Genistein Increases the Sensitivity of Cardiac Ion Channels to β-Adrenergic Receptor Stimulation

Livia C. Hool, Lisa M. Middleton, Robert D. Harvey

Abstract—The whole-cell patch-clamp technique was used to monitor the effects of genistein, a tyrosine kinase inhibitor, on membrane currents recorded from isolated guinea pig ventricular myocytes. Under control conditions, genistein (50 μmol/L) did not activate the latent cAMP-regulated Cl⁻ current (I_cl). However, in the presence of a subthreshold concentration (1 nmol/L) of the β-adrenergic agonist isoproterenol (Iso), genistein caused a near-maximal activation of this current. In the absence of genistein, Iso activated I_cl with an EC₅₀ of 5 nmol/L. In the presence of genistein, Iso activated I_cl with an EC₅₀ of 0.3 nmol/L. This facilitatory effect was not observed in the presence of daidzein (50 μmol/L), an analogue of genistein that only weakly inhibits tyrosine kinase activity. Furthermore, peroxovanadate, a potent inhibitor of phosphotyrosine phosphatase activity, inhibited I_cl activated by Iso alone, and it blocked the stimulatory effect of genistein in the presence of Iso. To determine whether the stimulatory effect of genistein was specific for I_cl, we also studied its action on the cAMP-regulated delayed rectifier K⁺ current (I_K) and L-type Ca²⁺ current (I_{Ca,L}) present in these cells. Basal I_K and I_{Ca,L} were partially (∼30% to 40%) inhibited by genistein. However, this inhibitory effect was mimicked by daidzein, suggesting that inhibition of tyrosine kinase activity is not involved. In addition to the nonspecific inhibitory effect, genistein also caused a significant increase in the β-adrenergic sensitivity of the unblocked cationic currents. In the absence of genistein, 1 nmol/L Iso had no effect on either I_K or I_{Ca,L}. However, in the presence of genistein, 1 nmol/L Iso significantly increased the magnitude of both currents. These results suggest that tyrosine kinase activity may play an important role in regulating β-adrenergic responsiveness of the heart. (Circ Res. 1998;83:33-42.)

Key Words: cardiac cystic fibrosis transmembrane conductance regulator Cl⁻ current ■ delayed rectifier K⁺ current ■ L-type Ca²⁺ current ■ tyrosine kinase ■ phosphotyrosine phosphatase

The role that protein kinases play in regulating ion channel function continues to be an area of intense research. Although serine/threonine kinases have received the most attention, it has become clear that tyrosine kinases also play an important role in regulating the activity of a variety of ion channels, including the CFTR, a cAMP/PKA-regulated Cl⁻ channel.1-6 Cystic fibrosis is due to mutations in the CFTR protein that result in a decrease in the Cl⁻ conductance and associated fluid transport of epithelial tissues. For that reason, a variety of alternative means of enhancing CFTR Cl⁻ channel activity have been investigated in an attempt to identify novel therapeutic approaches in treating this disease. As a result, a number of studies have obtained results suggesting that tyrosine kinase activity plays an important role in regulating epithelial CFTR function. The central piece of evidence supporting this idea is the fact that tyrosine kinase inhibitors like genistein can stimulate CFTR ion channel activity.1-7

It was originally suggested that the stimulatory effect that compounds like genistein have on CFTR function is mediated through a cAMP-independent mechanism. This conclusion was supported by reports that genistein does not affect basal cAMP levels in epithelial preparations.3,5 However, such conclusions were not consistent with the observation that genistein had no effect on channel activity in the presence of maximally stimulating concentrations of forskolin, which stimulates cAMP production by directly activating adenylyl cyclase.2,4,5 More recent reports have also demonstrated that the excitatory effect of genistein is observed only under conditions in which there is already some basal channel activity maintained by elevated cAMP and/or PKA activity.1,4,6 Furthermore, genistein was found to enhance PKA-dependent phosphorylation of the CFTR channel protein without directly stimulating PKA itself.4 cAMP-dependent activation of the channel was also found to be irreversible in the presence of genistein.1 On the basis of these results, it has been suggested that tyrosine kinase activity does not directly affect CFTR channel function but that it indirectly affects the channel by regulating the activity of a serine/threonine phosphatase.1,4,6

An alternatively spliced isoform of CFTR is also expressed in cardiac myocytes.4 In the heart, this channel is regulated through the same cAMP/PKA-dependent signaling pathway that regulates several other cardiac channels, including those contributing...
to $I_K$ and $I_{Ca-L}$. In the present study, we were interested in identifying the role that tyrosine kinase activity plays in the regulation of the cardiac CFTR Cl$^-$ current. Furthermore, we wanted to determine whether tyrosine kinases modulate the activity of other cAMP-regulated ion channels in a similar manner. This would inform us of whether tyrosine kinase activity plays a unique role in regulating Cl$^-$ channels or whether it has a more important role affecting the regulatory pathway shared by cation, as well as anion, channels in cardiac muscle. We have found that genistein alone, at concentrations of up to 50 μmol/L, did not significantly activate the Cl$^-$ current. However, genistein did potentiate the sensitivity of the current to activation by the β-adrenergic receptor agonist Iso. Genistein also increased the β-adrenergic sensitivity of $I_K$ and $I_{Ca-L}$. These results suggest that tyrosine kinases play an important role in regulating the β-adrenergic responsiveness of cardiac ion channels.

