

Protein Tyrosine Kinase–Dependent Modulation of Isoflurane Effects on Cardiac Sarcolemmal K_{ATP} Channel

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Background: Cardiac adenosine triphosphate–sensitive potassium (K_{ATP}) channels and protein tyrosine kinases (PTKs) are mediators of ischemic preconditioning, but the interaction of both and a role in myocardial protection afforded by volatile anesthetics have not been defined.

Methods: Whole cell and single channel patch clamp techniques were used to investigate the effects of isoflurane and the PTK inhibitor genistein on the cardiac sarcolemmal K_{ATP} channel in acutely dissociated guinea pig ventricular myocytes.

Results: At 0.5 mM internal ATP, genistein (50 μ M) elicited whole cell K_{ATP} current (22.5 ± 7.9 pA/pF). Genistein effects were concentration-dependent, with an EC_{50} of 32.3 ± 1.4 μ M. Another PTK inhibitor, tyrphostin B42, had a similar effect. The inactive analog of genistein, daidzein (50 μ M), did not elicit K_{ATP} current. Isoflurane (0.5 mM) increased genistein (35 μ M)-activated whole cell K_{ATP} current from 14.5 ± 3.1 to 32.5 ± 6.6 pA/pF. Stimulation of receptor PTKs with epidermal growth factor, nerve growth factor, or insulin attenuated genistein and isoflurane effects, and the protein tyrosine phosphatase inhibitor orthovanadate (1 mM) prevented their actions on K_{ATP} current. In excised inside-out membrane patches, and at fixed 0.2 mM internal ATP, genistein (50 μ M) increased channel open probability from 0.053 ± 0.016 to 0.183 ± 0.039 , but isoflurane failed to further increase open probability (0.162 ± 0.051) of genistein-activated channels. However, applied in the presence of genistein and protein tyrosine phosphatase 1B (1 μ g/ml), isoflurane significantly increased open probability to 0.473 ± 0.114 .

Conclusions: These results suggest that the PTK–protein tyrosine phosphatase signaling pathway may be one of the regulators of cardiac sarcolemmal K_{ATP} channel and may play a role in modulating its responsiveness to isoflurane. Relative importance of this modulation for cardioprotection by volatile anesthetics remains to be established.

PROTEIN tyrosine kinases (PTKs) play an important role in intracellular signaling by regulating various cellular events, including differentiation, growth, metabolism, and apoptosis.^{1–3} PTKs are also involved in regulation of ion channels^{4–6} via phosphorylation of tyrosine residues on the channel protein. The physiologic outcome of these events is determined by the interplay between PTKs and protein tyrosine phosphatases (PTP) that reverse kinase-dependent phosphorylation.⁷

A specific inhibitor of PTKs, isoflavone genistein,⁸ is widely used to investigate the role of PTKs in regulation of the ion channels. In the heart, genistein inhibition of PTKs attenuates the L-type Ca^{2+} current in guinea pig and rat ventricular myocytes,^{9–11} feline and human atrial myocytes,^{12,13} and rabbit sinoatrial myocytes.¹⁴ Genistein decreases β -adrenergic sensitivity of the L-type Ca^{2+} current in guinea pig ventricular cells¹⁵ and prevents activation of the cardiac swelling-activated Cl^- current in canine atrial myocytes.¹⁶ Yet genistein activates the cardiac cyclic adenosine monophosphate-dependent Cl^- channel and enhances its β -adrenergic responsiveness.^{9,17,18} Independent of PTK, direct actions of genistein on the cardiac delayed rectifier K channel have also been reported.¹⁹ Few studies assessed the role of tyrosine phosphorylation in regulation of cardiac adenosine triphosphate–sensitive K (K_{ATP}) channel. PTKs have been implicated in the inhibition of rabbit ventricular K_{ATP} channels by a cytokine interferon- α .²⁰ PTP1B has been reported to enhance the activity of rat ventricular K_{ATP} channels.²¹ Recently, the intracellular nucleotides have been shown to modify genistein activation of a recombinant, heterologously expressed K_{ATP} channel.²²

Following early studies from our laboratory,²³ additional evidence has been obtained to demonstrate that volatile anesthetics, particularly isoflurane, exert beneficial cardioprotective effects against myocardial ischemia and reperfusion injury. *In vivo*, isoflurane produces myocardial protection in part via activation of the K_{ATP} channel.^{24–29} In contrast to ischemic preconditioning, where the mitochondrial K_{ATP} channel appears to play a predominant role in the initiation of protection and the sarcolemmal K_{ATP} channel is thought to be protective during the reoxygenation phase of injury, the sarcolemmal K_{ATP} channel may play a pivotal role in the volatile anesthetic-induced preconditioning.^{27,28} Yet the mechanism of volatile anesthetic interaction with the cardiac sarcolemmal K_{ATP} channel is not well understood, and little is known about the conditions during which isoflurane may activate sarcolemmal K_{ATP} channels. Although isoflurane was reported to have no effect on K_{ATP} current (I_{KATP}) in human atrial cells²⁹ and to inhibit K_{ATP} channels in rabbit ventricular myocytes, the sensitivity to ATP was also decreased, suggesting a possibility of channel opening at higher intracellular ATP concentration.³⁰ Furthermore, recent patch clamp studies demonstrated that isoflurane facilitates cardiac sarcolemmal K_{ATP} channel preactivated by a metabolic inhibitor, 2,4-dinitrophenol,³¹ or a potassium channel opener, pinacidil.³² Both opening of the cardiac K_{ATP} channel and PTK

