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Genistein Inhibits Cardiac L-Type Ca^{2+} Channel Activity by a Tyrosine Kinase-Independent Mechanism

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ABSTRACT

It has been suggested that protein tyrosine kinase (PTK) activity can directly regulate cardiac L-type Ca^{2+} channels. This conclusion is based to a large extent on the observation that the PTK inhibitor genistein can inhibit the cardiac L-type Ca^{2+} current. The purpose of the present study was to determine whether the ability of genistein to inhibit cardiac L-type Ca^{2+} channel activity is due to inhibition of PTK activity. Genistein significantly reduced the magnitude of the L-type Ca^{2+} current in guinea pig ventricular myocytes recorded using the whole-cell patch-clamp technique. However, this effect was associated with extracellular, not intracellular, application of the drug. Peroxovanadate (PVN), a potent protein tyrosine phosphatase inhibitor, had no effect on the basal Ca^{2+} current. PVN was also ineffective in preventing the inhibitory effect of genistein. Internal perfusion of cells with a pipette solution containing ATPγS was used to prevent reversibility of phosphorylation-dependent processes. This treatment did not alter the inhibitory effect of genistein, although it did result in irreversible protein kinase A-dependent regulation of the Ca^{2+} current. Bath application of lavendustin A, a PTK inhibitor that is structurally unrelated to genistein, did not affect the Ca^{2+} current amplitude. The inhibitory effect of genistein was also associated with a hyperpolarizing shift in the voltage dependence of Ca^{2+} channel inactivation. These results are consistent with the conclusion that the cardiac L-type Ca^{2+} current is not directly regulated by PTK activity and that the inhibitory effect of genistein is due to direct non-catalytic blockade of the channels.

A growing body of experimental evidence accumulated over the last several years indicates that the activity of L-type Ca^{2+} channels can be directly regulated by protein tyrosine kinase (PTK)-dependent phosphorylation. Initially, this idea came from the finding that genistein, a specific PTK inhibitor, can inhibit basal L-type Ca^{2+} current in a number of preparations (Davis et al., 2001). Such observations are consistent with the conclusion that basal PTK activity produces a stimulatory effect on L-type Ca^{2+} channels. This conclusion has been substantiated by numerous studies involving smooth muscle and neuronal $Ca_v1.2$ channel isoforms. In vascular smooth muscle preparations, inhibitors of protein tyrosine phosphatases (PTPs) can stimulate the L-type Ca^{2+} channel current (Wijetunge et al., 1998; Wu et al., 2001). Furthermore, it has been reported that the activation of tyrosine kinases by platelet-derived growth factor (PDGF) or Src kinase-activating peptide as well as intracellular application of constitutively active Src (c-Src) kinase result in an augmentation of L-type Ca^{2+} channel current in smooth muscle cells (Wijetunge and Hughes, 1995a,b, 1996; Hu et al., 1998). In addition, the α_1 subunit of smooth muscle $Ca_v1.2$ channels was shown to coimmunoprecipitate with c-Src (Hu et al., 1998). This is consistent with the recent report by Bence-Hanulec et al. (2000) that insulin-like growth factor-1 (IGF-1) potentiates the L-type Ca^{2+} current in cultured cerebellar granule neurons through Src-mediated phosphorylation of a specific tyrosine residue (Tyr^{2122}) near the C terminus of the α_1 subunit of the neuronal $Ca_v1.2$ channel.

However, it remains unclear whether or not cardiac L-type Ca^{2+} channels can be directly regulated by PTK activity. On the one hand, in the study of Bence-Hanulec et al. (2000), IGF-1 did not potentiate activity of the cardiac L-type Ca^{2+} channel α_1 subunit. Furthermore, IGF-1 failed to produce an effect on the L-type Ca^{2+} current in ventricular myocytes (Sims et al., 2000). On the other hand, the ability of genistein to inhibit the basal L-type Ca^{2+} current in a variety of cardiac myocytes has been used as an argument to support the idea that these channels may be actually regulated by basal tyrosine kinase activity (Yokoshiki et al., 1996; Hool et al., 1998; Katsube et al., 1998; Wang and Lipsius, 1998; Ogura et al., 1999). Although genistein inhibits PTK activity with little or no effect on serine/threonine protein kinases, it can also produce effects that are unrelated to its ability to inhibit

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ABBREVIATIONS: PTK, protein tyrosine kinase; IGF-1, insulin-like growth factor-1; Iso, isoproterenol; PDGF, platelet-derived growth factor; PTP, protein tyrosine phosphatase; PVN, peroxovanadate; TEA, tetraethylammonium.

PTKs. For example, genistein has been reported to directly block ligand-gated (Huang and Dillon, 2000) and voltage-gated ion channels (Smirnov and Aaronson, 1995; Paillart et al., 1997; Washizuka et al., 1997). Therefore, the main objective of the present study was to investigate the contribution of PTK regulation to the inhibitory effect that genistein has on the L-type Ca^{2+} current in guinea pig ventricular myocytes.

Materials and Methods

Cell Isolation. Single ventricular myocytes were isolated from adult Hartley guinea pigs using a modification of a method described previously (Hool et al., 1998). The methods used in this study were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University. Briefly, animals were anesthetized by intraperitoneal injection of pentobarbital (150 mg kg^{-1}). Hearts were then quickly excised and the coronary arteries were perfused via the aorta with a solution containing 140 mM NaCl, 5.4 mM KCl, 2.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 11 mM glucose, and 5.5 mM HEPES, pH 7.4. The heart was perfused with this solution for 5 min, nominally Ca^{2+}-free solution for 5 min, and then nominally Ca^{2+}-free solution containing ~0.2 mg/ml collagenase (class B; Roche Diagnostics, Indianapolis, IN) for about 30 min. The ventricles were then removed and minced in a modified Kraft-Brühe solution containing 110 mM potassium glutamate, 10 mM KH_2PO_4, 25 mM KCl, 2 mM $MgSO_4$, 20 mM taurine, 5 mM creatine, 0.5 mM EGTA, 20 mM glucose, and 5 mM HEPES, pH 7.4. Single cells were obtained by filtering through nylon mesh. After settling, cells were resuspended in Ca^{2+}-containing solution and used on the day of isolation only.

Data Acquisition and Analysis. The L-type Ca^{2+} current was studied using the conventional whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Patch pipettes (1 to 2 MΩ) were filled with an intracellular solution containing 130 mM CsCl, 20 mM tetraethylammonium chloride (TEA-Cl), 5 mM MgATP, 5 mM EGTA, 0.1 mM Tris-GTP, and 5 mM HEPES, pH 7.2. In experiments employing ATPγS the following pipette solution was used: 120 mM CsCl, 20 mM TEA-Cl, 5 mM EGTA, 5 mM Li_4ATPγS, 5 mM $MgCl_2$, 0.1 mM Tris-GTP, and 5 mM HEPES, pH 7.2. Cells were bathed in a K^+-free control extracellular solution containing 140 mM NaCl, 5.4 mM CsCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 11 mM glucose, and 5.5 mM HEPES, pH 7.4. The voltage dependence and kinetics of L-type Ca^{2+} current inactivation were studied using the following extracellular solution: 100 mM TEA-Cl, 45.4 mM CsCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 11 mM glucose, and 5.5 mM HEPES, pH 7.4. Isolated myocytes were placed in a 0.5-ml chamber on the stage of an inverted microscope, where they were superfused with either control or drug containing extracellular solution at a rate of 1 to 2 ml/min. In some experiments, cells were exposed to different experimental solutions using a fast flow system. The system consisted of a cFlow 8 channel flow controller, cF-8VS valve assembly unit, and MPRE8 miniature manifold (Cell MicroControls, Norfolk, VA). This method allowed rapid (<1 s) changes in extracellular solutions bathing myocytes being voltage clamped. A 3 M KCl-agar bridge was used to ground the bath. All experiments were performed at room temperature.

Macroscopic currents were recorded using an Axopatch 200 voltage-clamp amplifier (Axon Instruments, Inc., Foster City, CA) and an IBM-compatible computer with a Digidata 1200 interface and pCLAMP software (Axon Instruments, Inc.). The voltage-clamp protocols employed a holding potential of −80 mV. The time course of changes in L-type Ca^{2+} current magnitude was monitored by applying a 50-ms prepulse to −30 mV and subsequent 100-ms test pulse to 0 mV once every 5 s. The magnitude of the L-type Ca^{2+} current evoked at test potentials of 0 mV was determined by measuring the peak inward current. Setting the Cl^- equilibrium potential equal to the test potential eliminated the cAMP-dependent Cl^- current from these Ca^{2+} current measurements. For current-voltage relationships, L-type Ca^{2+} currents were isolated by measuring the difference current obtained by subtracting currents recorded at each test potential in the absence and presence of 100 μM $CdCl_2$. Voltage dependence of Ca^{2+} current activation and inactivation were determined and analyzed by fitting data to Boltzmann equations as described previously (Belevych et al., 1999). All results are expressed as the mean ± S.E.M. of the results obtained from n number of cells. Statistical significance between two groups was defined by Student's t test P values of <0.05.

