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European Journal of Pharmacology 345 (1998) 309–314

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Inhibition of Ca^{2+} current in neonatal and adult rat ventricular myocytes by the tyrosine kinase inhibitor, genistein

Yasuhiro Katsube [a,b], Hisashi Yokoshiki [b], Lam Nguyen [b], Masao Yamamoto [a], Nicholas Sperelakis [b,*]

[a] *Department of Pediatrics, Nippon Medical School Hospital, 1-1-5 Sendagi, Bunkyo-ku, Tokyo, 113-0022, Japan*
[b] *Department of Molecular and Cellular Physiology, College of Medicine, University of Cincinnati, PO Box 670576 Cincinnati, OH, 45267-0576, USA*

Received 29 September 1997; revised 22 December 1997; accepted 30 December 1997

Abstract

Yokoshiki et al. (Yokoshiki, H., Sumii, K., Sperelakis, N., 1996. Inhibition of L-type calcium current in rat ventricular cells by the tyrosine kinase inhibitor, genistein and its inactive analog, daidzein. J. Mol. Cell. Cardiol. 28, 807–814) reported that genistein and daidzein inhibited L-type Ca^{2+} current ($I_{Ca(L)}$) in young rat ventricular cells. Therefore, we investigated the developmental differences in the effect of genistein, an inhibitor of tyrosine kinases, on $I_{Ca(L)}$ in freshly-isolated neonatal (3–7 days) and adult (2–5 months) rat ventricular myocytes using whole-cell voltage clamp and single-channel recordings (cell-attached configuration). For whole-cell voltage clamp, $I_{Ca(L)}$ was measured as the peak inward current at a test potential of +10 mV by applying a 300 ms pulse from a holding potential of −40 mV. To isolate $I_{Ca(L)}$, the pipette solution was Cs^+-rich and the bath solution was Na^+-, K^+-free. Ca^{2+} (1.8 mM) was used as charge carrier. Bath application of 100 μM genistein (sufficient for maximal effect) decreased the basal $I_{Ca(L)}$ by 43.3% ($n = 27$) in neonatal cells and by 30.6% ($n = 14$) in adult cells ($P < 0.05$). In the current/voltage relationships, the potential of peak $I_{Ca(L)}$ was shifted to the right by genistein by 8.6 mV in neonatal and by 9.3 mV in adult cells. Genistein produced a shift of the steady-state inactivation curve (to the left) in neonatal cells (from -16.0 ± 3.9 mV to -26.1 ± 4.2 mV; $P < 0.05$) and in adult cells (-15.9 ± 3.2 mV to -22.9 ± 3.3 mV; $P < 0.05$); the slope factor was not affected. For single-channel recordings in cell-attached patches, Ca^{2+} currents were evoked by applying a 150 ms pulse from a holding potential of −40 mV to a test potential of 0 mV. The pipette solution contained 110 mM Ba^{2+} (as charge carrier), and the bath solution contained 150 mM K^+ (to bring resting potential to near zero). Genistein (50 μM) decreased the open probability of the channels from 2.8% to 0.75% ($P < 0.05$) in absence of Bay K 8644, and from 24% to 7.9% ($P < 0.05$) in presence of Bay K 8644; the mean open time and the slope conductance of the currents were not affected. In conclusion, (1) genistein inhibits the basal $I_{Ca(L)}$ in rat ventricular cells and (2) the inhibition of $I_{Ca(L)}$ by genistein is greater in immature cells than in adult cells. © 1998 Elsevier Science B.V.

Keywords: Ca^{2+} current; Tyrosine kinase; Genistein; Heart, neonatal; Voltage clamp, whole-cell; Single-channel recording

1. Introduction

Tyrosine kinases play a role in regulating cellular growth and cell cycle (Shimokado et al., 1995; Glenney, 1992; Fantl et al., 1993). For example, tyrosine kinase activity was demonstrated at some receptors for growth factors, such as epidermal growth factor (Margolis et al., 1989), fibroblast growth factor (Lee et al., 1989), platelet-derived growth factor (Ek et al., 1982), and insulin (Kusuga et al., 1982). In addition, fibroblast growth factor signaling was impaired by tyrosine kinase inhibitors (Hawker and Granger, 1994).

