinase, and alteration of cyclic nucleotide level on the L-type Ca^{2+} channel current. We show that both the phosphorylation of L-type Ca^{2+} channels by PKC and tyrosine kinase increase the Ca^{2+}

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channel activity, whereas the increase in cyclic nucleotides such as cAMP and thus PKA suppresses the L-type Ca^{2+} channel currents.

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 passage between 18 and 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). The extracellular solution in the perfusion chamber contained (in mM): NaCl, 143; KCl, 5.4; NaH_2PO_4, 0.33; $BaCl_2$, 10; $MgCl_2$, 0.5; 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 5; glucose 11; adjusted to pH 7.4 with NaOH. In many cases, NaCl in the perfusing solution was substituted with NMDG-Cl to ensure elimination of Na^+-dependent currents. The patch pipettes were filled with a solution containing (in mM): CsCl, 120; HEPES, 10; ethyleneglycol-bis (β-aminoethylether) N, N, N', N'-tetraacetic acid (EGTA), 10; Mg-ATP 5; $MgCl_2$, 1 and pH was adjusted to 7.2 by CsOH.

Electrophysiological experiments were performed with the use of Axopatch-1D amplifier and pCLAMP software package from Axon Instruments (Foster City, CA). Ba^{2+} currents were elicited by depolarizing voltage steps from the holding potentials of −80 or −40 mV. Membrane currents were digitized online (1~2 kHz) being low-pass filtered by cut-off frequency of 1~5 kHz and stored in a computer. The patch pipettes were pulled from borosilicate capillaries (Clark Electromedical Instruments, Pangbourne, UK) using a Narishige puller (PP-83, Japan). We used patch pipettes with a resistance of 3~5 MΩ when filled with above pipette solutions. Results are shown as the mean±standard error of the mean. Student's t-test was used for the test of significance. A value of $p<0.05$ was considered to be statistically significant. All experiments were carried out at room temperature (22~25°C).

Drugs

Unless otherwise stated, all the chemicals were purchased from Sigma (MO, USA). Genistein, bay K 8644, and bisindolylmaleimide I (BIM I) were purchased from RBI (MA, USA). Forskolin, phorbol 12, 13-dibutyrate (PDBu), and isobutyl methylxanthine (IBMX) were purchased from Biomol (PA, USA). Nicardipine, genistein, bay K 8644, forskolin, BIM I, IBMX and PDBu were dissolved in dimethyl sulfoxide (DMSO) to yield stock solution and diluted in the extracellular bathing solution on the day of experiment. The final concentrations of the DMSO were always less than 0.1% and were without effect on the Ba^{2+} current.

RESULTS

Recording of L-type Ca^{2+} channel currents in GH_3 cells

Ba^{2+} currents through the Ca^{2+} channels were recorded by means of the whole-cell configuration of the patch-clamp technique (Hamill et al, 1981). From the holding potential of −80 mV, depolarizing step pulses above −40 mV elicited large inward Ba^{2+} currents. The current-voltage (I-V) relationship of the recorded Ba^{2+} current was bell-shaped and peaked around −10 mV (Fig. 1). The currents elicited by 700 ms-duration of step depolarization were composed of a relatively small portion of fast-inactivating component and large amplitude of less-inactivating component (inactivation usually less than 10%). Nicardipine (1 μM), a selective L-type Ca^{2+} channel inhibitor greatly suppressed the current, leaving only fast-inactivating component (Fig. 1B). The fast-