Drugs and Reagents. Genistein (Alexis Corp., San Diego, CA) and lavendustin A (Calbiochem, San Diego, CA) were prepared as stock solutions in dimethyl sulfoxide. To achieve the final concentrations used, these stock solutions were then diluted in external solution and sonicated prior to use. The final concentration of dimethyl sulfoxide in extracellular solutions was never more than 0.1%. It is important to note that in solutions containing 100 and 300 μM genistein, aggregates of crystals were clearly visible when viewed through the microscope. This suggests that under our experimental conditions, genistein at concentrations higher than 50 μM is not completely soluble in aqueous solution. PVN was prepared as previously described (Hool et al., 1998) by combining 10 mM H_2O_2 and 10 mM Na_3VO_4 in an aqueous solution containing 50 mM HEPES, pH 7.4. This mixture was allowed to stand at room temperature for 15 min, after which excess H_2O_2 was eliminated by adding catalase. The resulting stock solution contained a mixture of vanadate and peroxovanadate complexes (Posner et al., 1994). The final concentration of PVN used in our experiments is based on the concentration of Na_3VO_4 used in preparing the stock solution. All solutions containing genistein, PVN, and isoproterenol (Iso) were stored in light-resistant containers. All drugs were obtained from Sigma/RBI (Natick, MA), except where noted.

Results

Effect of Extracellular Genistein on the L-Type Ca^{2+} Current. Bath application of 50 μM genistein inhibited the amplitude of basal L-type Ca^{2+} current in guinea pig ventricular myocytes by 49 ± 1.9% (n = 34, Fig. 1). We used a fast flow system to rapidly exchange solutions bathing the cell and measure the onset of the inhibitory effect of genistein. The peak Ca^{2+} current measured within 5 s of exposure to genistein was already at a level equal to about 50% of the steady-state effect (Fig. 1A). The inhibitory effect of 50 μM genistein developed monoexponentially, with an average time constant of 11 ± 1.0 s (n = 11), and reached steady-state within 40 s. Upon washout of genistein, the amplitude of the L-type Ca^{2+} current returned to 88 ± 2.6% (n = 18) of its initial level with a time constant of 20 ± 0.4 s (n = 11). The apparent incomplete reversibility of the genistein effect can be explained by basal current rundown observed in some cells. It should be noted that 74% of the cells exposed to 50 μM genistein exhibited this type of inhibitory response. The remaining 26% exhibited an inhibitory response followed by a more slowly developing stimulatory reaction. This is consistent with previous reports that genistein can have both inhibitory and stimulatory effects (Hool et al., 1998; Wang and Lipsius, 1998). Cells displaying a biphasic response were not included in our analysis of the inhibitory effect of genistein.

The level of Ca^{2+} current inhibition produced by 50 μM genistein was independent of the voltage at which the effect was measured (Fig. 1C). Using the magnitude of the effect that genistein has on currents elicited by depolarizations to 0 mV, it is clear that this compound inhibited the L-type Ca^{2+}

current in a concentration-dependent manner (Fig. 1D). The relationship between the concentration of genistein used and the degree of inhibition observed is well described by a logistic equation where the concentration of genistein producing half-maximal inhibition (IC_{50}) is 20 ± 3.5 μM, the apparent Hill coefficient is 1.5 ± 0.34, and the maximal inhibitory effect is 68 ± 4.6%. However, it is important to point out that genistein at concentrations higher than 50 μM is poorly soluble in aqueous solutions (see *Materials and Methods*). Therefore, in solutions supposedly containing 100 and 300 μM genistein, the actual concentration in solution was most likely significantly less. Therefore, it is reasonable to assume that the estimated values for the IC_{50} and the maximal inhibitory effect are significantly underestimated.

Effect of Intracellular Genistein on the L-Type Ca^{2+} Current. If the genistein-induced reduction of the Ca^{2+} current is really due to inhibition of PTK activity, then one might expect that intracellular application of genistein would produce the same inhibitory response, and preempt any inhibitory effect produced by subsequent extracellular application of this compound. However, this was not the case (Fig. 2). The peak current density measured after 7 min of dialysis with a control pipette solution was 6.2 ± 0.50 pA/pF (n = 8). The peak current density measured after 7 min of dialysis with a pipette solution containing 50 μM genistein was 5.3 ± 0.44 pA/pF (n = 14). Although the L-type Ca^{2+} current density was 14% smaller in cells dialyzed with a pipette solution containing genistein, this difference was not statistically significant ($P > 0.2$, unpaired t test). Furthermore, cell dialysis with a pipette solution containing 50 μM genistein did not change the sensitivity of the L-type Ca^{2+} current to subsequent extracellular application of this compound (Fig. 2). In cells dialyzed with 50 μM genistein, exposure to 50 μM extracellular genistein resulted in inhibition of the Ca^{2+} current by 45 ± 1.9%. This is not significantly different from the magnitude of the response to the same concentration of genistein in cells dialyzed with a control pipette solution ($P > 0.2$, unpaired t test).

One possible explanation for the apparent lack of response to intracellular genistein is that the compound did not reach