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activation are indicated in ischemic preconditioning and are thought to play important roles in the volatile anesthetic-induced cardioprotection. We tested the hypothesis that PTKs contribute to regulation of the cardiac sarcolemmal K_{ATP} channel and modulate its responsiveness to isoflurane. Therefore, using whole cell and single channel patch clamp techniques on acutely isolated guinea pig ventricular myocytes, we investigated the role of the PTK-PTP signaling pathway for isoflurane interaction with the cardiac sarcolemmal K_{ATP} channel.

Materials and Methods

Cell Isolation

After we obtained approval from the Institutional Animal Use and Care Committee of the Medical College of Wisconsin, we isolated single ventricular myocytes from guinea pig hearts by an enzymatic dissociation procedure as described previously.³³ Briefly, either male or female guinea pigs weighing 150–300 g were injected intraperitoneally with heparin (1,000 U/ml) and anesthetized with sodium pentobarbital (275 mg/kg). Each heart was rapidly excised, mounted on a cannula of the Langendorff apparatus (Radnoti, Monrovia, CA), and perfused *via* the aorta with oxygenated (95% O_2 -5% CO_2) Joklik medium (Gibco BRL, Life Technologies, Grand Island, NY) containing heparin (2.5 U/ml), at a constant flow of 7 ml/min at 37°C. After washout of blood, hearts were perfused for 14 min with Joklik medium containing 0.4 mg/ml collagenase (Type II; Gibco BRL), 0.13 mg/ml protease (Type XIV; Sigma, St. Louis, MO), and 1 mg/ml bovine serum albumin (Serologicals, Kankakee, IL) at pH 7.23. The ventricles were then minced and incubated for additional 3–10 min in the enzyme solution. Isolated myocytes were washed in a Tyrode solution and used for patch clamp experiments within 10 h after isolation.

Solutions and Drugs

The modified Tyrode solution contained 132 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, and 5 mM glucose, at pH 7.4 adjusted with NaOH.

For the whole cell recordings, the external-bath solution contained 132 mM *N*-methyl-D-glucamine, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulphonic acid), and 0.0002 mM nisoldipine, at pH 7.4 adjusted with HCl. *N*-methyl-D-glucamine replaced sodium, and nisoldipine blocked the L-type Ca^{2+} channel currents. The intracellular-pipette solution contained 60 mM K glutamate, 50 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES, and 0.5 or 5 mM K_2ATP , at pH 7.4 adjusted with KOH.

For the single channel recordings in the excised inside-out patch configuration, the bath solution facing the cytosolic side of the membrane patch contained 140 mM KCl, 0.5 mM $MgCl_2$, 2 mM EGTA, 10 mM HEPES, 0.2 mM

K_2ATP , at pH 7.4 adjusted with KOH. The pipette solution facing the outer side of the membrane patch contained 140 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM HEPES, at pH 7.4 adjusted with KOH.

All standard chemicals, ATP, sodium orthovanadate (Na_3VO_4), and nerve growth factor (NGF; from *Vipera lebetina* venom) were obtained from Sigma. Nisoldipine was supplied by Miles-Pentex (West Haven, CT). Genistein (4',5,7-trihydroxyisoflavone), daidzein (4',7-dihydroxyisoflavone), tyrphostin B42 [AG490, α -cyano-(3,4-dihydroxy)-*N*-benzylcinnamide], and murine epidermal growth factor (EGF) were obtained from Calbiochem (Calbiochem-Novabiochem, San Diego, CA). Recombinant PTP1B was purchased from Upstate Biotechnology (Lake Placid, NY). Inhibitors of PTK and daidzein were dissolved in dimethyl sulfoxide. Nisoldipine was prepared as 1 mM stock in polyethylene glycol. Control experiments with the vehicles have shown that neither dimethyl sulfoxide (0.02–0.06%) nor polyethylene glycol (0.02%) at their final dilution in the recording buffers had any effect on whole cell I_{KATP} and single K_{ATP} channel activity (data not shown).

Measured volumes of isoflurane (Baxter Healthcare, Deerfield, IL) were added to appropriate bath solutions, and the anesthetic was dispersed by sonication. The solutions were then transferred to gas-tight glass syringe reservoirs and delivered to the recording chamber by a gravity-fed perfusion system. Isoflurane was used at a clinically relevant concentration of 0.5 ± 0.03 mM, equivalent to 1.05 vol% as determined at 20–23°C. Samples (1 ml) of anesthetic-containing perfusate were withdrawn from the recording chamber to determine effective millimolar concentrations of isoflurane by the headspace analysis method using a Shimadzu GC 8A flame ionization detection gas chromatograph (Shimadzu, Kyoto, Japan).