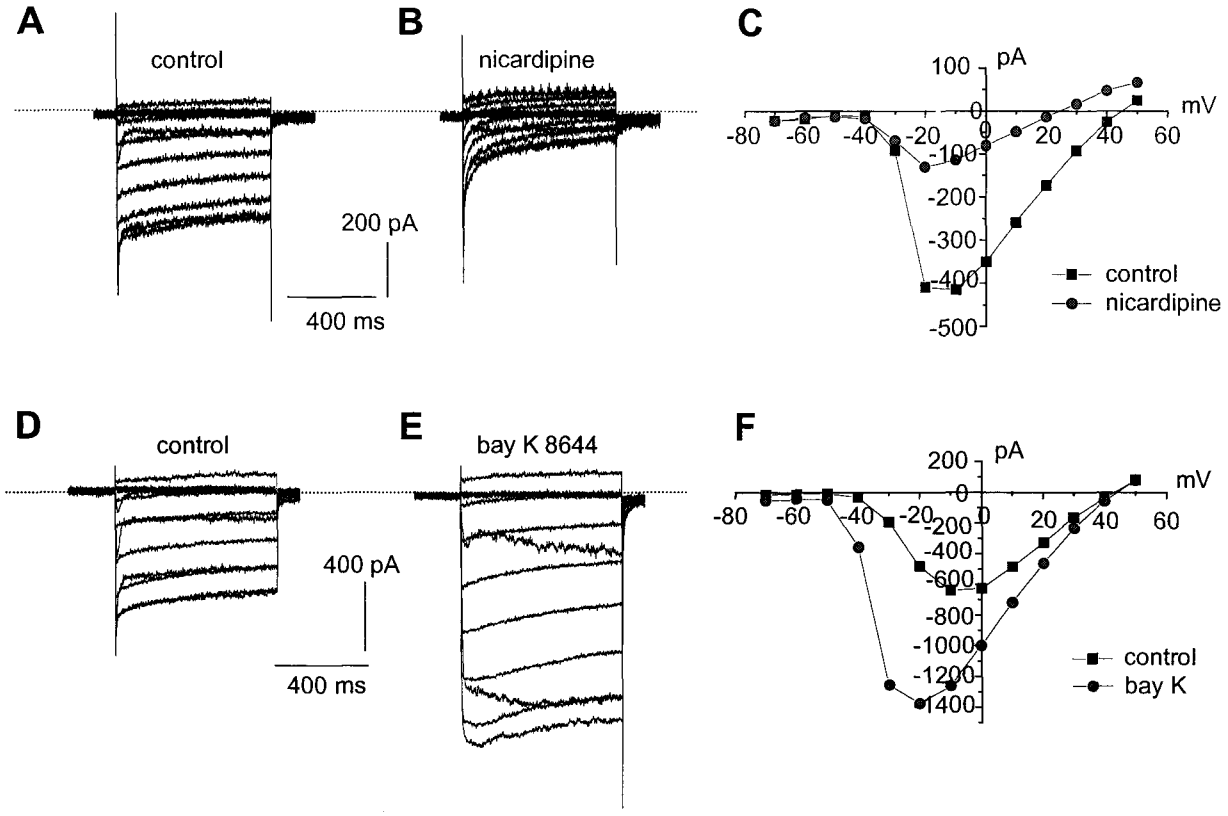

Fig. 1. Recordings of voltage-dependent Ba^{2+} currents. For recording of Ca^{2+} channel currents, the cells were dialyzed with Cs^{+}-rich pipette solution and bathed with modified Tyrode solution containing 10 mM $BaCl_2$. To elicit inward Ba^{2+} current through Ca^{2+} channels, step depolarizations were applied from the holding potential of −80 mV. 1 μM nicardipine greatly inhibited the inward Ba^{2+} current (A and B), whereas Bay K 8644 (1 μM) greatly increased the current and caused the left-shift of current-voltage (I-V) relationship (D-F). The I-V curve was drawn from the steady-state value of the currents. The dotted lines represent zero-current level. Results from upper and lower panels are from different cells.

inactivating component was not recorded either when extracellular Na^{+} was substituted with membrane-impermeable $NMDG^{+}$ or when holding potential was −40 mV (Fig. 2A). Application of the bay-K 8644, an L-type Ca^{2+} channel activator (1 μM), increased the inward Ba^{2+} current more than two-folds and shifted the I-V relationship to the left. These properties suggest the presence of a large population of L-type Ca^{2+} channels in GH_3 cells and they just slightly inactivate during depolarization. To ensure studying on modulation of L-type Ca^{2+} channels least contaminated with other type of ion channels, we usually used Na^{+}-free bathing solution and compared the steady-state I-V relationships. In some cases, holding potentials were −40 mV to completely inactivate the T-type Ca^{2+} channel current, though Gollasch et al (1991, 1993) reported that holding potential of −80 mV was sufficient for the inactivation of these channels.