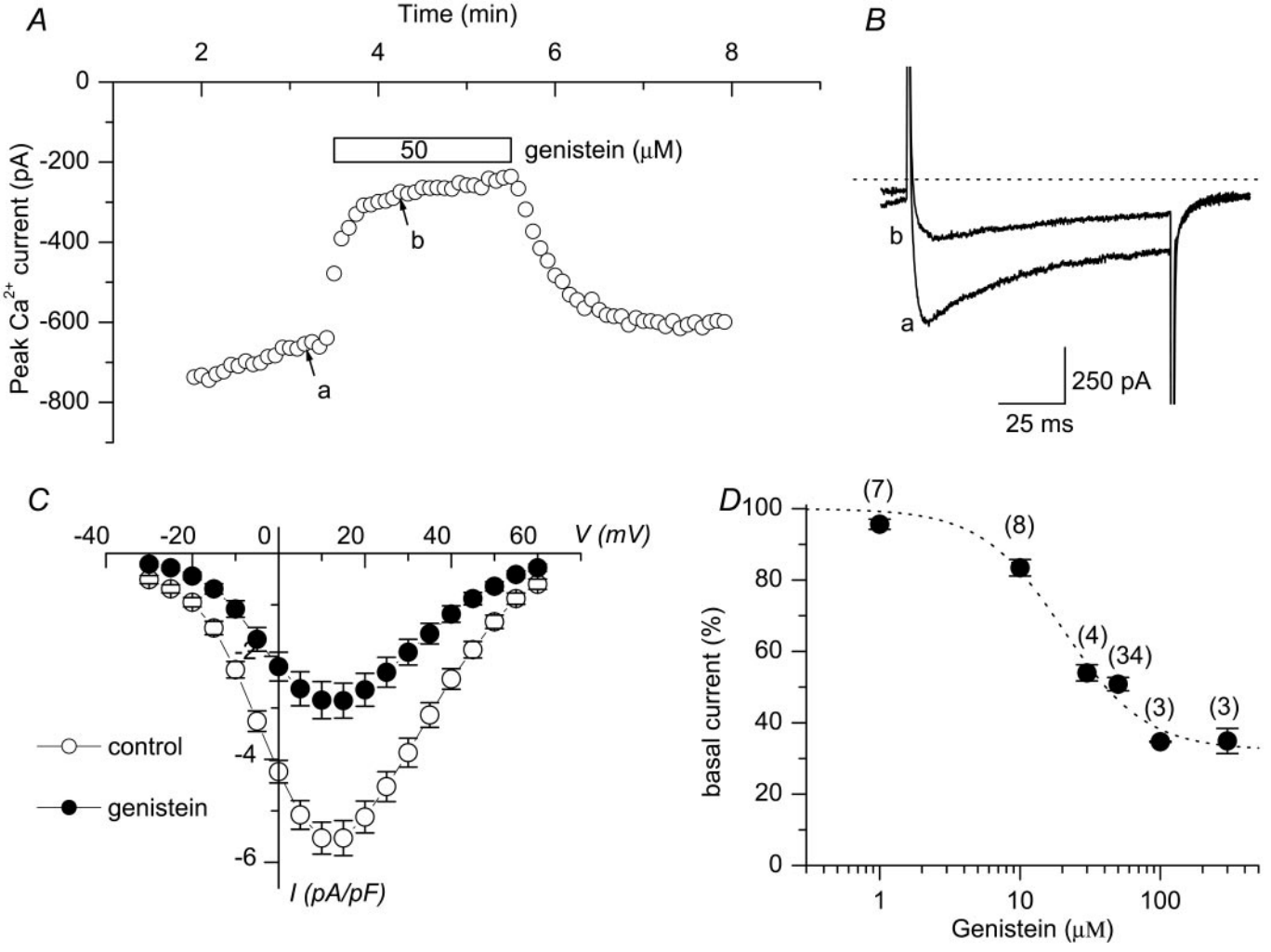

Fig. 1. Inhibitory effect of genistein on L-type Ca^{2+} current. A, time course of changes in amplitude of L-type Ca^{2+} current under control conditions (a) and during exposure to 50 μM genistein (b). B, examples of L-type Ca^{2+} current recorded under conditions indicated in panel A. The dotted line above current traces represents the zero current level. C, current-voltage relationships of peak L-type Ca^{2+} current density in the absence (○) and presence (●) of 50 μM genistein. Currents were evoked by 100 ms depolarizing steps to test potentials from −30 to 60 mV in 5 mV increments. D, dose-response relationship for the inhibitory effect (I) of genistein on L-type Ca^{2+} current. The dashed line represents the best fit of the data points to the equation: $(100 - I) = I_{max}/(1 + \{[genistein]/IC_{50}\}^k) + (100 - I_{max})$. The IC_{50}, apparent Hill coefficient (k), and maximal effect (I_{max}) were 20 ± 3.5 μM, 1.5 ± 0.34 and 68 ± 4.6%, respectively. The number of cells tested at each concentration of genistein is indicated in brackets.

significant levels within the cell. To ensure that genistein reached an effective level in the cytosol, we evaluated the response of the Ca^{2+} current to β-adrenergic stimulation using the agonist Iso (Fig. 2C). We previously reported that genistein significantly increases the sensitivity of L-type Ca^{2+} current to β-adrenergic stimulation in guinea pig ventricular myocytes (Hool et al., 1998). Therefore, if genistein in the pipette solution reached significant levels in the cytosol, we would expect to see an increased sensitivity to Iso. After 7 min of dialysis with a control pipette solution, exposure to 0.5 nM Iso increased the peak Ca^{2+} current amplitude by $42 \pm 12\%$ ($n = 8$) over baseline. When the same concentration of Iso was applied after 7 min of dialysis with a pipette solution containing 50 μM genistein, the peak Ca^{2+} current amplitude increased by $75 \pm 6.4\%$ ($n = 6$). This represents a significant increase in the sensitivity of the L-type Ca^{2+} current to β-adrenergic stimulation ($P < 0.05$, unpaired t test), suggesting that genistein had reached significant levels in the cytosol.

Effect of PVN on Genistein-Induced Inhibition of L-Type Ca^{2+} Current. Assuming that inhibition of PTK activity was responsible for inhibition of the Ca^{2+} current by extracellular genistein, then an increase in PTK-dependent phosphorylation might be expected to produce a stimulatory effect on this current. However, exposure to 100 μM PVN, a potent phosphotyrosine phosphatase (PTP) inhibitor (Posner et al., 1994), did not result in an increase in the amplitude of the L-type Ca^{2+} current. In fact, the current actually decreased by $15 \pm 4.0\%$ ($n = 5$, Fig. 3A). However, this small decrease was most likely due to current rundown and not a true inhibition of the current. The fact that the Ca^{2+} current did not respond to PVN suggests that either the L-type Ca^{2+} channels in guinea pig ventricular myocytes are not regulated by PTKs, or they have already been maximally stimu-

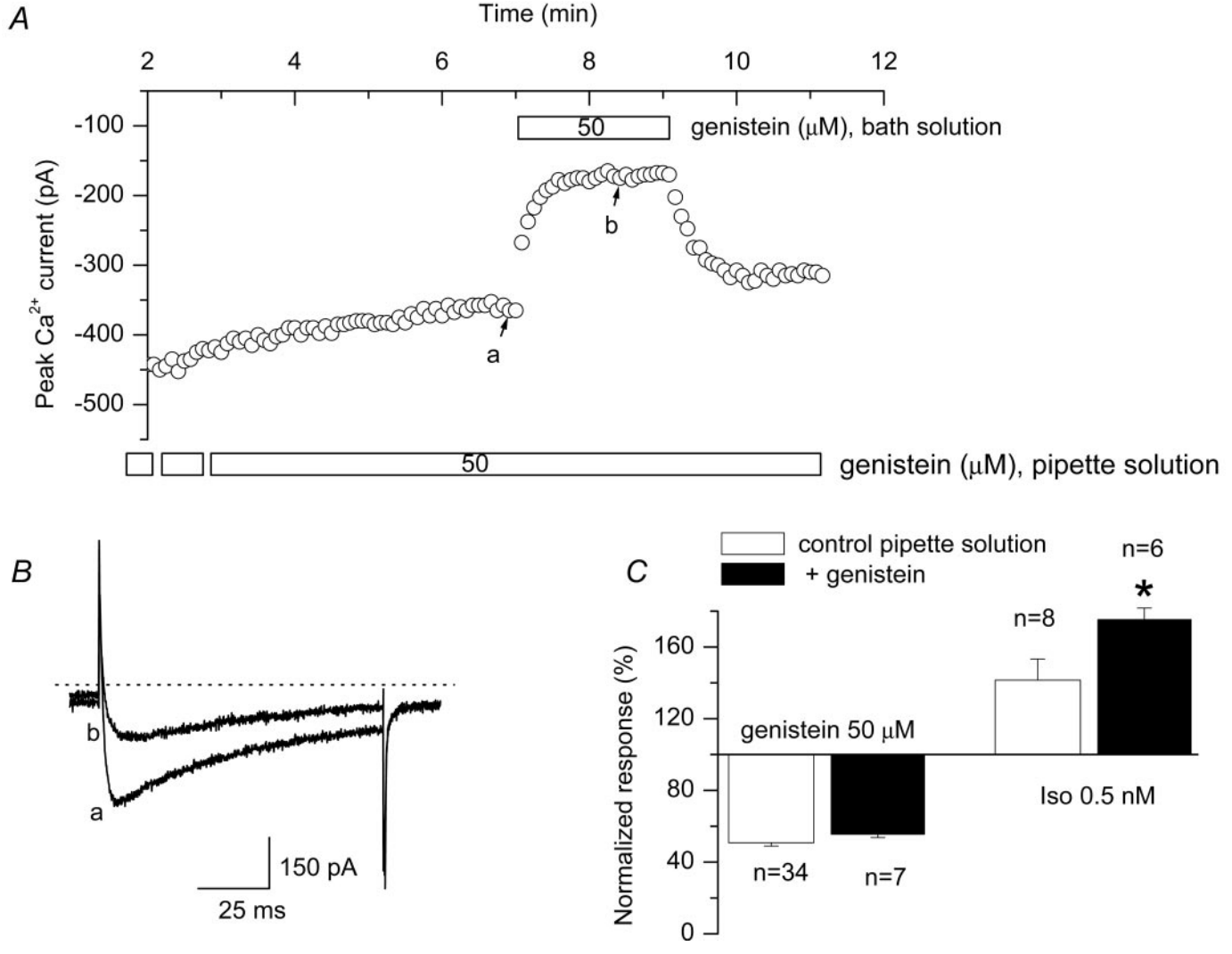

Fig. 2. Intracellular application of genistein does not prevent the inhibitory effect produced by extracellular application of genistein but increases the sensitivity of the Ca^{2+} current to β-adrenergic stimulation. A, time course of changes in amplitude of L-type Ca^{2+} current in cells dialyzed with a pipette solution containing 50 μM genistein before (a) and during (b) exposure to extracellular genistein (50 μM). Cells were dialyzed with a pipette solution containing 50 μM genistein for at least 7 min before extracellular application of 50 μM genistein. B, examples of L-type Ca^{2+} current recorded under conditions indicated in panel A. The dotted line above current traces represents the zero current level. C, inhibitory effect produced by extracellular application of genistein (50 μM) was not significantly different ($P > 0.2$, unpaired t test) in cells dialyzed with control pipette solution (□) and with a pipette solution containing 50 μM genistein (■). However, stimulatory effect of 0.5 nM Iso was significantly increased in cells dialyzed with pipette solution containing 50 μM genistein (⋆, $P < 0.05$, unpaired t test).

lated by basal PTK-dependent phosphorylation. If the latter were true, then inhibition of PTP activity would be expected to attenuate the inhibitory effect of genistein. However, application of genistein to cells pretreated with PVN was still able to inhibit the L-type Ca^{2+} current (Fig. 3, A and B). In the presence of 100 μM PVN, 50 μM genistein reduced the peak Ca^{2+} current by 56 $\pm$ 1.8% (n = 5), which is not significantly different from 49% inhibition observed under control conditions ($P > 0.1$, unpaired t test). Conversely, when cells were first exposed to genistein, subsequent addition of 100 μM PVN did not affect genistein-induced inhibition of the Ca^{2+} current (Fig. 3, C and D). The inhibitory effect of 50 μM genistein measured at the end of a 3- to 4-min application of 100 μM PVN was 45 $\pm$ 3.9% (n = 9), which was not significantly different from the 45 $\pm$ 2.0% reduction in L-type Ca^{2+} current amplitude measured just before PVN addition in the same cells ($P > 0.5$, paired t test).

