Genistein elicits biphasic effects on L-type Ca\(^{2+}\) current in feline atrial myocytes

YONG G. WANG AND STEPHEN L. LIPSIUS
Department of Physiology, Loyola University Chicago, Stritch School of Medicine, Maywood, Illinois 60153

Wang, Yong G., and Stephen L. Lipsius. Genistein elicits biphasic effects on L-type Ca\(^{2+}\) current in feline atrial myocytes. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H204–H212, 1998.—A perforated patch recording method was used to determine the effects of genistein (Gen), a protein tyrosine kinase (PTK) inhibitor, on basal L-type Ca\(^{2+}\) current (I\(_{\text{Ca,L}}\)) in feline atrial myocytes. Gen (50 µM) elicited biphasic changes in I\(_{\text{Ca,L}}\): an initial inhibition (−55 ± 4% phase 1) followed by a secondary stimulation (34 ± 9% phase 2) of I\(_{\text{Ca,L}}\). Withdrawal of Gen elicited a further potentiation of I\(_{\text{Ca,L}}\) (152 ± 19% phase 3) above control (n = 46). In general, phase 1 inhibition and phase 3 potentiation varied directly with Gen concentration, and phase 2 stimulation exhibited biphasic concentration-dependent changes compared with control. When cells were dialyzed using a ruptured patch recording method, Gen elicited only inhibition of I\(_{\text{Ca,L}}\); phases 2 and 3 were abolished. Vanadate (1 mM), an inhibitor of protein tyrosine phosphatase, abolished both Gen-induced inhibition and stimulation of I\(_{\text{Ca,L}}\). Daidzein (50 µM), a weakly active analog of Gen, exerted no significant effects on I\(_{\text{Ca,L}}\), and withdrawal of daidzein failed to potentiate I\(_{\text{Ca,L}}\). In a few cells, Gen elicited a prominent vanadate-sensitive stimulation of I\(_{\text{Ca,L}}\). In the absence of any significant inhibition of I\(_{\text{Ca,L}}\), Gen-induced changes in I\(_{\text{Ca,L}}\) were unaffected by either 100 µM 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA)-acetoxymethyl ester (AM) or 1 µM ryanodine, agents that alter intracellular Ca\(^{2+}\); 4 µM H-89 or 50 µM Rp diastereomer of adenosine 3’,5’-monophosphoester (RP-cAMPS), inhibitors of protein kinase A (PKA); 0.1 µM calphostin C or 2 µM chelerythrine, inhibitors of protein kinase C (PKC); or 100 µM N\(^6\)-monomethyl-L-arginine (L-NMMA), an inhibitor of nitric oxide (NO) synthase. We conclude that in feline atrial myocytes, Gen acts via membrane-bound PTks to inhibit I\(_{\text{Ca,L}}\) and via cytosolic PTks to stimulate I\(_{\text{Ca,L}}\). Gen-induced changes in I\(_{\text{Ca,L}}\) are not related to changes in intracellular Ca\(^{2+}\) or to secondary interactions with either PKA, PKC, or NO signaling pathways. These results indicate that in atrial myocytes I\(_{\text{Ca,L}}\) is regulated by two independent and competing PTK signaling mechanisms.

METHODS

Details of the isolation and recording methods have been published previously (35). Adult cats of either sex were anesthetized with pentobarbital sodium (70 mg/kg ip). Hearts were perfused via a Langendorff apparatus with a bicarbonate-buffered Tyrode solution for ~5 min followed by perfusion with a nominally Ca\(^{2+}\)-free Tyrode solution. After 5 min, the perfusion was switched to a low-Ca\(^{2+}\) (36 µM) Tyrode solution containing 0.06% collagenase (type II, Worthington Biochemical) for 30–40 min. After collagenase perfusion, both atria were cut into small pieces and agitated in fresh collagenase and 0.01% protease. Experiments were performed on either right or left atrial cells, with no discernible differences in responses. Cells studied were isolated on the morning of each experiment.