Materials and Methods

Cell Isolation

Adult Hartley guinea pigs were used as approved by the Institutional Animal Care and Use Committee. Ventricular myocytes were isolated using a modification of a previously described method.2 Hearts were excised from animals of either sex that had been anesthetized by intraperitoneal injection of pentobarbital. Coronary arteries were reintroduced to Ca$^{2+}$-containing KHB for 5 minutes, followed by Ca$^{2+}$-free KHB (Boehringer Mannheim) to achieve a final concentration of 0.5 to 0.7 μmol/L. After 30 minutes of digestion, both the left and right PVN simultaneous perfusions of 22 and 151.4 mmol/L, respectively, the predicted Cl$^-$ equilibrium potential was −50 mV. The Cl$^-$ current was isolated by blocking all K$^+$ channels with Cs$^+$ and/or tetraethylammonium-containing intracellular and extracellular solutions. L-type Ca$^{2+}$ channels were blocked by adding 1 μmol/L nisoldipine (Miles Laboratories) to all extracellular solutions. Na$^+$ channels were inactivated by using a holding potential of −30 mV. The time course of changes in Cl$^-$ conductance was monitored by applying 100-millisecond voltage steps to +50 mV once every 3 seconds. Current-voltage relationships were recorded by applying 100-millisecond voltage steps from a holding potential of −30 mV to test potentials from −120 to +50 mV in 10-mV increments. The Cl$^-$ current was defined as the agonist-induced difference current obtained by subtracting currents recorded in the absence of drug from those recorded in the presence of drug(s). Current magnitude was defined as the average current measured over a 15-millisecond span at the end of each 100-millisecond voltage-clamp step. The Cl$^-$ conductance was calculated by linear regression of the current-voltage relationship. However, genistein did potently increase the sensitivity of the β-adrenergic receptor agonist Iso. Genistein also increased the β-adrenergic sensitivity of $I_K$ and $I_{Ca-L}$. These results suggest that tyrosine kinases play an important role in regulating the β-adrenergic responsiveness of cardiac ion channels.

Data Acquisition

Membrane currents were recorded using the conventional whole-cell configuration of the patch-clamp technique.11 Microelectrodes with resistances between 0.5 and 1.5 MΩ were fabricated using borosilicate glass capillary tubing (Corning 7052, Garner Glass). Myocytes were placed in a 0.5-mL chamber, in which solutions were maintained at 37°C. A fast-flow system was used to rapidly (<1 second) change extracellular solutions bathing the myocyte from which membrane currents were being recorded.12 Currents were recorded using an Axopatch 200 voltage-clamp amplifier (Axon Instruments) and an IBM-compatible computer with a TL-1-125 interface and pCLAMP software (Axon Instruments). Junction potentials of ~10 mV were produced when the patch pipette was placed in the extracellular solution bathing the cell. Data were not corrected for this offset. A 3 mol/L KCl/agar bridge was used to ground the bath.

Experimental Protocols

When studying the Cl$^-$ current, pipettes were filled with the following intracellular solution (mmol/L): cesium glutamate 130, MgATP 5, Tris-GTP 0.1, HEPES 5, and EGTA 5. The pH was adjusted to 7.2 using CsOH. The control extracellular solution contained (mmol/L) NaCl 140, CsCl 5.4, CaCl$_2$ 2.5, MgCl$_2$ 0.5, HEPES 5.5, and glucose 11. The pH was adjusted to 7.4 using CsOH. With intracellular and extracellular Cl$^-$ concentrations of 22 and 151.4 mmol/L, respectively, the predicted Cl$^-$ equilibrium potential was −50 mV. The Cl$^-$ current was isolated by blocking all K$^+$ channels with Cs$^+$ and/or tetraethylammonium-containing intracellular and extracellular solutions. L-type Ca$^{2+}$ channels were blocked by adding 1 μmol/L nisoldipine (Miles Laboratories) to all extracellular solutions. Na$^+$ channels were inactivated by using a holding potential of −30 mV. The time course of changes in Cl$^-$ conductance was monitored by applying 100-millisecond voltage steps to +50 mV once every 3 seconds. Current-voltage relationships were recorded by applying 100-millisecond voltage steps from a holding potential of −30 mV to test potentials from −120 to +50 mV in 10-mV increments. The Cl$^-$ current was defined as the agonist-induced difference current obtained by subtracting currents recorded in the absence of drug from those recorded in the presence of drug(s). Current magnitude was defined as the average current measured over a 15-millisecond span at the end of each 100-millisecond voltage-clamp step. The Cl$^-$ conductance was calculated by linear regression of the current-voltage relationship. Genistein also increased the β-adrenergic sensitivity of $I_K$ and $I_{Ca-L}$. These results suggest that tyrosine kinases play an important role in regulating the β-adrenergic responsiveness of cardiac ion channels.

Selected Abbreviations and Acronyms

- CFTR = cystic fibrosis transmembrane conductance regulator
- $I_{Ca-L}$ = L-type Ca$^{2+}$ current
- $I_K$ = delayed rectifier K$^+$ current
- $I_{Ks}$ = inwardly rectifying K$^+$ current
- $I_{K1}$ = rapidly activating $I_K$
- $I_{Kw}$ = slowly activating $I_K$
- $I_{Iso}$ = isoproterenol
- KHB = Krebs-Henseleit buffer
- NBD = nucleotide-binding domain
- PKA = protein kinase A
- PVN = peroxovanadate