If the effect of genistein is actually due to inhibition of PTK activity, the ability of PVN to antagonize this response would depend on its ability to cross the membrane and enter the cell. Vanadate compounds can readily cross the plasma membrane of cells (Posner et al., 1994). Nevertheless, to eliminate the possibility that the lack of response to extracellularly applied PVN was due to its inability to achieve significant levels inside patch-clamped cells, we examined the effect of direct intracellular application of PVN. However, dialyzing cells with a pipette solution containing PVN had no effect on the response to genistein (Fig. 4). In the presence of 100 μM PVN, extracellular application of 50 μM genistein reduced the magnitude of the Ca^{2+} current by 46 $\pm$ 5.0% (n = 8). This is not significantly different than the magnitude of the response to the same concentration of genistein in cells dialyzed with a control pipette solution ($P > 0.4$). To verify the effectiveness of intracellular application of PVN, we studied the Ca^{2+} current responses to Iso under control conditions and in the presence of intracellular PVN. We previously demonstrated that PTP inhibitors such as PVN antagonize β-adrenergic stimulation of the L-type Ca^{2+} current in these cells (Sims et al., 2000). If PVN were reaching sufficient levels inside the cell, we would expect the sensitivity to Iso to be significantly decreased. As demonstrated in Fig. 4C, the sensitivity of the L-type Ca^{2+} current to Iso was significantly reduced in cells dialyzed with a pipette solution containing 100 μM PVN. Under control conditions 10 nM and 10 μM Iso increased peak Ca^{2+} current by 232 $\pm$ 13.2 and 231 $\pm$ 13.2% (n = 5), respectively. In the presence of PVN, stimulatory

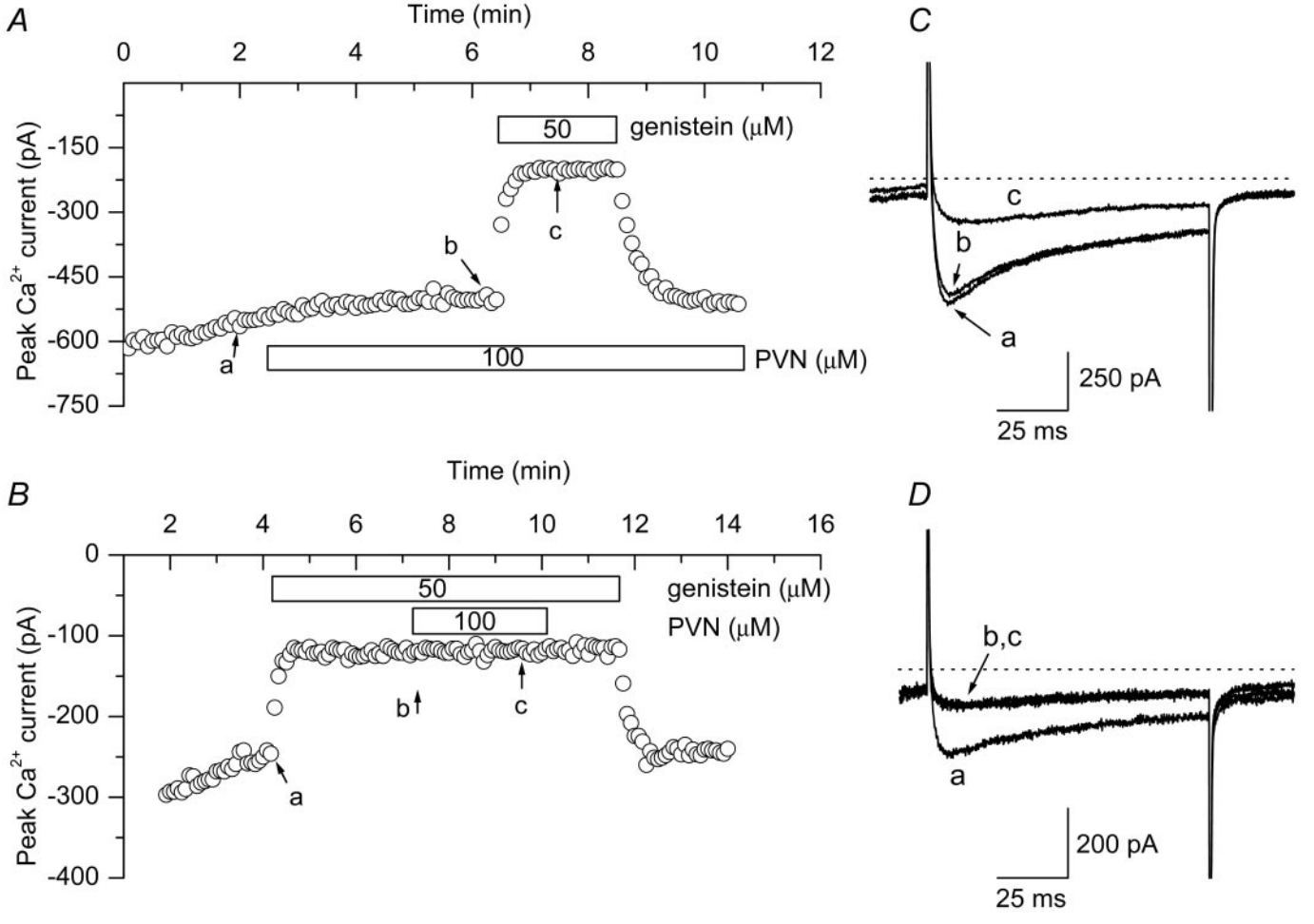

Fig. 3. The protein tyrosine phosphatase inhibitor PVN affects neither basal L-type Ca^{2+} current nor genistein-mediated inhibition of L-type Ca^{2+} current. A, time course of changes in amplitude of L-type Ca^{2+} current under control conditions (a), during exposure to 100 μM PVN alone (b), and 100 μM PVN plus 50 μM genistein (c). Cells were exposed to 100 μM PVN for at least 4 min before application of genistein. B, time course of changes in amplitude of L-type Ca^{2+} current under control conditions (a), during exposure to 50 μM of genistein alone (b), and 50 μM genistein plus 100 μM PVN (c). C and D, examples of L-type Ca^{2+} current recorded under conditions indicated in panels A and B, respectively. The dotted lines above current traces represent the zero current level.

effect of 10 nM Iso on L-type Ca^{2+} current was completely abolished, whereas 10 μM Iso increased amplitude of the current only by 97 $\pm$ 18% (n = 5). These data strongly suggest that the inhibitory effect that genistein has on the L-type Ca^{2+} current in guinea pig ventricular myocytes is not due to inhibition of PTK-dependent phosphorylation.

Effect of ATPγS on Genistein-Induced Inhibition of L-Type Ca^{2+} Current. Another piece of evidence arguing against the contribution of PTKs in genistein-mediated inhibition of cardiac L-type Ca^{2+} current was obtained from cells dialyzed with ATPγS, a non-hydrolyzable analog of ATP. ATPγS can substitute for ATP in kinase reactions. The product is thiophosphorylated proteins that are known to be poor substrates for both serine/threonine phosphatases (Gratecos and Fischer, 1974) and PTPs (Hiriyanna et al., 1994). Again, if the inhibition of basal PTK activity were the mechanism responsible for genistein's inhibitory effect, then dialysis of cells with a pipette solution containing ATPγS would be expected to produce irreversible, tyrosine thiophosphorylation of the Ca^{2+} channel protein (or other auxiliary proteins) and blunt the genistein-induced inhibition. However, in the presence of ATPγS, 50 μM genistein inhibited the amplitude of the L-type Ca^{2+} current by 53 $\pm$ 3.4% (n = 8, Fig. 5), which is not significantly different from the magnitude of the inhibitory effect observed in cells dialyzed with the control pipette solution ($P > 0.3$, unpaired t test). It should be noted that ATPγS alone produced a slowly developing stimulatory effect on the Ca^{2+} current. In myocytes dialyzed with a pipette solution containing ATPγS, the amplitude of the basal L-type Ca^{2+} current increased by 59 $\pm$ 22% (n = 8). The fact that the inhibitory response to genistein was not altered suggests that this increase in basal current was not due to PTK activity. As illustrated in Fig. 5, subsequent exposure to Iso produced further irreversible stimulation of the current, suggesting that ATPγS was effective in attenuating dephosphorylation associated with the activation of protein kinase A.