Cells used for study were transferred to a small tissue bath on the stage of an inverted microscope (Nikon Diaphot) and superfused with a modified Tyrode solution containing (in mM) 137 NaCl, 5.4 KCl, 1.0 MgCl\(_2\), 2.0 CaCl\(_2\), 5 HEPES, and 11 glucose and titrated with NaOH to a pH of 7.4. Solution was perfused through a small (0.3 ml) chamber by gravity at ~5 ml/min. The system required ~20 s to completely exchange the bath contents. All experiments were performed at 35 ± 1°C. Cells selected for study were elongated and quiescent. Ionic currents were recorded using a nystatin-perforated patch (14) whole cell recording method (12). This method minimizes dialysis of intracellular contents with the internal pipette solution, thereby maintaining physiological buffering of intracellular Ca\(^{2+}\) and second messenger signaling pathways. Nystatin was dissolved in DMSO at a concentration of 50 mg/ml and then added to the internal pipette solution to yield a final nystatin concentration of 150 µg/ml. The pipette solution containing nystatin was strongly sonicated before use. The internal pipette solution contained (in mM) 100 cesium glutamate, 40 CsCl, 1.0 MgCl\(_2\), 4 Na\(_2\)ATP,
0.5 EGTA, and 5 HEPES and was titrated with CsOH to a pH of 7.2. To record \( I_{\text{Ca,L}} \), \( K^+ \) currents were blocked by Cs\(^+\) in the internal pipette solution and 20 mM CsCl in the external solution. Addition of CsCl to the external solution was not osmotically compensated. In some experiments, a ruptured patch recording method was used to dialyze intracellular contents with internal pipette solution. When the ruptured patch method was used, the internal pipette solution was the same as indicated above except that EGTA concentration was 10 mM and CaCl\(_2\) concentration was 0.44 mM (pCa 7). In the ruptured patch configuration, the liquid junction potential (10 mV) measured between the internal pipette and bath solutions was subtracted from all voltage measurements. A single suction pipette recorded ionic currents (switch clamp) using an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA) in both perforated and ruptured patch recording configurations. The switch clamp precludes the need to compensate for series resistance. When filled with internal solution, the pipette tip resistance was \(-3\) M\(\Omega\). In the perforated patch configuration, access resistance was \(-15-20\) M\(\Omega\), and in the ruptured patch configuration, access resistance was \(-10\) M\(\Omega\). The sampling rate of the switch clamp was \(-10-12\) kHz, and a second oscilloscope was used to monitor the duty cycle to ensure that the voltage transient settled between cycles. Computer software (pCLAMP 6.2; Axon Instruments) was used to deliver voltage protocols and to acquire and analyze data. In addition, all signals were digitally recorded on videocassette recorder tape.

Generally, \( I_{\text{Ca,L}} \) was activated by clamp steps from a holding potential of \(-40\) to 0 mV for 200 ms every 5 s. This voltage protocol avoids activation of fast Na\(^+\) and T-type Ca\(^{2+}\) currents. In the experiments using a rupture patch recording method, rundown of \( I_{\text{Ca,L}} \) stabilized within \(-6\) min of rupturing the patch. In general, the extent of rundown was variable from cell to cell. In three cells, rundown decreased peak \( I_{\text{Ca,L}} \) by \(48\pm6\%\). The effects of Gen on \( I_{\text{Ca,L}} \) were recorded after rundown of \( I_{\text{Ca,L}} \) stabilized. Peak \( I_{\text{Ca,L}} \) was measured with respect to steady-state current and was not compensated for leak currents. The time constant of \( I_{\text{Ca,L}} \) inactivation was best fit as a single exponential using Clampfit (pCLAMP 6.2). Statistical significance of paired and unpaired data were determined by Student’s t-test at P values <0.05. Data are expressed as means \pm SE. The animal procedures used in this study were in accordance with the guidelines of the Animal Care and Use Committee of Loyola University Medical Center.

Drugs and chemicals used in this study include Gen, daidzein, sodium orthovanadate, and 1,2-bis(2-aminophenox) ethane-N,N,N',N'-tetraacetic acid (BAPTA)-acetoxymethyl ester (AM) (Sigma Chemical); H-89 (N-[2-bromoindinylamino]-5isoquinolinesulfonamide) (Seikagaku America); the Rp diastereomer of adenosine 3',5'-cyclic monophosphothioate (Rp-cAMPS) and chelerythrine chloride (LC Laboratories); nystatin (ProgressiveAgriSystems); and caphostin C (Kamiya Biomedical). Gen and H-89 were prepared as stock solutions in DMSO. Final concentration of DMSO was 0.05% and had no effect on basal \( I_{\text{Ca,L}} \). Sodium orthovanadate was prepared at its final concentration in buffered Tyrode solution.