The growth factors regulate the ion channels, not only of smooth muscle cells, but also of cardiomyocytes. For example, Kusaka and Sperelakis (1994) reported that a factor in fetal bovine serum increased the fast Na^+ current ($I_{Na(f)}$) in uterine leiomyosarcoma cells. In cultured neonatal rat ventricular myocytes, Gomez et al. (1994) reported that fetal calf serum containing several growth factors increased both cell capacitance and $I_{Ca(L)}$, and Guo et al. (1995) reported that basic fibroblast growth factor stimulated the transient outward current (I_{to}).

Therefore, tyrosine kinases may regulate, not only cellular growth, but also the activity of some ion channels. It

* Corresponding author. Tel.: +1-513-558-3126; fax: +1-513-558-2668; e-mail: spereln@ucbeh.san.uc.edu

was reported recently that tyrosine kinase inhibitors decreased the contractile force in heart (Taskinen et al., 1994), and affected the ionic currents in cardiomyocytes (Yokoshiki et al., 1996) and in smooth muscle cells (Wijetunge et al., 1992; Minami et al., 1994; Kusaka and Sperelakis, 1995, 1996; Smirnov and Aaronson, 1995). Because daidzein had a similar inhibitory effect on $I_{Ca(L)}$ as genistein, Yokoshiki et al. (1996) suggested that the effect of genistein was not mediated by phosphorylation. Since the rate of cell growth and cell cycling in immature cells is faster than in mature/senescent cells, this suggests that the activity of tyrosine kinase is greater in immature cells. Consistent with this, tyrosine kinase activity declines from embryo/neonate to adult in various tissues including heart of chick (Maher, 1991) and rat (Okada and Nakagawa, 1988). Therefore, the aim of the present study was to test whether tyrosine kinases may regulate the Ca^{2+} channels more strongly in immature than in mature cells.

2. Materials and methods

2.1. Cell preparation

Freshly isolated single cells were prepared from ventricles of neonatal (1–7-day-old) and adult (2–4-month-old) Sprague–Dawley rats. The rats were decapitated under the CO_2 anesthesia, and the hearts were removed and rinsed in oxygenated Tyrode solution, then immersed in Ca^{2+}-free Tyrode solution for 30 min at room temperature. After the spontaneous beatings had ceased, small pieces of the ventricles were enzymatically digested (for 20–40 min in neonatal and for 50 min in adult) in Ca^{2+}-free Tyrode solution (37°C) containing collagenase (0.3–0.5 mg/ml for neonatal and 0.9 mg/ml for adult; Worthington, USA). After incubation, the cells were mechanically dispersed in a modified KB (Kruftbrühe) solution using a Pasteur pipette. The cell suspension was stored in a refrigerator (4°C) until used.

2.2. Solutions and drugs

The normal Tyrode solution contained (in mM): NaCl 143, KCl 5.4, NaH_2PO_4 0.33, $MgCl_2$ 0.5, $CaCl_2$ 1.8, glucose 5.5, and HEPES 5; pH was adjusted to 7.4 with NaOH. The modified KB solution contained (in mM): K-glutamate 50, KOH 20, KCl 40, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 10, EGTA 0.5, and HEPES 10; pH was adjusted to 7.4 with KOH. For whole-cell voltage clamp, the external test solution (Na^+-free and K^+-free) contained (in mM): tetraethylammonium chloride 150, $CaCl_2$ 1.8, $MgCl_2$ 0.5, 4-aminopyridine 3, HEPES 5, and glucose 5.5; pH was adjusted to 7.4 with HCl. The pipette solution contained (in mM): CsOH 110, CsCl 20, L-glutamic acid 110, $MgCl_2$ 3, adenosine triphosphate disodium salt 5, creatine phosphate disodium salt 5, EGTA 10, and HEPES 5; pH was adjusted to 7.2 with CsOH.

For single-channel recordings, the external solution contained (in mM): KCl 150, EGTA 10, HEPES 5, and glucose 5.5 (pH 7.4). The pipette solution contained (in mM): $BaCl_2$ 110, HEPES 5, and tetrodotoxin 0.03; pH was adjusted to 7.4 with trizma base. 1.0 μM Bay K 8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4[2-(trifluoromethyl)phenyl]-3-pyridinecarboxylate methyl ester) was added in the internal and in the external solution when necessary.