Drugs and Chemicals

- (-)Iso = isoproterenol (Research Biochemicals International) to all extracellular solutions. Na$^+$ was defined as the time-dependent current elicited during the step to +50 mV. Under the conditions used in our experiments, $I_K$ actually consists of 2 components, $I_{K1}$ and $I_{Kw}$. However, only $I_{K1}$ is regulated by β-adrenergic stimulation.12 Furthermore, the contribution of $I_{Kw}$ to the current was measured by using 3-second depolarizing steps to +50 mV. Long test pulses maximize the relative magnitude of $I_{Kw}$ by allowing this current to more fully activate, and strong depolarizations minimize the relative contribution of $I_{K1}$ because this current exhibits pronounced inward rectification.13 When studying $I_{Ca-L}$, nisoldipine was excluded from the external solution and the holding potential was changed to −80 mV. Na$^+$ channels were inactivated by applying a 50-millisecond prepulse to −30 mV immediately before each test pulse. The time course of changes in Ca$^{2+}$ conductance was monitored by applying a 75-millisecond test pulse to 0 mV once every 10 seconds. The peak inward Ca$^{2+}$ current recorded during the step to 0 mV was measured relative to the zero current level. The pipette solution was identical to that used for studying Cl$^-$ currents except that cesium glutamate was replaced with an equimolar concentration of CsCl. This set the Cl$^-$ equilibrium potential near 0 mV, minimizing the contribution of any Cl$^-$ conductance to the current recorded during the test pulse to that potential.

Results are reported as mean±SE. Statistical comparisons of responses between groups of cells were conducted using paired or unpaired t tests where indicated. Multiple comparisons were performed using ANOVA and the Bonferroni t test (SigmaStat, Jandel Scientific Software).

Drugs and Chemicals

R(+)-isobutirrate (Research Biochemicals International) was prepared as a stock solution in distilled water; ascorbic acid (50 μmol/L) was added to all solutions to prevent its oxidative degradation. Genistein and daidzein (Alexis) and H-89 (Calbiochem)
were initially prepared as stock solutions in dimethyl sulfoxide and then diluted 1:1000 in external solution. Nisoldipine was prepared as a stock solution in polyethylene glycol (molecular weight, 400) and diluted 1:2000 in extracellular solution. These concentrations of dimethyl sulfoxide (0.1%) and polyethylene glycol (0.05%) did not affect β-adrenergic responsiveness in control experiments.

The PVN stock solution was prepared, as described previously, by adding H$_2$O$_2$ (30% [wt/wt] solution, Sigma) to an aqueous solution containing 10 mmol/L Na$_3$VO$_4$ (Fischer Scientific) and 50 mmol/L HEPES (pH 7.4). Enough H$_2$O$_2$ was added to achieve a final concentration of 10 mmol/L. This solution was mixed and allowed to stand at room temperature for 15 minutes. At the end of this time, excess H$_2$O$_2$ was eliminated by adding 200 μg/mL catalase (Sigma). The stock solution, which contains an aqueous mixture of vanadate and peroxovanadium complexes, was made immediately before use. The final concentration of PVN used in our experiments is based on the concentration of Na$_3$VO$_4$ used in preparing the stock solution. To control for potential nonspecific effects of catalase or any remaining H$_2$O$_2$, cells were exposed to solutions containing H$_2$O$_2$ and catalase alone before being exposed to PVN-containing solutions.

Results

Modulation of the cAMP-Regulated Cl$^-$ Current

In the absence of β-adrenergic stimulation, the Cl$^-$ conductance of guinea pig ventricular myocytes is negligible. Furthermore, not all myocytes functionally express cardiac CFTR Cl$^-$ channels. However, their presence in any particular cell can be verified by demonstrating that exposure to the β-adrenergic agonist Iso activates a macroscopic current that is time independent and outwardly rectifying and that reverses near the Cl$^-$ equilibrium potential (see Figure 1). We found that exposure to 50 μmol/L genistein had little or no effect on the Cl$^-$ conductance of guinea pig ventricular myocytes (Figure 1). In 13 experiments, the average change in conductance measured after exposure to genistein alone was only 3.5±6.7% of that observed after exposure to a maximally stimulating concentration of Iso in the same cell.

Despite the fact that this concentration of genistein appeared to be without effect under basal conditions, it did reversibly activate the Cl$^-$ current when myocytes were first exposed to a subthreshold concentration of Iso. Figure 1 illustrates the effect of 50 μmol/L genistein in the presence of 1 nmol/L Iso. This concentration of the β-adrenergic agonist Iso activates by itself does not activate the Cl$^-$ current. In 6 experiments, the average change in conductance measured after exposure to 1 nmol/L Iso alone was −0.3±5% of that observed after exposure to a maximally stimulating concentration of Iso in the same cell. Nevertheless, subsequent exposure to 50 μmol/L genistein in the continued presence of this subthreshold concentration of Iso resulted in maximal activation of the Cl$^-$ current. Even though neither 50 μmol/L genistein nor 1 nmol/L Iso was able to activate the Cl$^-$ current on its own, 50 μmol/L genistein in the presence of 1 nmol/L Iso activated the Cl$^-$ conductance to a level that was 121±19.8% (n=7) of that measured in the presence of a maximally stimulating concentration of Iso in the same cell. This is consistent with the idea that genistein was facilitating β-adrenergic activation of the cAMP-regulated Cl$^-$ current.

This conclusion is further supported by the fact that the current activated by genistein had the same biophysical characteristics as the current activated by Iso alone. It was time independent and outwardly rectifying, and it reversed near the Cl$^-$ equilibrium potential (see Figure 1). Furthermore, cells that did not respond to a maximally stimulating concentration of Iso did not respond to genistein in the presence of 1 nmol/L Iso.