Effect of Lavendustin A on L-Type Ca^{2+} Current. A common approach used to determine whether an effect produced by genistein is due to inhibition of PTK activity is to determine whether or not structural analogs such as daid-

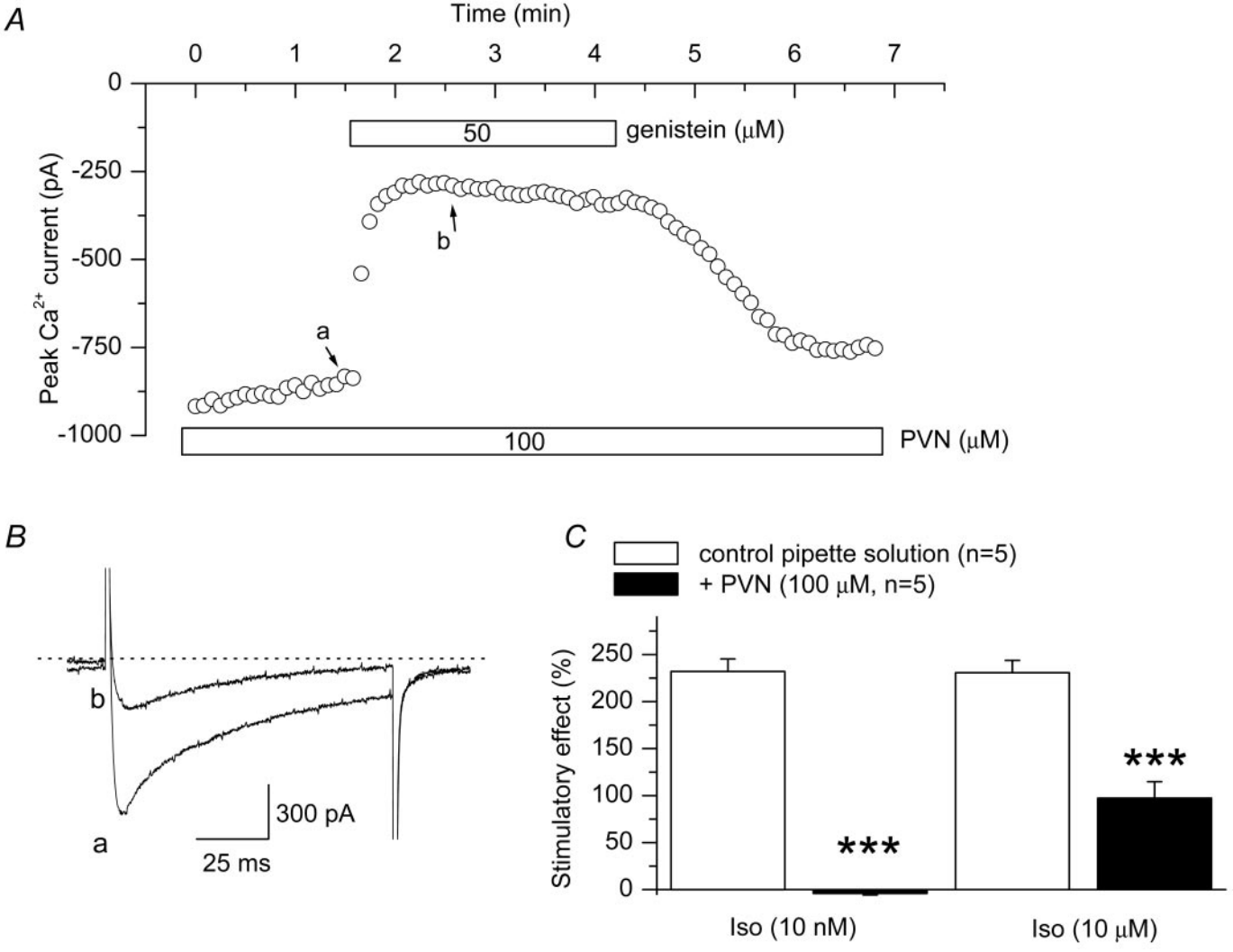

Fig. 4. Intracellular application of PVN does not prevent the inhibitory effect of genistein, but it significantly reduces β-adrenergic stimulation of the Ca^{2+} current. A, time course of changes in amplitude of L-type Ca^{2+} current in ventricular myocytes dialyzed with a pipette solution containing 100 μM PVN before (a) and during (b) exposure to 50 μM genistein. B, examples of L-type Ca^{2+} current recorded under conditions indicated in panel A. The dotted line above current traces represents the zero current level. C, stimulatory effects of 10 nM and 10 μM Iso recorded in myocytes dialyzed with a pipette solution containing 100 μM PVN (■) were significantly reduced (***, $P < 0.001$) when compared to those recorded under control conditions (□).

zein and/or genistin, which do not significantly inhibit PTK activity, produce the same type of response. However, neither daidzein nor genistin were sufficiently soluble in our extracellular solutions to allow us to attempt this type of experiment. As an alternative approach, we determined whether lavendustin A, a broad range PTK inhibitor that is structurally unrelated to genistein (Onoda et al., 1989), produces the same type of effect. As shown in Fig. 6, treatment with 5 μM lavendutsin A for 4 min did not significantly affect the basal L-type Ca^{2+} current. The magnitude of the Ca^{2+} current measured in the presence of lavendustin A (5 μM) was 96 $\pm$ 4.1% ($n = 7$) of that measured before exposure to lavendustin ($P > 0.4$, paired t test). We have previously demonstrated that this same concentration of lavendustin A can antagonize PTK-dependent inhibition of β-adrenergic responses associated with PVN treatment in guinea pig ventricular myocytes (Belevych et al., 2001). Therefore, the inability of lavendustin A to inhibit the basal Ca^{2+} current further supports the idea that basal PTK activity does not significantly contribute to the regulation of the basal L-type Ca^{2+} current in guinea pig ventricular myocytes.

Effect of Genistein on Kinetic and Voltage-Dependent Properties of Ca^{2+} Channels. It has previously been reported that PTK activity selectively enhances neuronal L-type Ca^{2+} channel activity at hyperpolarized membrane potentials, which is consistent with it causing a shift in the voltage dependence of channel activation. In addition, PTK activity increased the rate of current activation but only at more hyperpolarized test potentials, and it had no significant effect on the rate of current inactivation (Blair & Marshall, 1997; Bence-Hanulec et al., 2000). To determine whether genistein might affect cardiac L-type Ca^{2+} channel activity by inhibiting the same type of PTK-dependent regulatory responses, we studied the effect that genistein has on the kinetic and voltage-dependent properties of the current in guinea pig ventricular myocytes.

As illustrated in Fig. 7A, 50 μM genistein significantly slowed the rate of L-type Ca^{2+} current activation over a wide