Genistein (Sigma) was dissolved in dimethyl sulfoxide to provide a stock solution (100 mM). Maximal concentration of dimethyl sulfoxide (0.1%) did not affect Ca^{2+} currents as described by Yokoshiki et al. (1996).

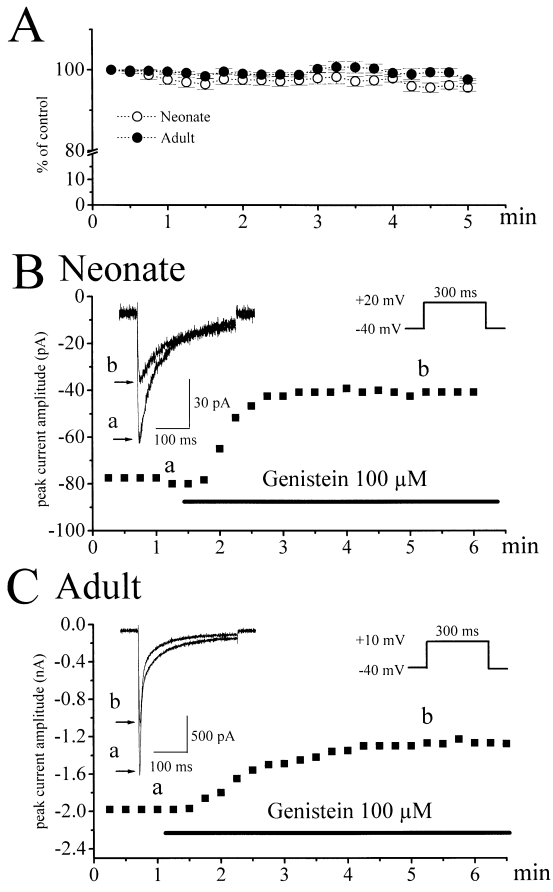

Fig. 1. Time-course of the rundown of Ca^{2+} currents and the effect of genistein on Ca^{2+} currents in neonatal and adult rat ventricular cells. $I_{Ca(L)}$ was measured as the peak current at a test potential of +20 mV in neonatal and +10 mV in adult cells by applying 300 ms duration pulse from a holding potential of −40 mV. A: After $I_{Ca(L)}$ was stabilized, the recording was started. There was no significant difference between neonatal and adult cells during 5 min after the recording was started. B and C: One example of the effect of genistein (100 μM) on 6-day-old neonate (C_m = 21 pF) and adult (C_m = 191 pF) are shown. The application of genistein decreased $I_{Ca(L)}$ by 49% (1Ba = 79 pA, 1Bb = 40 pA) in neonate and by 33% (1Ca = 1976 pA, 1Cb = 1316 pA) in adult. Selective current traces at points denoted are illustrated.

2.3. Whole-cell voltage clamp recordings

Whole-cell voltage clamp recordings were carried out using a patch clamp amplifier (Axopatch-1D, Axon Instruments, USA) and a heat-polished borosilicate glass pipette (World Precision Instruments, USA) with resistances of 2–6 MΩ when filled with the pipette solution. The cell suspension was placed into a small chamber (1.4 ml) on the stage of an inverted microscope (Diaphoto, Nikon, Japan), and perfused at a rate of 1.8 ml/min.

$I_{Ca(L)}$ was elicited from a holding potential of −40 mV to a test potential of +10 mV (or +20, depends on the peak current) for 300 ms (every 15 s). The currents were abolished completely by 2 mM Co^{2+} or 0.5 mM Cd^{2+}, consistent with the current being carried through a Ca^{2+} channel. Current/voltage (I/V) curves were obtained by applying voltage steps in 10 mV increments (from −40 mV to +60 mV) from a holding potential of −40 mV. The steady-state inactivation curves were determined by using a double-pulse protocol; conditioning pulse duration was 5 s and test pulse duration was 300 ms. The pulses were applied to a test potential of +10 mV from various conditioning pulse levels (from −80 mV to +20 mV). There was a 5 ms interval to allow for resetting of the activation gate between the end of conditioning pulse and the beginning of the test pulse (membrane potential returned to a holding potential of −80 mV).