If the stimulatory effect of genistein observed in the presence of subthreshold concentrations of Iso was due to inhibition of tyrosine kinase activity, this effect should not be mimicked by daidzein, a structural analogue of genistein commonly used as a negative control for genistein-mediated tyrosine kinase–dependent responses. The protocol for these experiments first involved exposure to 100 nmol/L Iso to verify that cAMP-regulated Cl$^-$ channels were present. The concentration of Iso was then reduced to 1 nmol/L, resulting in complete deactivation of the Cl$^-$ current, and 50 μmol/L daidzein was subsequently added. Figure 2 illustrates that in the presence of 1 nmol/L Iso, 50 μmol/L daidzein...
Daidzein, a structural analogue of genistein that is a less potent tyrosine kinase inhibitor, does not significantly activate the cAMP-regulated Cl\(^-\) current, even in the presence of a subthreshold concentration of Iso. A, Time course of changes in Cl\(^-\) conductance after exposure to a supramaximally stimulating concentration (100 nmol/L) of Iso, a subthreshold concentration (1 nmol/L) of Iso plus 50 \(\mu\)mol/L daidzein, and 1 nmol/L Iso plus 50 \(\mu\)mol/L genistein. B, Membrane potential (\(V_m\)) dependence of the difference current (\(\Delta I\)) obtained by subtracting current traces recorded under control conditions from those recorded under the conditions indicated at the time points illustrated in panel A.

Figure 2. Daidzein elicited a response that was negligible compared with that activated by either the maximally stimulating concentration of Iso or subsequent exposure to 50 \(\mu\)mol/L genistein in the presence of 1 nmol/L Iso. In 5 experiments, the change in membrane conductance observed after exposure to 50 \(\mu\)mol/L daidzein in the presence of 1 nmol/L Iso was only 7.2\(\pm\)14\% of that activated by a maximally stimulating concentration of Iso in the same cell. Although daidzein did elicit a small transient response in some cells, the magnitude of this effect was only \(\approx\)6\% of that activated by the same concentration of genistein. This small stimulatory effect is probably due to the fact that daidzein can weakly inhibit tyrosine kinase activity.22 The concentration of genistein that half-maximally inhibits tyrosine kinase activity (IC\(_{50}\)) is \(\approx\)25 \(\mu\)mol/L. Daidzein inhibits tyrosine kinase activity with an IC\(_{50}\) of \(>393\) \(\mu\)mol/L.13 Therefore, the results of these experiments are consistent with the conclusion that in the presence of a subthreshold concentration of Iso, 50 \(\mu\)mol/L genistein is activating the Cl\(^-\) current by inhibiting basal tyrosine kinase activity.

If basal tyrosine kinase activity plays an important role in regulating \(\beta\)-adrenergic responsiveness of the Cl\(^-\) current, one might also predict that inhibition of phosphotyrosine phosphatase activity would antagonize the response to Iso. To test this hypothesis, we investigated the effect that PVN has on the Cl\(^-\) current activated by a maximally stimulating concentration of Iso as well as its effect on the subsequent response to genistein (Figure 3). We found that 100 \(\mu\)mol/L PVN, which inhibits phosphotyrosine phosphatase activity with an IC\(_{50}\) of 1 \(\mu\)mol/L,23 almost completely inhibited the Cl\(^-\) current activated by 30 nmol/L Iso. The magnitude of the Cl\(^-\) conductance recorded in the presence of Iso plus PVN was 5.6\(\pm\)3.4\% (\(n=7\)) of that recorded in the presence of Iso alone in the same cell. An alternative explanation for the inhibitory effects of PVN is that Cl\(^-\) channels are being directly blocked. However, it has been previously shown that millimolar concentrations of vanadate do not block these Cl\(^-\) channels.22,24 Furthermore, even though PVN did not completely inhibit the Iso response, it did attenuate the stimulatory effect on subsequent exposure to genistein. The magnitude of the Cl\(^-\) conductance recorded after exposure to 50 \(\mu\)mol/L genistein in the continued presence of 30 nmol/L Iso plus 100 \(\mu\)mol/L PVN was only 11\(\pm\)2.7\% (\(n=4\)) of that measured in the presence of Iso alone in the same cell. These results are consistent with the hypothesis that basal tyrosine kinase activity regulates \(\beta\)-adrenergic stimulation of the Cl\(^-\) current and that genistein exerts its stimulatory effect by inhibiting basal tyrosine kinase activity.

The fact that genistein was able to activate the cAMP-regulated Cl\(^-\) current in the presence, but not the absence, of subthreshold concentrations of Iso indicates that inhibition of tyrosine kinase activity increases the sensitivity of the Cl\(^-\) current to \(\beta\)-adrenergic stimulation. To quantify this effect,
we examined the concentration dependence of Cl\(^-\) current activation by Iso. A, Time course of changes in Cl\(^-\) conductance after exposure to varying concentrations of Iso in the presence of 50 μmol/L genistein. B, Concentration dependence of Iso activation of the cAMP-regulated Cl\(^-\) current measured in the presence (n=4) and absence (n=5) of 50 μmol/L genistein. The data points were fit to the following equation: 

\[
G_{Cl} = G_{max} / \left(1 + \left(\frac{EC_{50}}{[Iso]}\right)^n\right),
\]

where \(G_{Cl}\) is the magnitude of the Cl\(^-\) conductance measured in the presence of a given concentration of Iso (±genistein) normalized to \(G_{max}\), the magnitude of the Cl\(^-\) conductance activated by a supramaximally stimulating concentration (100 nmol/L) of Iso in the same cell. Under control conditions, the concentration of Iso producing half-maximal activation (EC\(_{50}\)) of the Cl\(^-\) current was 5.0±0.05 nmol/L, and the slope factor (n) was 1.6±0.2. In the presence of 50 μmol/L genistein, EC\(_{50}\) was 0.31±0.02 nmol/L, and n was 1.2±0.1.