Effect of PKC inhibitor and activator

Since most of the secretagogues including TRH act via the activation of G-protein coupled phospholipase C (PLC) and subsequent release of inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG), the modulation of L-type Ca^{2+} channels by the PKC can be very important in the regulation of hormone secretion in GH_3 cell. Previous studies suggest that the sustained activation of the L-type Ca^{2+} channels is important mechanism of the prolactin and growth hormone secretion by TRH (Albert & Tashjian, 1984; Dubinsky & Oxford, 1985; Gershengorn, 1986; Mollard et al, 1990). To explore the relationship between

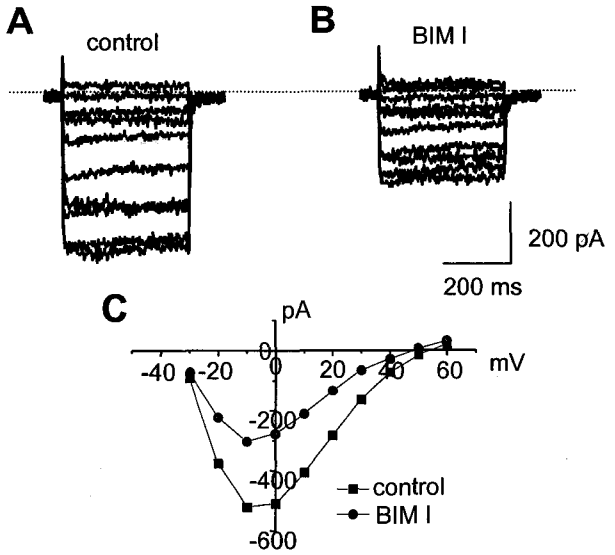

Fig. 2. Effect of bisindolylmaleimide I (BIM I), a selective PKC inhibitor. The current were recorded under Na^+-free condition at holding potential of −40 mV to ensure elimination of Na^+-dependent currents and T-type Ca^{2+} channel currents. Ba^{2+} currents were elicited by applying 350-ms depolarizing step pulses from −30 to +60 mV. Bath application of the BIM I (1 μM) caused the inhibition of the inward Ba^{2+} currents at all tested potentials. Dotted line denotes the zero-current level. Panel C shows the I-V relationship for the BIM I-induced inhibition of the voltage-dependent Ba^{2+} currents.

PKC and Ca^{2+} channel activity, we applied bisindolylmaleimide I (BIM I, 1 μM), a selective PKC inhibitor. At all test potentials, BIM I suppressed the inward Ba^{2+} current (Fig. 2). This result suggests that activation of the PKC might increase the L-type Ca^{2+} channels. Consistent with this expectation, the PKC activator phorbol 12,13-dibutyrate (PDBu, 100 nM), increased the Ba^{2+} currents (Fig. 3). Though the increasing effect of the PDBu on the Ba^{2+} current was not large, it was significant ($p<0.05$; see Fig. 7). These results indicate that the activation of PKC, which is in the downstream of the TRH-mediated signal transduction, can contribute to the sustained increase of the cytosolic Ca^{2+} and hormone secretion in GH_3 cells.

Effect of protein-tyrosine kinase (PTK) inhibitor and protein-tyrosine phosphatase (PTP) inhibitor

Next we tested the effect of tyrosine phosphorylation on the L-type Ca^{2+} channels. The phosphorylation of the tyrosine is thought to be important together with the phosphorylation of serine/threonine (Cataldi et al, 1996) in the regulation of the Ca^{2+}

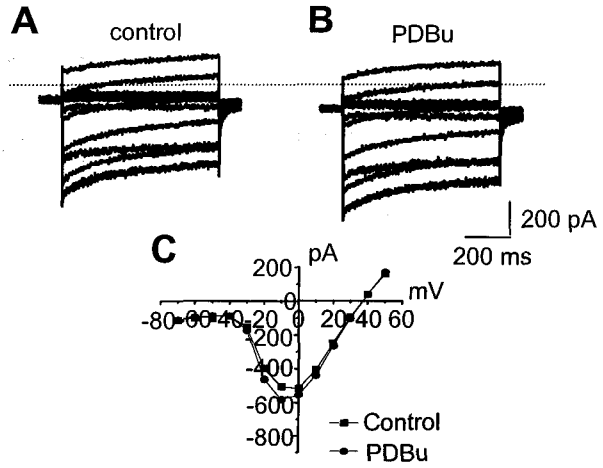

Fig. 3. Effect of phorbol 12, 13 dibutyrate (PDBu), a selective PKC activator. The holding potential was −80 mV, and 700-ms depolarizing steps from −70 to +50 mV were applied. The data are shown without leak correction. Panel C shows the I-V relationship for the control and PDBu (100 nM)-treated Ba^{2+} currents.