**RESULTS**

Figure 1, A–C, shows the effects of 50 \(\mu M\) Gen on selected original records of basal \( I_{\text{Ca,L}} \) (A), consecutive measurements of peak \( I_{\text{Ca,L}} \) amplitude (B), and mean percent changes in peak \( I_{\text{Ca,L}} \) (C). Exposure to Gen elicited an initial inhibition of \( I_{\text{Ca,L}} \) (Fig. 1Aa, phase 1; Ba) that was followed by stimulation of \( I_{\text{Ca,L}} \) above control levels (Fig. 1Ab, phase 2; Bb). Phase 2 stimulation of \( I_{\text{Ca,L}} \) remained relatively constant during exposure to Gen (Fig. 1Bb). After 2 min of exposure, removal of Gen elicited a prominent additional increase or potentiation in \( I_{\text{Ca,L}} \) amplitude (Fig. 1Ac, phase 3; Bc) and then returned \( I_{\text{Ca,L}} \) to control levels. Gen had no effect on the holding current. In a total of 46 cells (Fig. 1C), Gen-induced changes in peak \( I_{\text{Ca,L}} \) elicited during phase 1 inhibition, phase 2 stimulation, and phase 3 potentiation were \(-55\pm4\%\) (P < 0.001), 34 \pm 9\% (P < 0.01), and 152 \pm 19\% (P < 0.001), respectively, compared with control. Phase 3 potentiation represents an additional increase of 118\% above the level of phase 2 stimulation. Measured in 14 cells, from the onset of...
exposure to Gen, phase 1 inhibition and phase 2 stimulation reached their peaks by 30 ± 5 and 69 ± 5 s, respectively. From the withdrawal of Gen, phase 3 potentiation of I_{Ca,L} reached its peak at 40 ± 6 s, and I_{Ca,L} required 118 ± 11 s to return to control levels. These results indicate that exposure to Gen elicits a biphasic effect on I_{Ca,L} amplitude, an initial inhibition followed by a secondary stimulation. The biphasic nature and the fact that the stimulatory component is potentiated when Gen is withdrawn suggests that Gen is acting via two separate and competing signaling mechanisms. Because phase 2 stimulation is the net result of two opposing effects, its magnitude was more variable than either phase 1 or phase 3. Hence, the amplitude of I_{Ca,L} during phase 2 was usually, but not always, larger than control I_{Ca,L}. We have adopted the terminology phase 2 stimulation because after phase 1 inhibition, I_{Ca,L} amplitude invariably increased with time during continued exposure to Gen, even though it did not always become larger than control I_{Ca,L}.

Next, we determined a dose-response relationship for Gen-induced changes in peak I_{Ca,L}. The graphs in Fig. 2, A–C, show the phase 1 (A), phase 2 (B), and phase 3 (C) responses to different Gen concentrations ranging from 0.1 to 100 µM. In general, phase 1 inhibition (Fig. 2A) was directly related to the Gen concentration, phase 2 stimulation showed biphasic changes in relation to control, and phase 3 potentiation (Fig. 2C) increased with Gen concentrations. More specifically, Gen concentrations <1 µM had no significant effects on I_{Ca,L}. Exposure to 1 µM Gen failed to elicit significant phase 1 or phase 2 changes, although withdrawal of 1 µM Gen elicited a significant phase 3 increase in I_{Ca,L}. At 10 µM Gen, phase 1 inhibition became significant and phase 3 potentiation increased. At 100 µM Gen, phase 1 inhibition and phase 3 potentiation were further increased and phase 2 stimulation became significant. The direction and amplitude of phase 2 depended on the net Gen-induced effects on I_{Ca,L}. At Gen concentrations ≤10 µM, the net stimulatory effect resulted in phase 2 stimulation above control levels. At 100 µM Gen, the more prominent inhibitory effect resulted in phase 2 stimulation that did not exceed control levels. These results indicate that Gen induces a dose-dependent inhibition and stimulation of I_{Ca,L} and that phase 2 was biphasic as a result of the net effect of these two opposing responses. It is worth mentioning that because of the apparent competition between the stimulatory and inhibitory components, the response to each Gen concentration and therefore the sensitivity of each component is probably underestimated in these experiments.