**Figure 4.** Genistein shifts the concentration dependence of Cl\(^-\) current activation by Iso. A, Time course of changes in Cl\(^-\) conductance after exposure to Iso (100 nmol/L) of Iso in the same cell. Under control conditions from those recorded under the conditions indicated at the time points illustrated in panel A.

The observation that inhibition of tyrosine kinase activity significantly increases the sensitivity of the Cl\(^-\) current to activation by β-adrenergic stimulation.

The ability of genistein to increase the sensitivity of the cAMP-regulated Cl\(^-\) current to β-adrenergic stimulation is quite striking, and it has important implications with respect to the role that tyrosine kinase activity may play in regulating cardiac function. However, if inhibition of tyrosine kinase activity were to have a similar effect on other cAMP-regulated ion channels, this would not only provide important insight into the mechanism mediating such responses, it would also demonstrate that tyrosine kinase activity may play a much more profound role in regulating the β-adrenergic responsiveness of the heart. To investigate this possibility, we
looked to see whether genistein also affected the sensitivity of $I_{K}$ and $I_{Ca,L}$ to stimulation by Iso.

First, we looked to see what effect, if any, genistein had on $I_{K}$. We found that 50 μmol/L genistein alone actually caused a rapid and reversible partial inhibition of all outward $K^+$ currents in these cells. This included not only the time-dependent $I_{K}$ elicited during depolarizing steps to +50 mV but also the time-independent $I_{K1}$ recorded at a holding potential of −30 mV (Figure 6). The fact that genistein caused a very rapid inhibition of more than one type of $K^+$ channel suggested that this might be a nonspecific effect, unrelated to inhibition of tyrosine kinase activity. This idea was supported by the fact that 50 μmol/L daidzein also rapidly blocked $I_{K}$ as well as $I_{K1}$ (Figure 6). Genistein (50 μmol/L) inhibited the magnitude of $I_{K}$ activated during depolarizing steps to +50 mV by 44±3.0% (n=6); daidzein (50 μmol/L) inhibited this current by 21±2.4% (n=5).

Continued exposure to genistein also seemed to have a slight stimulatory effect on $I_{K}$ that manifested itself as a slow increase in the magnitude of the time-dependent current after the initial rapid inhibition. Exposure to 50 μmol/L genistein for 5 minutes increased the magnitude of $I_{K}$ by 34±7.1% (n=6) over its minimum value measured immediately after the initial inhibitory effect of the drug. This stimulatory effect also appeared as a rebound increase in the magnitude of the current after washout of the drug. The rebound response can be explained by the difference in the rate with which the inhibitory and stimulatory effects of genistein are reversed on washout. The inhibitory effect reverses rapidly, revealing the stimulatory effect that then returns to baseline more slowly. Unlike genistein, daidzein did not exhibit any stimulatory effect. After exposure to 50 μmol/L daidzein for 5 minutes, the magnitude of the current actually decreased by 7.5±5.1% (n=6) from its minimum value measured immediately after exposure to the drug, and there was no rebound response after washout of daidzein. The slight decrease in current magnitude observed with continued exposure to daidzein most likely reflects current rundown.

To determine whether genistein also increased the sensitivity of $I_{K}$ to β-adrenergic stimulation, we compared the effect of 1 nmol/L Iso in the absence and presence of 50 μmol/L genistein. In the absence of genistein, 1 nmol/L Iso had no stimulatory effect. In 5 experiments, the magnitude of the current recorded after exposure to this concentration of Iso actually decreased by 7.5±5.1%. Again, this slight decrease was probably due to current rundown. When the same cell was then treated with 50 μmol/L genistein, subsequent exposure to 1 nmol/L Iso significantly increased the magnitude of $I_{K}$ (Figure 7). This stimulatory effect appeared as an increase in the magnitude of the current observed after exposure to Iso, as well as an increase in the magnitude of the rebound response observed after washout of genistein in the continued presence of Iso. In 6 experiments, exposure to 1 nmol/L Iso alone had no detectable effect on the

---

**Figure 6.** Genistein and daidzein inhibit $I_{K}$ in a nonspecific manner. A, Time course of changes in magnitude of the time-dependent $K^+$ current elicited by depolarizing voltage-clamp steps to +50 mV after exposure to 50 μmol/L genistein and 50 μmol/L daidzein. B, Example of $K^+$ currents recorded in the absence (1) and presence (2) of 50 μmol/L genistein and $K^+$ currents recorded in the absence (3) and presence (4) of 50 μmol/L daidzein. Current traces were recorded at time points indicated in panel A. Note that both genistein and daidzein inhibited $I_{K}$ recorded during the voltage-clamp step to +50 mV as well as $I_{K1}$ recorded at the holding potential of −30 mV.