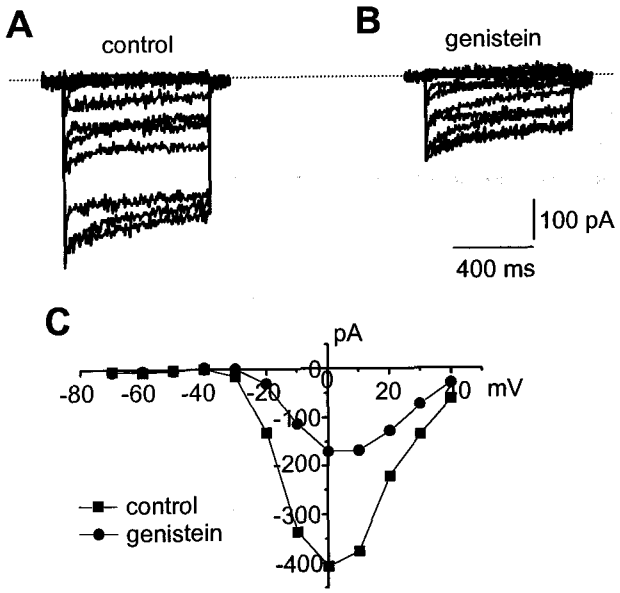

Fig. 4. Effect of genistein, a selective PTK inhibitor. The holding potential was −80 mV, and 700-ms depolarizing steps from −70 to +50 mV were applied. Superfusion of genistein-containing bath solution caused inhibition of the Ba^{2+} currents (A and B). Panel C shows the I-V relationship for the genistein (50 μM)-induced inhibition of the voltage-dependent Ba^{2+} currents.

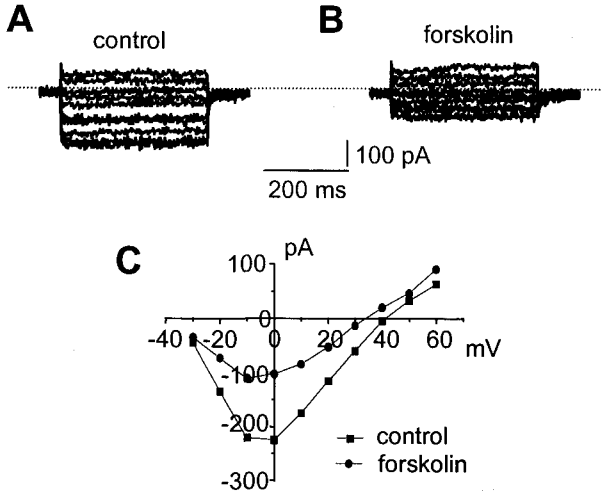

Fig. 5. Effect of forskolin, an adenylate cyclase activator. The holding potential was −40 mV, and 350-ms depolarizing step pulses from −30 mV to +60 mV were delivered to elicit inward Ba^{2+} currents. Forskolin (10 μM) induced the inhibition of the Ba^{2+} currents at all tested potentials. Panel C shows the I-V relationship of the forskolin-induced inhibition of the voltage-dependent Ba^{2+} currents.