Leak and residual capacitative currents were subtracted using currents elicited by small hyperpolarizing pulses (P/4 protocol). Series resistance was compensated electrically (about 80%). Current and voltage signals were filtered with cut-off frequency of 1 kHz, and digitized by an A/D converter (TL-1, Axon) and sampled at 2.5 kHz. Current and voltage signals were stored and analyzed on an IBM-AT personal computer with a pCLAMP software (Axon). Membrane capacitance was determined by applying ramp pulses (first phase; depolarize to −30 mV from a holding potential of −50 mV, second phase; return to a holding potential) at a rate of 0.8 V/s to avoid interference by any time-dependent ionic currents. Currents recordings were obtained after $I_{Ca(L)}$ became stabilized (usually 5–10 min after breaking the membrane). All experiments were carried out at room temperature (22–24°C).

2.4. Single-channel recordings

Single-channel currents were recorded by the cell-attached configuration. The patch pipettes were coated with Sylgard (Dow Corning, USA) with resistances of 5–12 MΩ. The $I_{Ca(L)}$ currents were elicited from a holding potential of −40 mV to a test potential of 0 mV for 150 ms. Current and voltage signals were filtered with a cut-off frequency of 1 kHz and sampled at 10 kHz every 3 s. The recordings were carried out at room temperature.

2.5. Statistical analysis

All data are presented as mean ± S.E.M. Statistical analyses were performed using Student's paired or un-

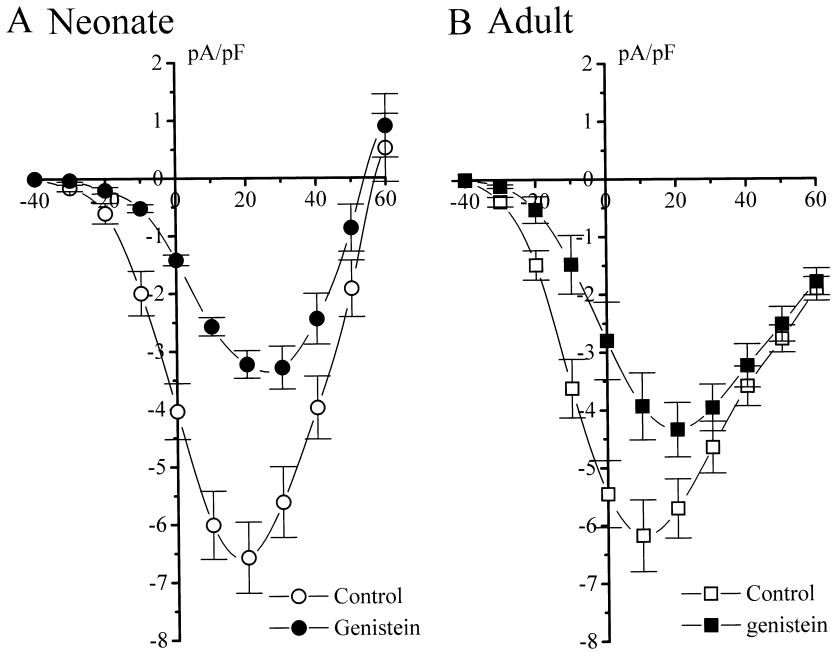

Fig. 2. Current/voltage relationships taken from before (open symbols) and after (closed symbols) application of genistein (100 μM) in neonatal ($n = 7$) and adult ($n = 6$) rat ventricular cells. $I_{Ca(L)}$ was measured as a test potential (from −40 mV to +60 mV) by applying 300 ms duration pulse from a holding potential of −40 mV. Ordinate represents current density (pA/pF) obtained by normalizing the currents to the membrane capacitances. Note that genistein inhibits the $I_{Ca(L)}$ to a greater extent in neonatal cells than in adult cells. The potential for the peak current was shifted to the right by 8.6 mV in neonatal ($P < 0.05$) and by 9.3 mV in adult ($P < 0.05$) cells.

paired *t*-test, and $P < 0.05$ was defined as statistically significant.

3. Results

$I_{Ca(L)}$ in cardiac myocytes decreases (called 'rundown phenomenon') after starting the internal dialysis (Belles et al., 1988). The time-course of the rundown may be faster in neonatal than in adult myocytes because of the smaller cell size. Thus rundown could complicate the results. Therefore, the current was stabilized for 5 min before the drug was added. $I_{Ca(L)}$ in both age groups ($n = 6$ in both neonatal and adult myocytes) showed a slight rundown with almost same time-course, suggesting that rundown had no influence on the results (Fig. 1A).