If one of the component changes in I_{Ca,L} induced by Gen is related to a soluble cytosolic factor(s), then dialysis of intracellular contents may provide a means of separating the two effects of Gen. We therefore tested 50 µM Gen while recording I_{Ca,L} using a ruptured patch, rather than a perforated patch, recording method. Figure 3, A–C, shows the effect of Gen on selected original I_{Ca,L} traces (A), consecutive measurements of peak I_{Ca,L} (B), and mean percent changes in peak I_{Ca,L} (C). In these experiments, Gen was tested after the rundown of I_{Ca,L} had stabilized. Gen elicited an initial phase 1 inhibition of −85% (Fig. 3, Ab and Bb) that diminished only slightly during continued exposure to Gen (Fig. 3, Ac and Bc). As a result, during phase 2, I_{Ca,L} amplitude still was inhibited significantly compared with control. Withdrawal of Gen returned I_{Ca,L} to control levels with no phase 3 potentiation (Fig. 3, Ad and Bd). In other words, withdrawal of Gen simply removed the inhibitory component. In a total of six cells tested (Fig. 3C), Gen-induced changes during phase 1 and “phase 2” were −68 ± 9 and −57 ± 8%, respectively, and withdrawal of Gen simply returned I_{Ca,L} to baseline. In two additional cells when Gen was tested
within 3 min of rupturing the membrane patch, i.e., before rundown of $I_{\text{Ca,L}}$ had stabilized, the stimulatory components were still evident. These results indicate that dialysis of the cell interior eliminated the phase 2 stimulation and phase 3 potentiation typically induced by Gen when recordings are performed using a perforated patch method. In addition, they indicate that both stimulatory phases (2 and 3) induced by Gen are due to the same mechanism that involves a soluble cytosolic factor(s). On the other hand, the inhibitory effects of Gen result from a membrane-bound mechanism.

To assess the relative specificity of Gen action, we examined the effects of 50 µM daidzein, a weakly active analog of Gen (1), on $I_{\text{Ca,L}}$. The effects of 50 µM Gen and 50 µM daidzein were tested in the same atrial myocytes to ensure that cells exhibited a typical response to Gen before being tested with daidzein. In a total of four cells studied, Gen elicited phase 1 inhibition (−24 ± 3%), phase 2 stimulation (212 ± 52%), and phase 3 potentiation (303 ± 43%) of $I_{\text{Ca,L}}$. Daidzein exerted no significant inhibitory or stimulatory effects on $I_{\text{Ca,L}}$, and withdrawal of daidzein had no effect on $I_{\text{Ca,L}}$.

Effects elicited by Gen-induced inhibition of PTK activities depend on intact protein tyrosine phosphatase (PTPase) activity to dephosphorylate tyrosine residues. Vanadate (Van) enhances tyrosine phosphorylation by inhibiting PTPase activities (32, 34) and thereby can prevent the effects of Gen that are mediated by PTK inhibition. As shown Fig. 4, A and B, we therefore tested Gen in the absence and then presence of 1 mM Van to determine whether the inhibitory and/or stimulatory effects of Gen are mediated via inhibition of PTK activities. Cells were exposed to Van for 4 min between the first and second exposure to Gen. The graph in Fig. 4A shows consecutive measurements of peak $I_{\text{Ca,L}}$ obtained from a single atrial myocyte. Under control...
conditions, 50 µM Gen elicited phase 1 inhibition (−33%), a prominent phase 2 stimulation (110%), and phase 3 potentiation (143%) of I_{Ca,L}. Van alone decreased I_{Ca,L} by −8%. It should be noted, however, that after the initial exposure to Gen, I_{Ca,L} stabilized at a level somewhat higher than control. As a result, Van simply decreased I_{Ca,L} back to the control level, probably by inhibiting a residual stimulatory effect of Gen. In the presence of Van, Gen-induced phase 1 inhibition was abolished, phase 2 stimulation was significantly attenuated, and phase 3 potentiation of I_{Ca,L} was abolished. As summarized in Fig. 4B, in the five cells studied, under control conditions Gen induced phase 1 inhibition (−30 ± 5%), phase 2 stimulation (100 ± 15%), and phase 3 potentiation (134 ± 34%) of I_{Ca,L}. In the presence of Van, Gen-induced changes in phases 1, 2, and 3 were −3 ± 2, 18 ± 5, and 17 ± 6%, respectively (P < 0.05). In two control cells, in two consecutive exposures to Gen separated by a 4-min period elicited typical changes in I_{Ca,L} that were not significantly different from one another (data not shown). On the basis of the mean value for the five cells, Van alone had no significant effect on peak I_{Ca,L} amplitude (−2 ± 6%). This mean value, however, was obtained from individual experiments where Van elicited variable changes in I_{Ca,L} among the different cells tested. Specifically, Van decreased I_{Ca,L} in three cells (−8, −15, and −6%), increased I_{Ca,L} in one cell (22%), and in a fifth cell, Van had no effect compared with control. Van also elicited small and variable changes in I_{Ca,L} inactivation that were not significant (2 ± 4%; n = 5). Because Van always was administered after an initial exposure to Gen, it seems likely that the variable effects of Van alone were influenced by the residual effects resulting from the initial exposure to Gen (see Fig. 4A). Nevertheless, the present results indicate that regardless of the effects of Van on I_{Ca,L}, Van blocked both the inhibitory and stimulatory effects of Gen.