**Figure 7.** Genistein increases the sensitivity of $I_{K}$ to stimulation by Iso. A, Time course of changes in magnitude of the time-dependent $K^+$ current elicited by depolarizing voltage-clamp steps to +50 mV after exposure to a subthreshold concentration (1 nmol/L) of Iso alone, 50 μmol/L genistein alone, and 50 μmol/L genistein plus 1 nmol/L Iso. B, Example of $K^+$ currents recorded in the presence (1) and absence (2) of 1 nmol/L Iso under control conditions and $K^+$ currents recorded in the absence (3) and presence (4) of 1 nmol/L Iso after exposure to 50 μmol/L genistein. Current traces were recorded at the time points indicated in panel A.
magnitude of $I_k$, but the magnitude of the current measured after exposure to 1 nmol/L Iso was increased by 42±6.9% over that observed in the presence of 50 μmol/L genistein alone. These results clearly demonstrate that genistein increased the sensitivity of $I_k$ to β-adrenergic stimulation. This is analogous to the effect that genistein had on the β-adrenergic sensitivity of the Cl⁻ current. Like the response of the Cl⁻ current, the effect of genistein on $I_k$ was also blocked by H-89. In cells preexposed to 10 μmol/L H-89, genistein alone produced no stimulatory effect, and 1 nmol/L Iso did not enhance $I_k$ in the continued presence of genistein. The magnitude of the current after exposure to Iso in the presence of 50 μmol/L genistein was 5.7±12% (n=6) smaller than that observed in the presence of genistein alone (data not shown). This result supports the idea that the stimulatory effect of genistein on $I_k$ also involves a cAMP-dependent mechanism.

**Modulation of the cAMP-Regulated Ca²⁺ Current**

Next, we examined the effect of genistein on $I_{Ca-L}$. Exposure to 50 μmol/L genistein alone rapidly inhibited the peak inward current recorded during depolarizing steps to 0 mV, and this effect was readily reversed on washout of the drug (Figure 8A). Again, the speed with which this inhibitory effect occurred suggested that it might be a nonspecific blocking effect. Consistent with this idea, 50 μmol/L daidzein produced a similar rapid and reversible inhibition of $I_{Ca-L}$ (Figure 8B). Genistein inhibited the peak current by 16±2.3% (n=10). These observations are consistent with a previous report that both genistein and daidzein inhibit $I_{Ca-L}$ in cardiac myocytes. Unlike its effect on $I_k$, genistein in the absence of β-adrenergic stimulation only infrequently produced a stimulatory effect on the Ca²⁺ current. In the examples illustrated in Figures 8 and 9, genistein alone produced no evidence of a stimulatory effect, but on average, exposure to 50 μmol/L genistein for 5 minutes increased the magnitude of $I_{Ca-L}$ by 11±12% (n=12) over its minimum value measured immediately after the initial inhibitory effect of the drug.

We then examined the effect that a subthreshold concentration of Iso had on $I_{Ca-L}$ in the absence and presence of 50 μmol/L genistein (Figure 9). As with the cAMP-regulated Cl⁻ and K⁺ currents, 1 nmol/L Iso alone had no effect on the Ca²⁺ current. The magnitude of the current recorded after exposure to this concentration of Iso actually decreased by 4.4±2.3%. This slight decrease can be accounted for by current rundown. However, when cells were first treated with 50 μmol/L genistein, subsequent exposure to 1 nmol/L Iso increased the magnitude of the Ca²⁺ current by an average of 74±14% (n=12). These results clearly demonstrate that genistein increased the sensitivity of $I_{Ca-L}$ to β-adrenergic stimulation. This is analogous to the effect that genistein had on the β-adrenergic sensitivity of the Cl⁻ and K⁺ currents.

**Discussion**

In the present study, we have demonstrated that genistein increases the sensitivity of the cAMP-regulated Cl⁻ current to β-adrenergic receptor stimulation in guinea pig ventricular myocytes. This effect appears to be due to the ability of genistein to inhibit tyrosine kinase activity. This conclusion is
Genistein Facilitates β-Adrenergic Responses

supported by the fact that daidzein, a structural analogue of genistein that only weakly inhibits tyrosine kinase activity, did not exert this type of stimulatory effect. These results support the idea that basal tyrosine kinase activity inhibits β-adrenergic regulation of this Cl\(^{-}\) current. This conclusion is further reinforced by the fact that PVN, an inhibitor of phosphotyrosine phosphatase activity, inhibits β-adrenergic stimulation of the Cl\(^{-}\) current and blocks the stimulatory effects of genistein.

We did not find any evidence that genistein alone is able to significantly activate this Cl\(^{-}\) current. This is in contrast to a previous study by Shuba et al\(^{22}\) in which genistein alone was reported to directly activate this current in the same preparation. However, even though that group found that 50 μmol/L genistein (the same concentration used in the present study) elicited a response in some cells, the actual EC\(_{50}\) for direct activation of the cardiac Cl\(^{-}\) current by genistein was >100 μmol/L.\(^{29}\) Therefore, we might have seen effects with genistein alone had we used higher concentrations. Unfortunately, higher concentrations of genistein were not soluble in our solutions. It is also possible that high concentrations of genistein alone have direct effects on the cardiac CFTR Cl\(^{-}\) channel that are distinctly different from the effects that lower concentrations of genistein have on β-adrenergic responsiveness of cardiac ion channels in general. For example, Shuba et al\(^{22}\) reported that the effects of high concentrations of genistein alone were mediated through a cAMP-independent process. However, our results strongly suggest that the response to genistein that we observed depends on cAMP-dependent activation of PKA. This conclusion is supported by the fact that we detected a genistein response only in the presence of β-adrenergic receptor stimulation. Furthermore, genistein facilitated β-adrenergic stimulation of cAMP-regulated K\(^{+}\) and Ca\(^{2+}\) channels, in addition to cAMP-regulated Cl\(^{-}\) channels. More important, we found that the effect of genistein was blocked by H-89, an inhibitor of cAMP-dependent protein kinase activity.