3.1. Whole-cell voltage clamp

Yokoshiki et al. (1996) reported that genistein inhibited the $I_{Ca(L)}$ in young rat cardiomyocytes in a dose-dependent manner. The maximal inhibition of $I_{Ca(L)}$ was attained at a concentration of 100 μM. Therefore, in the present study, 100 μM genistein was used to obtain the maximal inhibitory effect on $I_{Ca(L)}$. The genistein-induced decrease in $I_{Ca(L)}$ was monophasic, and reached a steady-state at 3–3.5 min in both neonatal and adult cells. The inhibition of $I_{Ca(L)}$ by genistein was 43.3% ($n = 27$) in neonatal cells and 30.6% ($n = 14$) in adult cells. Fig. 1B and C show one example of the time-course of the effect of genistein on $I_{Ca(L)}$ in neonatal and adult myocytes. After $I_{Ca(L)}$ became stabilized, the application of genistein inhibited the current by 49% in neonatal myocyte and by 33% in adult myocyte.

The current/voltage relationships were obtained from neonatal ($n = 7$) and adult ($n = 6$) cells (Fig. 2). The potential giving the peak current was shifted in the positive direction significantly (from 19.1 ± 1.1 mV to 27.7 ± 1.2 mV ($P < 0.05$) in neonatal myocytes and from 9.6 ± 1.2 mV to 18.9 ± 2.6 mV ($P < 0.05$) in adult myocytes).

To investigate the effects of genistein on the voltage-dependence of inactivation, the steady-state inactivation curves were obtained before and after application of genistein from neonatal ($n = 7$) and adult ($n = 6$) cells (Fig. 3). The inactivation curves were obtained by the double-pulse protocol (Fig. 3 inset). The peak current elicited by test pulses (from a prepulse of −80 mV) was normalized and plotted against the conditioning potential. These points were fitted to the Boltzmann equation: $1/\{1 + \exp[(V_m - V_h)/k]\}$, where V_m is the conditioning potential, V_h is the potential required for half-inhibition of current, and k is the slope factor. The V_h was shifted significantly from −16.0 ± 3.9 mV to −26.1 ± 4.2 mV ($P < 0.05$) in neonatal cells, and from −15.9 ± 3.2 mV to −22.9 ± 3.3 mV ($P < 0.05$) in adult cells without affecting the slope factor k. The degree of the shift of V_h was not statistically significant ($P = 0.086$) between neonatal (10.1 ± 1.3 mV) and adult (7.0 ± 1.0 mV) cells.