Although Van is a potent inhibitor of PTPase activity (32, 34), it has been reported to exert other effects as well (3, 10, 23). For example, in relation to the present study, Van may inhibit serine/threonine phosphatase activity and/or act as a P1 analog, thereby interfering with cellular mechanisms involving serine/threonine phosphorylation or ATP hydrolysis. To access these possibilities, we tested Van in cells where I_{Ca,L} had been prestimulated by isoproterenol (Iso) at a concentration (0.02 µM) that submaximally stimulates I_{Ca,L}. If Van significantly inhibits serine/threonine phosphatase activity or interferes with ATP hydrolysis, it should significantly alter β-adrenergic stimulation of I_{Ca,L}. Figure 5 shows a typical experiment in which Iso alone increased peak I_{Ca,L} by 177%. The addition of 1 mM Van to the Iso-stimulated cell had no effect on peak I_{Ca,L} amplitude. When Iso was withdrawn, leaving the cell in the presence of Van, I_{Ca,L} amplitude returned to within 28% of control. This return of I_{Ca,L} toward control would not be expected if Van acted to significantly inhibit serine/threonine phosphatase activity or stabilize the transition state for phosphotransfer. Removal of Van returned I_{Ca,L} back to control. In a total of three cells, the effects of Iso on I_{Ca,L} amplitude in the absence and presence of Van were 123 ± 34 and 120 ± 32%, respectively, and the withdrawal of Iso returned I_{Ca,L} amplitude to within 30% of control. These findings provide support for our interpretation that under the present experimental conditions Van is blocking the effects of Gen on I_{Ca,L} primarily via inhibition of PTPase activities.

In a few cells studied, Gen elicited an atypical effect on I_{Ca,L} that may provide insight into the underlying mechanisms of Gen action. Figure 6 shows selected recordings of I_{Ca,L} (A) and consecutive measurements of peak I_{Ca,L} (B) recorded from the same atrial myocyte. Exposure to 50 µM Gen failed to induce phase 1 inhibition of I_{Ca,L} and instead elicited only marked stimulation of I_{Ca,L} (109%) above control. Withdrawal of Gen induced a small potentiation of I_{Ca,L} (129%) above control, an additional increase of 20%. These Gen-induced stimulatory effects on I_{Ca,L} were blocked by 1 mM Van (data not shown). In a total of five cells that showed this type of response, Gen stimulated I_{Ca,L} by 123 ± 35%, and withdrawal of Gen potentiated I_{Ca,L} to 150 ± 42% above control, an additional increase of 27%. In the presence of Van, Gen-induced stimulation (7 ± 3%) and potentiation (19 ± 2%) were abolished (n = 2). This type of response was selected to demonstrate that Gen could elicit a prominent stimulation of I_{Ca,L} via PTK signaling in the absence of any significant inhibition of I_{Ca,L}.