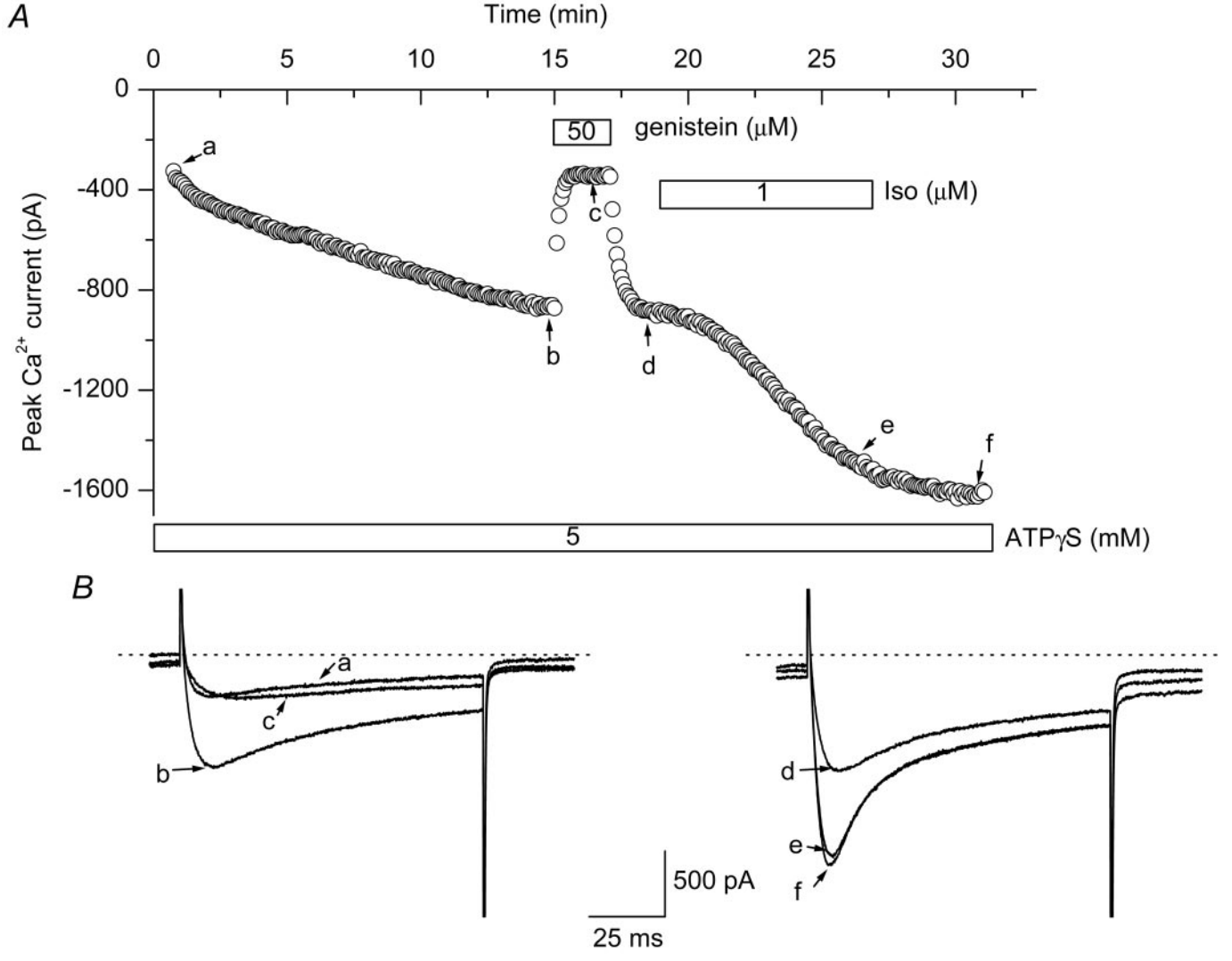

Fig. 5. Intracellular ATPγS has no effect on genistein-induced inhibition of the L-type Ca^{2+} current. A, time course of changes in amplitude of L-type Ca^{2+} current in ventricular myocytes dialyzed with a pipette solution containing 5 mM ATPγS at the beginning of cell dialysis (a), after 14 minutes of cell dialysis (b), following exposure to 50 μM genistein (c), following washout of genistein (d), following exposure to 1 μM Iso (e), and after washout of Iso (f). B, examples of L-type Ca^{2+} current recorded under conditions indicated in panel A. The dotted lines above current traces represent the zero current level.

range of test potentials. In the presence of 50 μM genistein, the latency from the onset of the voltage-clamp step to the peak of the Ca^{2+} current was increased by 10 to 30%. However, the voltage dependence of Ca^{2+} channel activation was not significantly affected by 50 μM genistein ($P > 0.3$) (Fig. 7B). The voltage dependence of channel activation was derived from the I-V relationships shown in Fig. 1C. For these calculations, the reversal potential of the current was estimated by extrapolating the linear portion of the I-V curves. Under control conditions, the reversal potential was 59 ± 1.1 mV, which was not significantly different from the reversal potential of 57 ± 0.64 mV observed in the presence of 50 μM genistein ($P > 0.05$, paired t test, $n = 8$). Using this information, the Ca^{2+} conductance was computed and plotted as a function of the test potential. The resulting data points were then fit to a Boltzmann relationship. The membrane potential at which half-maximal activation occurred was −1.6 ± 0.26 mV under control conditions and −1.2 ± 0.25 mV in the presence of genistein. The slope factor of the relationship was 7.3 ± 0.23 mV under control conditions and 7.2 ± 0.22 mV in the presence of genistein.

To determine whether or not genistein affects the kinetics of channel inactivation, we analyzed the decay phase of the Ca^{2+} current evoked by 1000-ms depolarizing steps to 0 mV from a holding potential of −80 mV. Under control conditions, inactivation of the current was best described by the sum of two exponentials with time constants of 21 ± 2.7 and 160 ± 6.2 ms ($n = 10$). In the presence of genistein (50 μM), the time constant of the fast component increased to 31 ± 4.4 ms ($P < 0.01$, paired t test), whereas the time constant of the slow exponential remained unaffected (151.7 ± 7.5 ms, $P > 0.3$ paired t test). This slowing of inactivation is unlikely to be explained by attenuation of Ca^{2+}-dependent inactivation secondary to the decrease in current amplitude caused by genistein, since similar results were obtained when Ca^{2+} was replaced with an equimolar concentration of Ba^{2+}. Under control conditions, inactivation of the Ba^{2+} current at 0 mV was best described by the sum of two exponentials with time constants of 70 ± 4.3 and 358 ± 19.0 ms. Subsequent exposure to 50 μM genistein inhibited the magnitude of the peak inward current by 56 ± 3.0% and increased the time constants for inactivation to 79 ± 5.7 and 429 ± 23.5 ms ($n = 7$, $P < 0.05$, paired t test), respectively.

The effect of genistein on the voltage dependence of L-type Ca^{2+} current inactivation was studied using the voltage protocol shown in Fig. 8. A conditioning pulse to membrane potentials between −90 and 30 mV was followed by a test pulse to 0 mV. The normalized amplitude of the Ca^{2+} current measured during the test pulse was plotted as a function of the conditioning potential. The resulting data points were then fit to a Boltzmann relationship. In the presence of 50 μM genistein, there was a significant shift in the voltage dependence of L-type Ca^{2+} current inactivation to more negative potentials. The membrane potential at which half-maximal inactivation occurred shifted from −23 ± 0.10 mV ($n = 6$) under control conditions to −36 ± 0.88 mV ($n = 6$, $P < 0.001$) in the presence of genistein. The slope factor of this

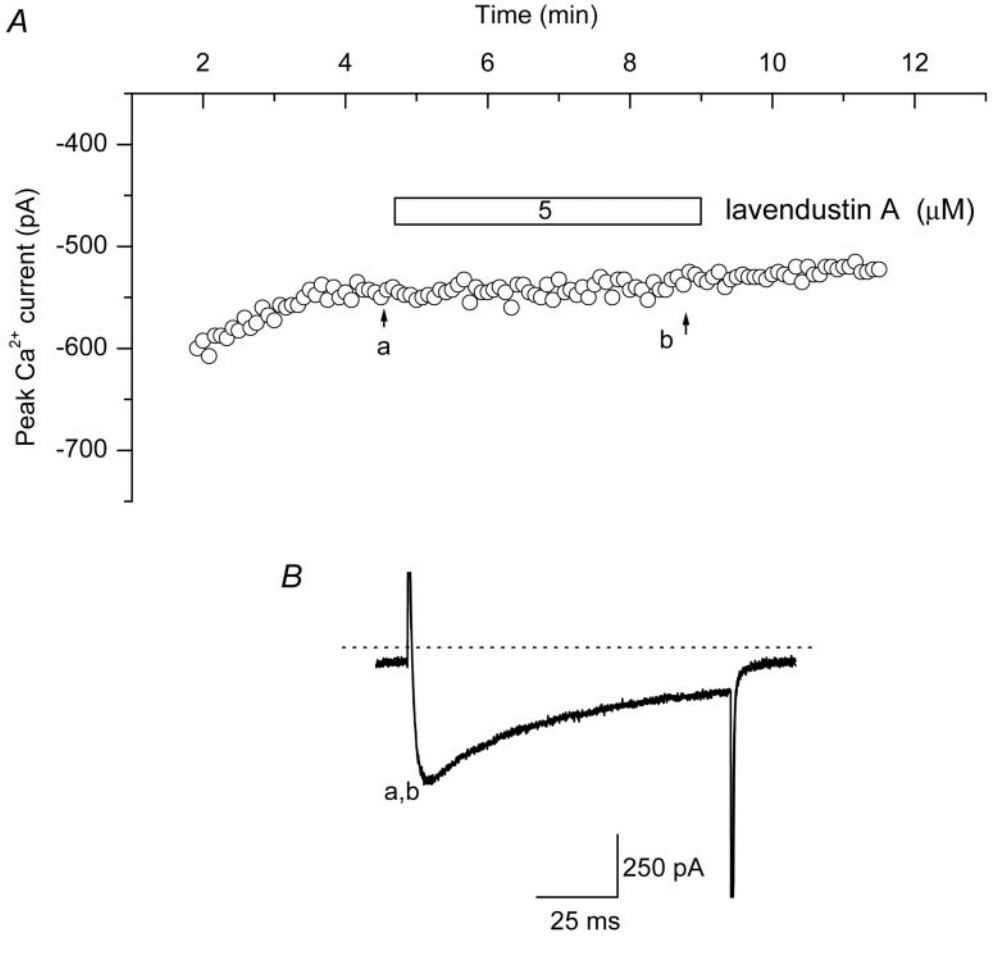

Fig. 6. The tyrosine kinase inhibitor lavendustin A has no effect on the basal L-type Ca^{2+} current. A, time course of changes in amplitude of L-type Ca^{2+} current under control conditions (a) and in the presence of 5 μM lavendustin A (b). B, examples of L-type Ca^{2+} current recorded under conditions indicated in panel A. The dotted line above current traces represents the zero current level.

relationship was 5.1 ± 0.10 mV under control conditions and 10 ± 0.75 mV ($P < 0.001$) in the presence of genistein.