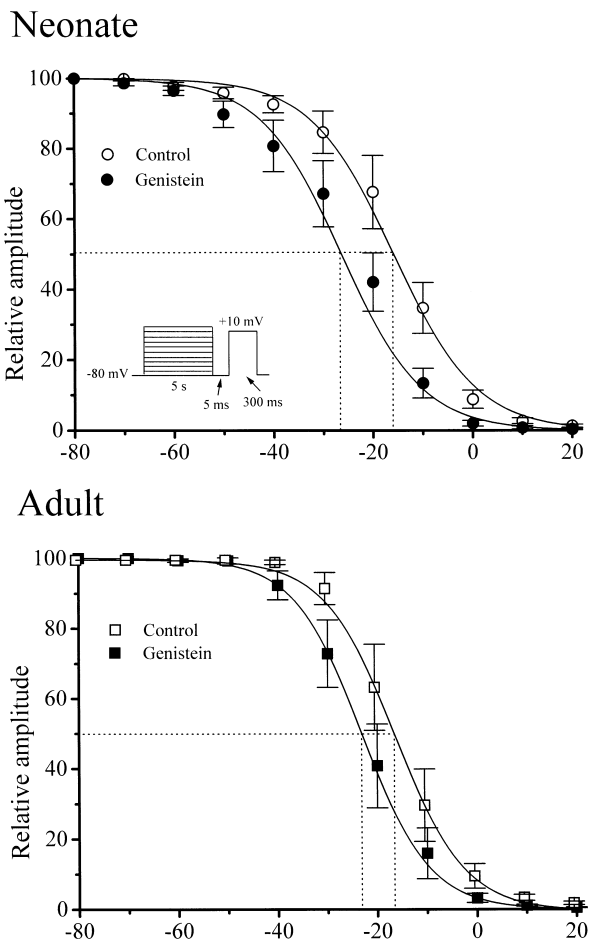

Fig. 3. Steady-state inactivation curves taken from before (open symbols) and after (closed symbols) application of genistein (100 μM) in neonatal ($n = 7$) and adult ($n = 6$) rat ventricular cells. Pulse protocol is given in the inset. In neonatal cells, the value of the half-inactivation potential (V_h) was shifted to the left by 10.13 ± 1.3 mV (from 19.1 ± 1.1 mV to 27.7 ± 1.2 mV) ($P < 0.05$), while the slope factor (k) was not shifted after application of genistein. In adult cells, the V_h was shifted to the left by 6.97 ± 0.98 mV (from 9.6 ± 1.2 mV to 18.9 ± 2.6 mV) ($P < 0.05$), while the value of k was not shifted as well as neonatal cells.

3.2. Single-channel recordings

We investigated the effects of genistein on the kinetic properties of single Ca^{2+} channels. Single-channel recordings from adult cells (in the absence of Bay K 8644) was difficult; therefore, we could use only neonatal cells for the single-channel recordings. Single-channel currents were elicited by applying test potential to 0 mV (150 ms duration) from a holding potential of −40 mV. Slope conductance was obtained by applying various test potentials (from −20 mV to +20 mV in increments of 10 mV) from a holding potential of −40 mV in the presence of 1.0 μM Bay K 8644. Bay K 8644 was used to increase the open probability. Table 1 gives a summary of the data from

Table 1
Summary of the effect of genistein (50 mM) on the properties of single Ca^{2+} channels in neonatal rat ventricular myocytes

		Slope conductance (pS)	P_o (%)	m_o (ms)	m_c (ms)
Without Bay K 8644 ($n = 4$)	Control	–	2.8 ± 0.4	1.2 ± 0.1	5.8 ± 0.5
	Genistein	–	0.75 ± 0.48[a]	1.2 ± 0.1	10.0 ± 2.3
With Bay K 8644 ($n = 3$)	Control	24.0 ± 0.8	24.0 ± 3.8	10.0 ± 2.3	5.2 ± 0.7
	Genistein	24.0 ± 0.3	7.9 ± 0.0[a]	13.0 ± 0.4	5.9 ± 0.3

Mean ± S.E.M.
P_o: open probability, m_o: mean open time, m_c: mean closed time.
A hundred sweeps were obtained from one cell in both absence and presence of genistein.
[a] $P < 0.05$ compared to control.

single-channel recordings. In the absence of Bay K 8644, open probability (P_o), mean open time (m_o) and mean closed time (m_c) of the channels was 2.8%, 1.2 ms and 5.8 ms in control, and 0.75%, 1.2 ms and 10.0 ms after application of genistein. In the presence of Bay K 8644, those values were 24.0%, 10.0 ms and 5.2 ms in control, and 7.9%, 13.0 ms and 5.9 ms after genistein. Genistein decreased P_o without affecting m_o and m_c both in absence and in presence of Bay K 8644. Furthermore, genistein did not affect the slope conductance of the channel. The single-channel conductance was 24 pS both in control and in presence of genistein (Table 1).

4. Discussion

The present study was designed to compare the effect of genistein on $I_{Ca(L)}$ in neonatal and adult rat ventricular myocytes. The major findings of our study are as follows. (1) The tyrosine kinase inhibitor, genistein, inhibited the basal $I_{Ca(L)}$. (2) The inhibitory effect of genistein on $I_{Ca(L)}$ was significantly greater in neonatal than in adult cells. (3) In both groups, the steady-state inactivation curve was shifted to the left and potential of the peak $I_{Ca(L)}$ was shifted to the right. (4) In single-channel recordings, genistein decreased the open probability without affecting the mean open time and slope conductance of the channels.