PTK signaling may interact with protein kinase A (PKA) or protein kinase C (PKC) signaling mechanisms that regulate I_{Ca,L}. Therefore, in Fig. 7, A and B, we tested the effect of H-89, an inhibitor of PKA (5), and calphostin C, a specific inhibitor of PKC (19), respectively, on Gen-induced changes in I_{Ca,L}. Previous work from this laboratory has shown that H-89 (35) and calphostin C (36) are effective in blocking PKA- and PKC-mediated regulation of ion channels, respectively, in these atrial myocytes. Exposure to 50 µM Gen in the
absence and presence of 4 µM H-89 showed no differences in Gen-induced changes in $I_{Ca,L}$ (Fig. 7A). Likewise, cells exposed to 50 µM Gen in the absence and then presence of 0.1 µM calphostin C showed no differences in Gen-induced changes in $I_{Ca,L}$ (Fig. 7B). In three additional cells, 50 µM Rp-cAMPS, a more specific inhibitor of PKA (6), failed to affect either the inhibition or stimulation of $I_{Ca,L}$ induced by Gen (data not shown). Previous work has shown that superfusion of cat atrial myocytes with Rp-cAMPS blocks cAMP-mediated stimulation of $I_{Ca,L}$ (35). Moreover, 2 µM chelerythrine, another specific PKC inhibitor (13), had no significant effect on Gen-induced changes in $I_{Ca,L}$ ($n = 3$) (data not shown). The present results, therefore, indicate that the effects of Gen on basal $I_{Ca,L}$ are not mediated via secondary interactions between PTK signaling and PKA or PKC signaling mechanisms.

Nitric oxide (NO) also can modulate atrial $I_{Ca,L}$ amplitude via second messenger cGMP signaling mechanisms (17, 38). To determine whether the effects of Gen may be mediated via NO signaling, Gen was tested in the absence and presence of 100 µM N(G)-monomethyl-L-arginine (L-NMMA), an inhibitor of NO synthase activity (18). Under control conditions, 50 µM Gen elicited typical phase 1 ($-62 \pm 3\%$), phase 2 ($43 \pm 21\%$), and phase 3 ($55 \pm 18\%$) changes in $I_{Ca,L}$. After recovery from Gen, exposure to L-NMMA alone slightly decreased $I_{Ca,L}$ ($-7 \pm 2\%$). In the presence of L-NMMA, Gen-induced changes in $I_{Ca,L}$ during phase 1 ($-55 \pm 18\%$), phase 2 ($68 \pm 25\%$), and phase 3 ($78 \pm 34\%$) were not significantly different from control responses.

Because these experiments are performed with the perforated patch method, it is possible that secondary alterations in intracellular Ca$^{2+}$ may contribute to the effects of Gen on $I_{Ca,L}$. We therefore tested the effects of Gen in cells exposed to 100 µM BAPTA-AM, a cell-permeable Ca$^{2+}$ chelator. BAPTA-AM abolished visible contractile activity associated with activation of $I_{Ca,L}$, slowed $I_{Ca,L}$ inactivation ($-18 \pm 7\%$), and increased peak $I_{Ca,L}$ amplitude ($16 \pm 4\%$). These changes are consistent with the effect of BAPTA to bind intracellular Ca$^{2+}$ and reduce Ca$^{2+}$ concentration close to the channel. In a total of seven cells tested, in the presence of BAPTA-AM, 50 µM Gen elicited a typical phase 1 inhibition ($-28 \pm 4\%$), phase 2 stimulation ($+26 \pm 14\%$), and phase 3 potentiation ($+69 \pm 20\%$) of $I_{Ca,L}$. In three additional cells, we found that 1 µM ryanodine, an alkaloid that depletes intracellular Ca$^{2+}$ stores (7) and abolished visible contractile activity, had no effect.
on Gen-induced changes in $I_{Ca,L}$ (data not shown). Another consideration is that cells held at $-40$ mV are close to the theoretical equilibrium potential for Na$^+$/$Ca^{2+}$ exchange. This could inhibit Ca$^{2+}$ efflux, allowing intracellular Ca$^{2+}$ to increase. We therefore performed a voltage-damp protocol where the cell was held at $-80$ mV between pulses and then ramped to $-40$ mV immediately before activation of $I_{Ca,L}$. With the use of this protocol in a total of five cells, Gen elicited typical phase 1 inhibition ($-43 \pm 9\%$), phase 2 stimulation ($+30 \pm 7\%$), and phase 3 potentiation ($+134 \pm 40\%$) of $I_{Ca,L}$. Together, these results indicate that changes in intracellular free Ca$^{2+}$ or intracellular Ca$^{2+}$ release from internal stores are not factors in either the inhibitory or stimulatory effects of Gen on $I_{Ca,L}$.