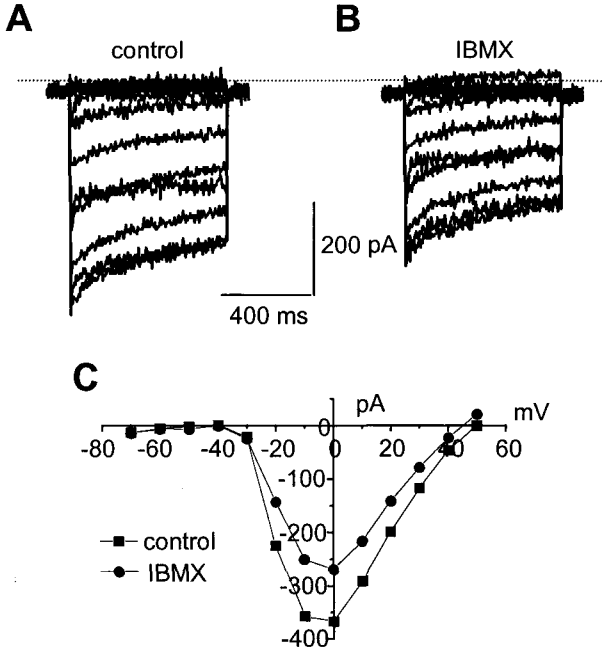

Fig. 6. Effect of isobutyl methylxanthine (IBMX), a non-specific phosphodiesterase inhibitor. The holding potential was −80 mV, and 700-ms depolarizing steps from −70 to +50 mV were applied. IBMX (100 μM) caused the inhibition of the Ba^{2+} currents at all tested potentials. Panel C shows the I-V relationship of the IBMX-induced inhibition of the voltage-dependent Ba^{2+} currents.

channel activity. Genistein (50 μM), which inhibits PTKs by competing with ATP for the binding on these enzymes, caused distinct inhibition of the Ba^{2+} current at all tested potentials (Fig. 4). By contrast, when the PTP inhibitor vanadate (100 μM) was applied to the bathing solution, the Ba^{2+} currents slightly increased (not shown). These results suggest that the phosphorylation of the tyrosine can activate the L-type Ca^{2+} channels in GH_3 cells.

Effect of activator of adenylate cyclase and phosphodiesterase inhibitor

The modulation of L-type Ca^{2+} channel by protein kinase A (PKA) is well-known (Curtis & Catterall, 1985). Usually the PKA-dependent serine/threonine phosphorylation of the L-type Ca^{2+} channel is thought to be important in the normal voltage-dependent activation of the L-type Ca^{2+} channel (Armstrong & Eckert, 1987; Kamp & Hell, 2000). We tested the effect of elevation of the cAMP, which may activate PKA, by applying an adenylate cyclase activator, forskolin (10 μM). Forskolin significantly suppressed the Ba^{2+} currents at all potentials tested (Fig. 5). To further confirm that the effect of forskolin was through the elevation of the cyclic nucleotide, we tested the effect of isobutyl methylxanthine (IBMX,

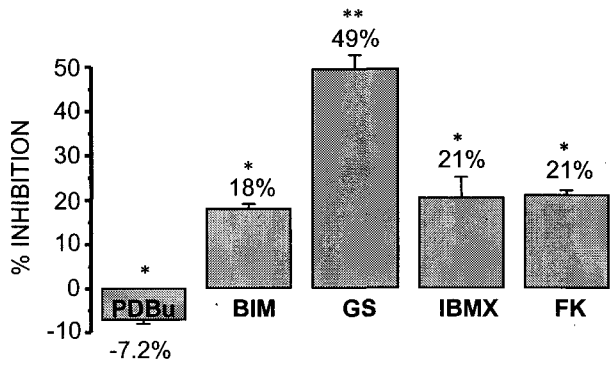

Fig. 7. Summary of the kinase activator and inhibitors on the voltage-dependent Ba^{2+} currents. The figure shows the percent inhibition Ba^{2+} currents at 0 mV by PDBu (100 nM), BIM I (1 μM), genistein (GS; 50 μM), IBMX (100 μM), and forskolin (FK; 10 μM). The negative sigh of value denotes increase of the Ba^{2+} currents (PDBu). Each point is the mean±S.E. of at 3~10 separate experiments. *represents $p<0.05$ and **$p<0.01$.

100 μM), a non-selective phosphodiesterase inhibitor on the Ba^{2+} currents. The inhibitory effect of the forskolin was mimicked by IBMX (Fig. 6). The re-

sults indicate that the increase in cyclic nucleotides such as cAMP may suppress the L-type Ca^{2+} channel currents in GH_3 cells.