The significant shift in the voltage dependence of inactivation and the lack of any effect on the voltage dependence of activation suggests that genistein does not produce its effect on the cardiac L-type Ca^{2+} current by inhibiting the same type of PTK-dependent mechanism that regulates neuronal L-type Ca^{2+} channel activity.

Discussion

In the present study, we demonstrated that genistein is able to inhibit the L-type Ca^{2+} current in native cardiac myocytes. This is consistent with what has been previously reported (Chiang et al., 1996; Yokoshiki et al., 1996; Hool et al., 1998; Katsube et al., 1998; Wang and Lipsius, 1998; Ogura et al., 1999). The basal current was inhibited by 68% in the presence of maximally effective concentrations of genistein, and the apparent IC_{50} was 20 μM. Although the accuracy of these values is most likely limited by the insolubility of genistein at higher concentrations, our results are in line with maximal inhibitory effects of 40 to 79% and IC_{50} values of 11 to 47 μM, which have been reported by others (Yokoshiki et al., 1996; Ogura et al., 1999).

Although genistein's ability to inhibit L-type Ca^{2+} channel activity has been a consistent observation, the conclusion as to whether or not this is due to changes in PTK activity has not. Several approaches have been used to investigate the underlying mechanism. One that has been employed by most groups involves the use of daidzein, a structural analog of genistein that does not inhibit PTK activity. The ability of daidzein to at least partially mimic the inhibitory effect of genistein has been reported by some, but not all, investigators (Chiang et al., 1996; Yokoshiki et al., 1996; Wang and Lipsius, 1998; Ogura et al., 1999). In the present study, we did not examine the effects of daidzein, because it was only partially soluble in our experimental solutions. However, we previously reported that daidzein could inhibit the L-type Ca^{2+} current in guinea pig ventricular myocytes but that the magnitude of its inhibitory effect was only about 50% of that produced by genistein (Hool et al., 1998). It is conceivable that the lower potency of daidzein might be explained at least in part by its lower solubility reducing the effective amount of this compound that is actually in solution.

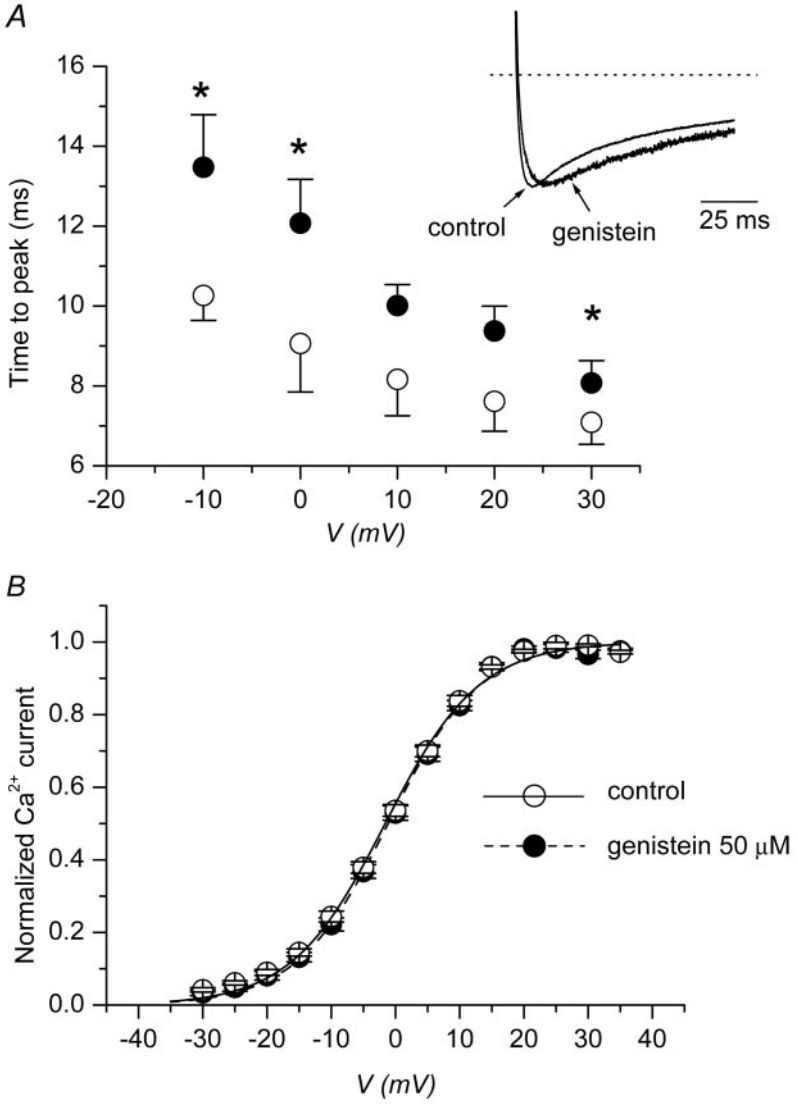

Fig. 7. Genistein slows the rate of L-type Ca^{2+} current activation but does not affect the voltage dependence of peak current activation. A, the time to peak current following depolarization to indicated membrane potentials under control conditions (○) and in the presence of 50 μM genistein (●) (⋆, $P < 0.05$, paired t test, $n = 8$). Inset, examples of Ca^{2+} current recorded at 0 mV under control conditions and in the presence of 50 μM genistein. The traces were normalized to peak current amplitude. B, voltage dependence of L-type Ca^{2+} current activation observed under control conditions (○) and in the presence of 50 μM genistein (●). Activation curves were derived from I-V relationships (Fig. 1) by calculating the conductance at each test potential. Lines through data points represent a least-squares fit to a Boltzmann equation. The membrane potential at which activation was half-maximal was −1.6 and −1.2 mV, and the slope factor was 7.3 and 7.2 mV in the absence and presence of genistein, respectively ($P > 0.3$).

Another approach that has been used to address the potential role of changes in tyrosine phosphorylation in mediating genistein-induced inhibition of the cardiac L-type Ca^{2+} current has been to determine whether PTP inhibitors can block or reverse such responses. Wang and Lipsius (1998) reported that 1 mM Na_3VO_4 was able to completely block the inhibitory effect of genistein. This is at least partially consistent with the work of Ogura et al. (1999) who reported that this same concentration of Na_3VO_4 antagonized the ability of low, but not high, concentrations of genistein to inhibit the Ca^{2+} current in guinea pig ventricular myocytes. On the contrary, in the present study, we found that the PTP inhibitor PVN had no such effect on the inhibitory response to genistein. The explanation for this apparent discrepancy is unclear. The negative response to PVN is unlikely to be explained by the inability of this compound to actually inhibit PTP activity in cardiac myocytes. Even though we used a concentration of only 100 μM, PVN contains a mixture of peroxovanadate derivatives that are significantly more potent inhibitors of PTP activity than Na_3VO_4 (Posner et al., 1994). The lack of response to externally applied PVN is also unlikely to be due to the inability of these compounds to cross the plasma membrane and reach an effective concentration inside the cell, because dialysis with a PVN-containing pipette solution did not alter the response to genistein, even though it did inhibit the response to β-adrenergic receptor stimulation. Furthermore, we have previously demonstrated that exposure of isolated guinea pig ventricular myocytes to bathing solutions containing this concentration of PVN produces a profound increase in the level of protein tyrosine phosphorylation (Belevych et al., 2001).