Slow-type Ca^{2+} channels are regulated by several mechanisms (for review; McDonald et al., 1994; Sperelakis et al., 1996). The phosphorylation by cAMP/protein kinase A (PK-A) is a now well-accepted mechanism for the regulation of $I_{Ca(L)}$ in heart cells (Osterrieder et al., 1982; Irisawa and Kokubun, 1983). Besides cAMP/PK-A regulation, $I_{Ca(L)}$ is regulated by cGMP/PK-G (Sumii and Sperelakis, 1995; Haddad et al., 1995), PK-C (Dösemeci et al., 1988; Lacerda et al., 1988), and Ca^{2+}/calmodulin dependent PK (Bkaily et al., 1984).

The inhibition of $I_{Ca(L)}$ by genistein in young (10–17 days) rat ventricular cells was recently reported (Yokoshiki et al., 1996), but daidzein exerted a similar effect. Genistein inhibited $I_{Ca(L)}$ in a dose-dependent manner, but the maximal inhibition was only about 40%. Genistein shifted the steady-state inactivation curve to the left, while the steady-state activation curve was not shifted. Consistent with their data, in the present study also, genistein shifted the steady-state inactivation curve to the left. This shift was greater in neonatal (-10.1 ± 1.3 mV) than in adult (-7.0 ± 1.0 mV) cells, but not statistically significant ($P = 0.086$). The leftward shift of the steady-state inactivation curve may be one of the mechanisms of the inhibition of $I_{Ca(L)}$ by genistein, but is insufficient to account for the 30–43% inhibition. The predicted inhibitory effect due to the shift of the steady-state inactivation (at a holding potential of -40 mV) was about 13% in neonatal and only 4% in adult cells.

The single-channel recordings in neonatal cells showed that genistein inhibited the currents by decreasing the open probability of the channels. However, genistein did not affect the mean open time and conductance of the channel. The open probability of the channel was decreased by genistein about 30% of control (in absence or presence of Bay K 8644). Therefore, the genistein inhibition may be explained by the decrease of the open probability of the channel.

We cannot exclude the possibility that genistein inhibits $I_{Ca(L)}$ directly by binding to the outer surface of the channel, because daidzein, an inactive analogue of genistein, was reported to also inhibit $I_{Ca(L)}$ (Yokoshiki et al., 1996) and $I_{Na(f)}$ (Kusaka and Sperelakis, 1996). However, the following facts may support the view that genistein acts inside the cell. (1) After exposure to genistein (e.g., for over 5 min), the $I_{Ca(L)}$ could not be easily recovered after washing. (2) In single-channel recordings, the application of genistein inhibited the Ca^{2+} currents even in the cell-attached configuration, in which it would be very difficult for genistein to diffuse to the outer surface of the patch.

The basal $I_{Ca(L)}$ may be regulated by tonic phosphorylation of the channels. For example, Kameyama et al. (1986) reported that, in adult guinea pig ventricular myocytes, a PK-A inhibitor (PKI) inhibited the basal $I_{Ca(L)}$ by about 20%, suggesting that there is tonic regulation of the basal $I_{Ca(L)}$ by PK-A. In our recent preliminary data, H-7 (30 μM), a non-specific protein kinase inhibitor, inhibited the basal $I_{Ca(L)}$ by 24.4 ± 4.29 ($n = 12$) in neonatal and by $25.2 \pm 4.64\%$ ($n = 13$) in adult cells. Basal $I_{Ca(L)}$ may also be regulated by tonic phosphorylation by tyrosine kinases.

In addition, in biochemical studies, the tyrosine kinase activity in neonatal rat (1–3 days) was about 170% of the adult level (Okada and Nakagawa, 1988) and, in embryonic chick (7 days), was about 210% of adult (Maher, 1991). Our present results showed that the inhibition of $I_{Ca(L)}$ was 43.3% in neonatal and 30.6% in adult ($43.3/30.6 = 142\%$). This value is close to the value obtained in the biochemical study.

In summary, the present study demonstrated that tyrosine kinase inhibitor, genistein, inhibited the basal $I_{Ca(L)}$ by a maximum of 31–43%. The inhibition was greater in neonatal cells (43.3%) than in adult cells (30.6%). There may be tonic phosphorylation of the Ca^{2+} channels by tyrosine kinases, and this phosphorylation may be greater in *immature* cells.

Acknowledgements

We thank Mrs. Sheila Blank for preparation of the cells. This study was supported by National Institute of Health grant HL-31942.

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