The results of the present study are summarized and shown in Fig. 7. The results shown in Fig. 7 are averages of 3~10 cells for each case. Increase in the phosphorylation activity by PKC and PTK slightly increased the L-type Ca^{2+} channels (result for vanadate not shown), whereas inhibitors of these kinases distinctly suppressed the currents. The elevation of the cyclic nucleotide including cAMP by forskolin and IBMX inhibited the L-type Ca^{2+} channel current, indicating the cyclic nucleotide-dependent protein kinases such as PKA might suppress the Ba^{2+} currents. Genistein, a PTK inhibitor was most potent in suppressing the Ca^{2+} channel currents among the drugs tested in this study.

DISCUSSION

The results of the present study demonstrate that the Ca^{2+} channel currents in GH_3 cells can be modulated by PKC, PTK and possibly by PKA. PKC and PTK increased the Ba^{2+} currents through the L-type Ca^{2+} channels but elevation of the cyclic nucleotide by forskolin or IBMX inhibited the currents.

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. Stimulation of prolactin release by TRH in GH_3 cells is biphasic and linked to similar biphasic changes in electrical activity, consisting of an initial phase of transient hyperpolarization and a sustained increase in action potential frequency (Gershengorn, 1986; Bjoro et al, 1990). The initial hyperpolarizing phase is due to the IP_3-dependent Ca^{2+} release and subsequent activation of Ca^{2+}-activated K^+ channels (Dubinsky & Oxford, 1985; Lang & Ritchie, 1987). The initial phase is followed by a sustained stimulation of the Ca^{2+} influx (Gershengorn, 1986; Cohen et al, 1988; Hescheler et al, 1988; Rosenthal et al, 1988; Gollasch et al, 1991). Though the nature of this retarded and prolonged Ca^{2+} entry is not clearly known, activation of the voltage-dependent L-type Ca^{2+} channels were suggested to be involved (Aizawa et al, 1985; Gershengorn, 1986; Gollash et al, 1993). However, the effect of TRH on the Ca^{2+} channel itself is somewhat complicated. Gollash et al (1991) reported that the TRH caused opposite effect on Ca^{2+} channel currents by two separate pathways: Ca^{2+}-induced inhibition and direct stimulation. The stimulatory effect of TRH on the Ca^{2+} channel currents was shown to require protein kinase C and the G-protein (G_{i2}), which is different from that for IP_3 receptor activation and Ca^{2+} mobilization (Gollash et al, 1993). The result of the present investigation, however, shows that PKC activation alone can increase L-type Ca^{2+} channel currents, suggesting that activation of G_{i2} may not be necessarily required for the facilitation of L-type Ca^{2+} channel by TRH.

Our results also show that PTK activation can influence the L-type Ca^{2+} channel current. Since the inhibition of the Ba^{2+} current by genistein was most distinct of all the tested kinase modulators (Fig. 7), the regulation of the Ca^{2+} channel activity by PTK activity might significantly contribute to GH_3 cell function in physiologic condition. The results of the PTK activation and inhibition of the present study are consistent with those of Cataldi et al (1996), which also clearly demonstrated the activating effect of PTK on the L-type Ca^{2+} channels.

Generally speaking, the phosphorylation by PKA is thought to be indispensable for the normal voltage-dependent activation of the L-type Ca^{2+} channels (Armstrong & Eckert, 1987). The results of the present study, however, show that both forskolin, an adenylate cyclase activator and IBMX, a non-specific phosphodiesterase inhibitor suppressed the Ba^{2+} currents, indicating activation of cyclic-nucleotide dependent protein kinases including PKA probably inhibit the L-type Ca^{2+} channel currents in GH_3 cells. However, there are still some possibilities that the cyclic nucleotide directly inhibited the channels independent from the kinase activation. For the understanding of the more precise mechanism of the forskolin and IBMX-induced inhibition of the Ca^{2+} channel, further study using more specific drugs for separate cyclic nucleotide-dependent kinase activator/inhibitor is required.

In conclusion, the L-type Ca^{2+} channels are targets for the phosphorylation by PKC, PTK, and possibly PKA. Since the L-type Ca^{2+} channels seem to play an indispensable role in the regulation of the intracellular Ca^{2+} concentration and hormone secretion in GH_3 cells, these modulations of the L-type Ca^{2+} channels by protein phosphorylation probably offer some mechanisms for the regulation of GH_3 cell func-

tion by kinase under various physiologic conditions.

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