The fact that it was necessary to use 10 μmol/L H-89 to obtain near-maximal inhibition of the genistein response is not surprising. Even though this compound is reported to inhibit PKA activity with an IC\(_{50}\) of 30 nmol/L, this is only in the presence of micromolar concentrations of ATP.\(^{25}\) The ability of H-89 to inhibit kinase activity is competitively antagonized by ATP.\(^{25}\) Our experiments were conducted in the presence of millimolar concentrations of ATP. Under these conditions, H-89 still selectively inhibits PKA, but only at concentrations of 10 to 20 μmol/L.\(^{25}\) Furthermore, we have found that PKA-dependent activation of the Cl\(^{-}\) current by β-adrenergic receptor stimulation alone is inhibited only by H-89 at concentrations >1 μmol/L, with 10 μmol/L producing near-maximal effects (L.C. Hool and R.D. Harvey, unpublished data, 1997). This is identical to the sensitivity of the genistein-activated Cl\(^{-}\) current to H-89 reported in the present study. This further supports the idea that H-89 was acting by inhibiting PKA and that the effect of Iso in the presence of genistein is cAMP dependent.

In addition to increasing the sensitivity of Cl\(^{-}\), K\(^{+}\), and Ca\(^{2+}\) channels to β-adrenergic stimulation, genistein also exerted an inhibitory effect on several different currents in guinea pig ventricular myocytes. The ability of genistein to inhibit \(I_{\text{Ca-L}}\) in cardiac myocytes has been reported previously.\(^{39}\) However, we also found that genistein inhibited \(I_{\text{K}}\) and \(I_{c}\). The fact that the onset of the inhibitory effect occurred as fast as the drug was applied and that it reversed equally as fast, regardless of the current type, suggested that it might be due to a nonspecific block of the channel pore rather than the modification of an enzyme-dependent process, namely, inhibition of tyrosine kinase activity. This conclusion is supported by the fact that daidzein exerted a similar inhibitory effect. Genistein and daidzein have also been reported to exert nonspecific blocking effects on Na\(^{+}\) channels as well.\(^{20,25}\) Despite the fact that genistein appears to be a promiscuous ion channel blocker, there was no evidence that it blocked the cardiac CFTR Cl\(^{-}\) current. With \(I_{c}\) and \(I_{c+1}\), the inhibitory effect of genistein washed in and out much more rapidly than the stimulatory effect. As a result, washout of genistein in the presence of a subthreshold concentration of Iso resulted in a rebound increase in the magnitude of both currents (see Figures 7 and 9). By analogy, if this drug had exerted inhibitory as well as stimulatory effects on the cardiac CFTR Cl\(^{-}\) current, we would have expected to see a rebound increase in the magnitude of the Cl\(^{-}\) current immediately after washout of genistein. However, there was no evidence for such a phenomenon (see Figure 1). We only observed a slight delay between the time that genistein was washed out and the time that the current deactivated. This suggests that the site at which genistein exerts its inhibitory influence is unique to cation channels.

The idea that the stimulatory effect that genistein has on cardiac ion channels involves a cAMP-dependent mechanism is consistent with recent reports demonstrating that genistein also stimulates epithelial CFTR Cl\(^{-}\) channel activity in a cAMP-dependent manner.\(^{1,4,6}\) In cells expressing the epithelial isoform of CFTR, genistein alone does not activate Cl\(^{-}\) channel activity unless basal PKA activity is high or unless adenylate cyclase activity is stimulated with forskolin. Reports that genistein does not directly affect PKA or adenylate cyclase activity has led to the hypothesis that genistein regulates channel function by inhibiting the activity of a serine/threonine phosphatase.\(^{1,4,6}\) This hypothesis implies that the activity of this phosphatase is maintained by basal tyrosine kinase activity. It is possible that genistein could be regulating CFTR channel activity in cardiac myocytes through such a mechanism. However, we found that genistein did not prevent complete deactivation of the cardiac Cl\(^{-}\) current on washout of Iso (see Figures 1 and 4). Although this might argue against the idea that inhibition of tyrosine kinase activity enhances β-adrenergic sensitivity of the cardiac Cl\(^{-}\) channel by preventing serine/threonine dephosphorylation, it should be noted that activation of CFTR Cl\(^{-}\) channels is believed to involve phosphorylation of at least 2 functionally distinct sites.\(^{26}\) It has been postulated that genistein may be preventing dephosphorylation of a site that by itself does not maintain channel activity but, instead, modifies responses to phosphorylation of other sites.\(^{3}\) If this were true, preventing dephosphorylation of this modulatory site would not necessarily prevent complete deactivation of the current.
Gating of CFTR Cl\(^{-}\) channels also depends on the interaction of ATP with 2 NBDs that are an integral part of the channel protein. When the channel is in a phosphorylated state, ATP hydrolysis at one of these NBDs is necessary for channel opening, whereas ATP hydrolysis at the other is believed to result in channel closing.\(^{27}\) A more recent study has suggested that the effect of genistein on epithelial CFTR channel function is due to inhibition of ATPase activity at the second NBD, which slows channel closing, increasing its open probability.\(^{7}\) Although we cannot rule out the possibility that this mechanism contributes to the effect that genistein has on \(\beta\)-adrenergic regulation of the cardiac CFTR Cl\(^{-}\) channel, it is unlikely to explain the changes in \(\beta\)-adrenergic sensitivity of the cAMP-regulated K\(^{+}\) and Ca\(^{2+}\) currents, because these channels do not possess NBDs. High concentrations (mmol/L) of vanadate have also been reported to alter CFTR channel activity by affecting ATP hydrolysis at an NBD. However, it is unlikely that PVN was acting in this manner in our experiments, since vanadate has been reported to increase the open probability of this channel by inhibiting ATP hydrolysis at the NBD that is responsible for channel closing.\(^{24}\)