**DISCUSSION**

The present study indicates that Gen elicits a biphasic effect on $I_{Ca,L}$ that is mediated via two competing PTK signaling mechanisms: an initial inhibitory component that is overcome by a secondary stimulatory component. These two components appear to result from independent signaling mechanisms. Thus, during intracellular dialysis, Gen could elicit the inhibitory component in the absence of the stimulatory response. Moreover, in some cells, a prominent stimulatory response could be elicited by Gen in the absence of any significant inhibitory response. Competition between these two opposing mechanisms also is evident in the variable phase 2 changes in $I_{Ca,L}$ induced by any given concentration of Gen, and in the biphasic changes in phase 2 that were dose dependent. Furthermore, when the stimulatory component was eliminated by cell dialysis, phase 1 inhibition changed from a transient to a sustained response. In addition, cell dialysis eliminated both phase 2 and 3 stimulatory components and showed that the withdrawal of Gen simply removed a sustained inhibitory component, returning $I_{Ca,L}$ to baseline. These results clearly indicate that the phase 3 potentiation of $I_{Ca,L}$ typically elicited by withdrawal of Gen resulted from the rapid removal of an inhibitory component competing with a more sustained stimulatory component. This also is consistent with the relatively small potentiation of $I_{Ca,L}$ elicited by withdrawal of Gen in the few cells in which Gen failed to elicit an inhibitory component. This last observation raises an interesting point; although Gen failed to elicit any noticeable inhibition of $I_{Ca,L}$, withdrawal of Gen still induced a small potentiation of $I_{Ca,L}$. This suggests that the relatively large stimulatory response of these particular cells masked a smaller inhibitory effect of Gen and that upon withdrawal of Gen the underlying inhibitory signal induced by Gen was removed, resulting in a small potentiation of $I_{Ca,L}$. The present findings also indicate that the mechanisms underlying Gen-induced inhibition of $I_{Ca,L}$ are more rapid in onset and more rapidly removed than those underlying stimulation of $I_{Ca,L}$. However, at Gen concentrations $\geq 50 \mu$M, the stimulatory component is more potent and can overcome the inhibitory component. Therefore, at relatively low Gen concentrations, the stimulatory component is able to attenuate or even mask the inhibitory effect on $I_{Ca,L}$.

These findings can be interpreted in terms of several different PTKs that could potentially be inhibited by Gen. PTKs are categorized into two general groups: receptor-operated and non-receptor-operated (33). Receptor-operated PTK activities are considered membrane bound, whereas non-receptor-operated PTK activities are soluble, cytosolic components that may be compartmentalized within the cell. Also, there are membrane-associated PTKs such as the Src (9) and Jak families (16) that can dissociate from the membrane once activated and affect cytosolic signaling mechanisms. Several findings support the idea that Gen-induced inhibition and stimulation of $I_{Ca,L}$ are both mediated via inhibition of PTK activities. First, the effects of Gen could not be mimicked by daidzein, an analog of Gen but weak inhibitor of PTK activity. Moreover, the dose-response relationship suggests that the half-maximal inhibitory and stimulatory concentrations of Gen are $\approx 50 \mu$M, which is well within the concentration range for specific PTK inhibition (1). In addition, Van, a potent inhibitor of PTPase activities, essentially abolished both the inhibitory and stimulatory effects of Gen. As discussed earlier, Van can exert various nonspecific effects (3, 10, 23). The present results, however, suggest that under our experimental conditions, the ability of Van to block Gen-induced changes in $I_{Ca,L}$ was due to inhibition of PTPase activities. Thus Van failed to affect Iso-induced stimulation of $I_{Ca,L}$. This finding would not be expected if Van acted nonspecifically as an inorganic phosphate analog or via inhibition of serine/threonine activity. Moreover, this last observation is consistent with the present findings that the effects of Gen are not mediated via PKA- or PKC-mediated signaling. Although Van probably does exert various effects, the most likely explanation of its actions in the present experiments is via inhibition of PTPase activities. Taken together, the present results suggest that Gen-induced inhibition of $I_{Ca,L}$ is mediated via inhibition of membrane-bound or -associated PTKs, and Gen-induced stimulation of $I_{Ca,L}$ is mediated via inhibition of cytosolic PTK activities.