The ability of other tyrosine kinase inhibitors to mimic the inhibitory response to genistein has also been used to support the idea that tyrosine kinase activity actually does regulate cardiac L-type Ca^{2+} channel activity. Ogura et al. (1999) demonstrated that the inhibitory effect of genistein could be mimicked by tyrphostins A23 and A25, which are also broad range PTK inhibitors. However, tyrphostin A1, a structural analog with no PTK inhibitory activity, has also been reported to block L-type Ca^{2+} channel activity in vascular

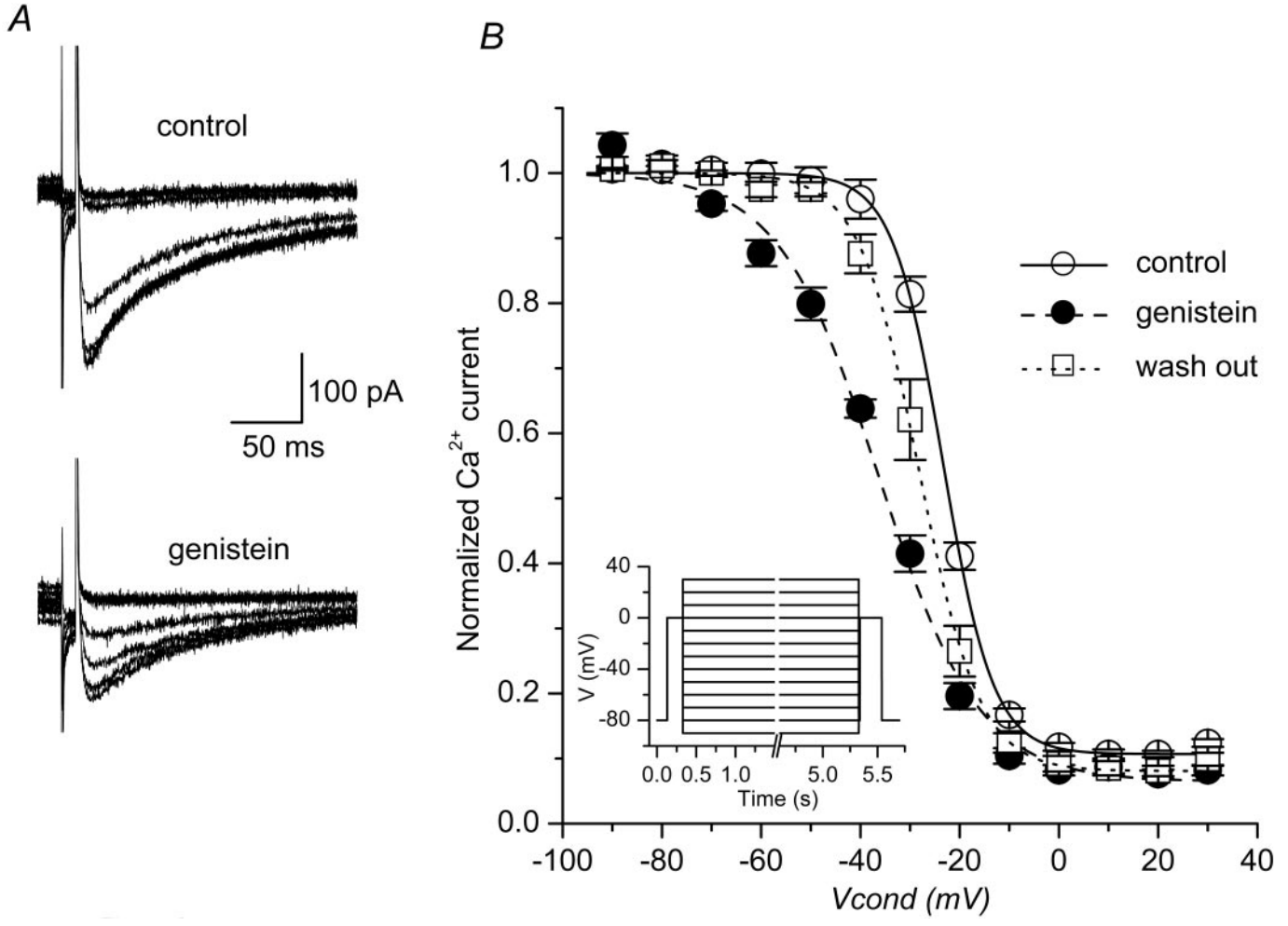

Fig. 8. Genistein significantly alters the voltage dependence of L-type Ca^{2+} current inactivation. A, L-type Ca^{2+} currents evoked by 200-ms test pulses following 5-s conditioning steps from −90 to 30 mV in 20 mV increments under control conditions and in the presence of 50 μM genistein. B, voltage dependence of L-type Ca^{2+} current inactivation before (○), during (●), and after (□) exposure to 50 μM genistein. Lines through the data points represent least-squares fit to a Boltzmann equation. The membrane potential at which inactivation was half-maximal ($V_{0.5}$) was −23, −36, and −28 mV, and the slope factor was 5.1, 10, and 6.1 mV before, during, and after cell exposure to genistein, respectively. There is a statistically significant difference in $V_{0.5}$ and slope factor obtained in the absence and presence of genistein ($P < 0.001$). Inset, the voltage-clamp protocol used to define the voltage dependence of inactivation. Each conditioning pulse was preceded by a 200-ms pretest pulse to 0 mV and followed by a 10-ms step to −80 mV before depolarizing to the test pulse at 0 mV. The magnitude of the current recorded during the pretest pulse to 0 mV was used to normalize responses and correct for changes in current magnitude due to rundown.

smooth muscle cells, as well as cardiac myocytes (Wijetunge et al., 1992; Ogura et al., 1999). For that reason, we did not use any of the tyrphostins. Instead, we tested the response to lavendustin A, another broad range inhibitor of PTK activity that is significantly more potent than genistein (Onoda et al., 1989). Because of its greater potency, we could use lower concentrations, potentially avoiding any nonspecific effects. However, at a concentration that we previously found to antagonize other PTK-dependent responses in these cells (Belevych et al., 2001), lavendustin A had no effect on the basal L-type Ca^{2+} current. This observation supports the idea that the inhibitory effect of genistein has nothing to do with inhibition of PTK activity. However, this interpretation is based on the assumption that both genistein and lavendustin A are able to inhibit the same contingent of PTKs.

In the present study, we attempted to shed new light on the mechanism responsible for the inhibitory effect that genistein has on cardiac L-type Ca^{2+} channel activity by determining whether there is any sidedness to its effect. If genistein does produce its inhibitory effect by inhibiting PTK activity, then it must be acting at a site inside the cell. However, we found that the inhibitory effect of genistein was only observed when the compound was applied from the outside. Introduction of genistein intracellularly, through cell dialysis, did not produce obvious inhibition of the basal current. Furthermore, it did not alter the magnitude of the response to subsequent external application of genistein (Fig. 2). The lack of an inhibitory response to genistein added to the pipette solution is unlikely to be explained by ineffective dialysis of genistein into these cells since, as expected, the response to β-adrenergic stimulation was enhanced (Hool et al., 1998). This supports the idea that genistein produces its inhibitory effect by acting at an external site, which is not consistent with a mechanism involving regulation of PTK activity.

Another new approach employed in the present study addressed the mechanism responsible for the inhibitory effect of genistein by using ATPγS. It is well established that ATPγS can substitute for ATP in protein kinase-dependent phosphorylation reactions. The result is a thiophosphorylated protein that is resistant to dephosphorylation by both serine/threonine and tyrosine phosphatases (Gratecos and Fischer, 1974; Hiriyanna et al., 1994; Sorota, 1995). However, dialyzing cells with a pipette solution containing ATPγS did not alter the inhibitory response to genistein. Again, the absence of an effect on the genistein response cannot be attributed to inadequate dialysis with ATPγS, since exposure of these cells clearly produced irreversible PKA-dependent enhancement of the L-type Ca^{2+} current following exposure to Iso. Therefore, these results argue against the possibility that the response to genistein is due to PTK inhibition, which then allows basal PTP activity to dephosphorylate a site responsible for maintaining basal channel activity.

In the final set of experiments, we demonstrated that genistein shifts the voltage dependence of Ca^{2+} channel inactivation. This confirms the effect reported by Yokoshiki et al. (1996). It is also consistent with the ability of genistein to shift the voltage dependence of L-type Ca^{2+} channel inactivation in smooth muscle cells (Wijetunge et al., 2000). However, PTK-dependent regulation of this current in smooth muscle cells is not necessarily associated with a change in the voltage dependence of channel inactivation (Wijetunge and Hughes, 1996; Wijetunge et al., 1998). This suggests that the ability of genistein to affect the voltage dependence of channel gating may be independent of its ability to inhibit PTK activity. Consistent with this idea, PTK-dependent regulation of the neuronal and smooth muscle isoforms of the L-type Ca^{2+} channel are believed to involve phosphorylation of the tyrosine residue located at position 2122 of the α_1 subunit (Bence-Hanulec et al., 2000; Davis et al., 2001). However, the cardiac isoform of the L-type Ca^{2+} channel lacks this tyrosine residue. This could then explain why genistein inhibition of the L-type Ca^{2+} current in cardiac myocytes is associated with a change in voltage dependence but not inhibition of PTK activity.

The most likely explanation for the ability of genistein to inhibit the cardiac L-type Ca^{2+} current is that the drug directly blocks the channel. It should be noted that such an effect is not restricted to L-type Ca^{2+} channels. It has been reported that genistein exerts a direct, non-catalytic blocking effect on glycine receptors (Huang and Dillon, 2000), voltage-gated Na^+ channels, (Paillart et al., 1997), and voltage-gated K^+ channels (Smirnov and Aaronson, 1995; Washizuka et al., 1997; Zhang and Wang, 2000). Thus our study provides further support for the conclusion that genistein is actually a promiscuous ion channel blocker, and its use as a probe for assessing the role of PTK activity in regulating ion channel activity should be avoided.

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