The actions of vanadate are related to its structural similarity to inorganic phosphate. It can inhibit ATPase activity, like that at the NBD of CFTR, by binding tightly, in place of inorganic phosphate, the normal product of ATP hydrolysis.\(^{24}\) By acting as an inorganic phosphate analogue, vanadate may also compete for phosphate binding to phosphatases. However, such an effect is specific for inhibiting tyrosine phosphatases.\(^{28}\) If PVN had inhibited serine/threonine phosphatase activity in our experiments, Cl\(^{-}\) channel activity would have been inhibited, not inhibited. Previous studies have also demonstrated that high concentrations of vanadate do not prevent PKA-dependent activation of the Cl\(^{-}\) channel in cardiac myocytes.\(^{24}\) This would argue that the inhibitory effect of PVN that we observed is not due to inhibition of channel phosphorylation by this kinase.

One potential limitation of the present study is the fact that membrane currents were recorded using a cell-dialyzing, whole-cell, patch-clamp technique. This approach can lead to problems associated with current rundown. We previously characterized the effect that cell dialysis has on \(\beta\)-adrenergic responsiveness and found that there is a slight decrease in the sensitivity (EC\(_{50}\)) of the Cl\(^{-}\) current to activation by Iso.\(^{11}\) However, this shift is insignificant during the first 15 to 20 minutes of dialysis. The Cl\(^{-}\) current is activated by Iso with an EC\(_{50}\) of 3.8 nmol/L when the perforated patch-clamp technique is used. Consistent with our previous finding, this is not significantly different from the EC\(_{50}\) of 5.0 nmol/L observed, in the absence of genistein, in the present study. It is, however, an order of magnitude greater than the EC\(_{50}\) of 0.3 nmol/L found in the presence of genistein. Therefore, the fact that we used the conventional whole-cell patch-clamp technique does not affect our main conclusion, which is that genistein increases the sensitivity cardiac ion channels to \(\beta\)-adrenergic receptor stimulation.

The simplest explanation for the fact that genistein increases the \(\beta\)-adrenergic sensitivity of cardiac Cl\(^{-}\), K\(^{+}\), and Ca\(^{2+}\) channels is that basal tyrosine kinase activity exerts a tonic inhibitory influence on some element in the cAMP-signaling pathway that is involved in regulating all of these channels. The regulation of phosphatase activity is one possibility. However, the possibility that genistein increases \(\beta\)-adrenergic sensitivity by inhibiting the effect of tyrosine kinases acting at other points in the cAMP-signaling pathway cannot be ruled out. For example, the effects of genistein may be at the level of the \(\beta\)-adrenergic receptor itself. It is known that tyrosine phosphorylation of \(\beta\)-adrenergic receptors inhibits their ability to activate adenylate cyclase.\(^{20,29}\) Although regulation of ion channels in guinea pig ventricular myocytes is mediated solely through the activation of \(\beta\)-adrenergic receptors,\(^{31}\) there is evidence that tyrosine phosphorylation of this receptor also inhibits its ability to stimulate cAMP production.\(^{21}\) Investigation of this hypothesis awaits further study.

\(\beta\)-Adrenergic regulation of ion channels is a ubiquitous mechanism for controlling both cardiac electrical and mechanical activity.\(^{22}\) However, it is yet to be determined whether tyrosine kinase–dependent regulation of these \(\beta\)-adrenergic responses occurs in all species, especially humans. It would also be important to determine whether this pathway is part of a receptor-mediated signaling process. If such a mechanism for affecting \(\beta\)-adrenergic regulation of ion channels does exist in human myocardium, it would be interesting to investigate the possibility of whether or not alterations in this signaling process could contribute to the changes in \(\beta\)-adrenergic responsiveness and susceptibility to arrhythmias that occur under conditions such as heart failure.\(^{13,34}\) Furthermore, identifying the specific mechanism by which genistein enhances \(\beta\)-adrenergic responsiveness might provide a target for developing novel therapeutic approaches in the treatment of this disease state.

Acknowledgments
This study was supported by grants from the National Institutes of Health and the Northeast Ohio Affiliate of the American Heart Association, an Established Investigatorship from the American Heart Association (Dr Harvey), and a postdoctoral fellowship from the Northeast Ohio Affiliate of the American Heart Association (Dr Hool). The authors also wish to acknowledge the technical assistance of Montelle Sanders.

References
Genistein Facilitates β-Adrenergic Responses

12. Sanguinetti MC, Jurkiewicz NK, Scott A, Siegl PKS. Isoproterenol antag-
13. Sanguinetti MC, Jurkiewicz NK. Two components of cardiac delayed rectifier K+ current: differential sensitivity to block by class III antiar-
18. Paillart C, Carlier E, Guedin D, Dargent B, Couraud F. Direct block of voltage-sensitive sodium channels by genistein, a tyrosine kinase inhib-
25. Chijwara T, Mishima A, Hagisawa M, Sano M, Hayashi K, Inoue T, Naito K, Toshikoa T, Hidaka H. Inhibition of forskolin-induced neurite out-
29. Karoor V, Baltensperger K, Paul H, Czech MP, Malbon CC. Phosphory-
31. Hool LC, Harvey RD. Role of β1- and β2-adrenergic receptors in regu-
Genistein Increases the Sensitivity of Cardiac Ion Channels to β-Adrenergic Receptor Stimulation
Livia C. Hool, Lisa M. Middleton and Robert D. Harvey

Circ Res. 1998;83:33-42
doi: 10.1161/01.RES.83.1.33

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/83/1/33

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/