As alluded to earlier, receptor-operated or membrane-associated PTKs also may interact with cytosolic PTKs to affect downstream signal transduction mechanisms that could stimulate $I_{Ca,L}$. Although this possibility is not excluded by these experiments, several of the present observations make it unlikely. First, prominent stimulatory responses could be elicited by Gen in the absence of any significant inhibitory effects on $I_{Ca,L}$. In addition, this mechanism is not consistent with the present finding that removal of the inhibitory component by withdrawal of Gen potentiated the stimulatory response. Moreover, Gen-induced changes in $I_{Ca,L}$ were not mediated via secondary interactions with PKA or PKC pathways or regulated by intracellular Ca$^{2+}$ signaling. This makes it unlikely that certain PTK activities that are activated by intracellular Ca$^{2+}$ and/or PKC activities, such as PYK2 (21), are involved in the effects of Gen. Likewise, other Ca$^{2+}$-dependent signaling...
mechanisms such as activation of Ca^{2+}/calmodulin-dependent protein kinase II (8) or Ca^{2+}-dependent NO synthase (18) are probably not involved either. The latter statement is further supported by the present finding that inhibition of NO synthase activity by L-NMMA had little effect on Gen-induced changes in I_{Ca, L}. That intracellular Ca^{2+} does not play a role in the effects of Gen also makes it unlikely that intracellular dialysis eliminated the stimulatory component by buffering intracellular Ca^{2+}. Of course, the present experiments are not exhaustive, and therefore, it is possible that other signaling pathways not examined in the present study and modulated by PTK signaling may contribute to the effects of Gen on I_{Ca, L}.

In contrast to the present findings, there are reports indicating that Gen may directly block membrane channels by a mechanism unrelated to PTK inhibition. For example, in vascular smooth muscle cells, Gen blocks K^-currents by a mechanism independent of ATP utilization and insensitive to inhibition by vanadate (29). In rat brain neurons, Gen inhibited Na^-influx and Na^-current, but daidzein also elicited similar inhibitory effects as Gen, albeit at higher concentrations than Gen (22). In cardiac ventricular myocytes, Gen and daidzein both elicited similar inhibition of I_{Ca, L} (4, 40). Clearly, these findings differ significantly from those presented here, where the effects of Gen on I_{Ca, L} were not mimicked by daidzein and were effectively blocked by vanadate.

An important aspect of the present study is that we used a perforated patch recording method to study PTK-mediated regulation of I_{Ca, L}. The studies cited above each used whole cell ruptured patch methods. The importance of maintaining the intracellular milieu is evident from the present experiments where cell dialysis using the ruptured patch method abolished the stimulatory component, leaving only Gen-induced inhibition of I_{Ca, L}. This finding may have bearing on the interpretation of studies in ventricular myocytes where recordings performed with the ruptured patch method indicated that Gen elicits only inhibition of I_{Ca, L} (4, 40). This raises the question of whether the ruptured patch recording method influenced the results obtained in ventricular myocytes and the possibility that Gen may also elicit a stimulatory effect on I_{Ca, L} in those cells. In fact, in cat ventricular myocytes, Gen elicited a phase 1 inhibition (\( -54 \pm 6 \)%), phase 2 stimulation (\( -33 \pm 4 \)%) and phase 3 potentiation (\( 23 \pm 8 \%) (n = 10), indicating that the stimulatory components were present but significantly smaller in magnitude than in cat atrial myocytes (37).

The present results suggest that Gen elicits both an inhibitory and stimulatory effect on I_{Ca, L} via dephosphorylation of different tyrosine residues that compete in the regulation of I_{Ca, L}. Others have reported that dual regulation of cardiac I_{Ca, L} also can be achieved by phosphorylation of serine/threonine residues where cGMP enhances cAMP/PKA activity to stimulate or protein kinase G activity to inhibit I_{Ca, L} (26, 35). In the present experiments, the net steady-state effect of Gen on I_{Ca, L} appears to be a balance between two opposing PTK signaling mechanisms. These results therefore suggest that atrial muscle functions mediated by I_{Ca, L} may be strongly regulated by neurotransmitters or hormones that act via receptor-operated and/or non-receptor-operated PTK signaling mechanisms.

We thank the late Christine E. Rechenmacher for expert technical assistance with these studies. In addition, we thank Drs. Pamela Lucchesi and Alan Samarel for very helpful discussions regarding these experiments.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-27652. Address for reprint requests: S. L. Lipsius, Dept. of Physiology, Loyola University Medical Center, 2160 S. First Ave., Maywood, IL 60153.

Received 9 October 1997; accepted in final form 2 April 1998.

REFERENCES