Membrane Current Measurements

Conventional configurations of the patch clamp technique were used to measure whole cell K_{ATP} current and single K_{ATP} channel activity. Currents were recorded using a LIST EPC-7 patch clamp amplifier (ALA Scientific Instruments, Westbury, NY) and a Digiata 1200B (Axon Instruments, Foster City, CA) interfaced to a PC computer. Data were acquired using pClamp8 software (Axon Instruments). The recording chamber (RC-16; Warner, Hamden, CT) was mounted on the stage of an inverted IMT-2 microscope (Olympus, Tokyo, Japan). Patch pipettes were pulled from borosilicate glass tubing (Garner Glass, Claremont, CA) with a PC-84 micropipette puller (Sutter Instruments, Novato, CA), and the pipette tips were heat-polished with a MF-83 microforge (Narishige, Tokyo, Japan). The resistance of pipettes filled with the appropriate pipette solution was 2–3 M Ω for the whole cell recordings and 7–12 M Ω for single

channel recordings. All experiments were conducted at room temperature (20–23°C).

For the whole cell $I_{K_{ATP}}$ measurements, the series resistance was compensated electronically to obtain the fastest cell capacity transient. Whole cell currents were monitored over time by applying a 100-ms voltage step to 0 mV from a holding potential of –40 mV every 15 s. Current amplitude, measured at the end of the voltage step, was normalized to cell capacitance for $I_{K_{ATP}}$ density (pA/pF) determination.

Single K_{ATP} channel currents were recorded from the excised inside-out patches at the transmembrane patch potential of +40 mV. Currents were sampled at 1 kHz and low-pass filtered at 500 Hz through an eight-pole Bessel filter. The 120-s-long recordings were made at each experimental step. The K_{ATP} channels were identified by single channel conductance, sensitivity to inhibition by intracellular ATP, and blockade by 1 μ M glibenclamide. The threshold for detecting the open state was set at half of the single channel amplitude. Amplitude of single channel current was determined from the all-points amplitude histograms. The number of channels (N) in the patch was estimated from the mean single channel amplitude and the maximal current. The channel open probability (P_o) was calculated as a fraction of the total time the active channels in the patch were in the open state during the recording. P_o was determined from the ratios of the area under the peaks in the all-points amplitude histograms fitted with a Gaussian function. Because of a variable number (N) of channels in the patches reported here, P_o is expressed as cumulative P_o . The experimental protocols were completed within 10–15 min after patch excision to minimize the influence of rundown. Recordings from patches exhibiting large differences in channel activity between the control and the final washout that suggested a significant rundown were excluded from further analyses.

Statistics

Whole cell and single channel data were analyzed using pClamp8 software (Axon Instruments) and Origin6 software (ORIGINLAB, Northampton, MA). Data were presented as means \pm SEM. Comparisons between two groups of means were made using a paired or unpaired Student *t* test. Multiple group means were compared using one-way analysis of variance with a Student–Newman–Keuls test. Differences with a *P* < 0.05 were considered statistically significant.

Results

Genistein Activates Whole Cell K_{ATP} Current at Low Internal Adenosine Triphosphate

To assess a possible role of the PTK-PTP signaling pathway for isoflurane interaction with the cardiac sar-

colemmal K_{ATP} channel, we first evaluated the effects of genistein, an inhibitor of PTKs, on the whole cell K^+ current in ventricular myocytes. Applied in the external solution, genistein consistently activated whole cell outward K^+ current in myocytes dialyzed for 20 min with the pipette solution containing low (0.5 mM) but no high (5 mM) ATP. Genistein-activated current was identified as $I_{K_{ATP}}$ because of sensitivity to blockade by 1 μ M glibenclamide. Activation of $I_{K_{ATP}}$ by 50 μ M genistein was biphasic, with a rapidly rising peak followed by a more gradually established plateau. Figure 1 shows the effects of genistein on whole cell $I_{K_{ATP}}$: sample traces of glibenclamide-sensitive current recorded during a 100-ms depolarizing voltage step from –40 mV to 0 mV in the absence and presence of 50 μ M genistein (fig. 1A), a time course of genistein activation of $I_{K_{ATP}}$ (fig. 1B), and mean current density data from 10 myocytes (fig. 1C). The density of genistein-activated $I_{K_{ATP}}$ was 37.4 ± 11.3 pA/pF at peak and 22.5 ± 7.9 pA/pF at plateau. Because of high variability in the magnitude of genistein-activated current and a lack of significant differences between mean peak and plateau values, only plateau values were subsequently used in the evaluation of genistein effects on $I_{K_{ATP}}$. In another group of experiments, daidzein, an inactive structural analog of genistein, was used to determine whether genistein effects were mediated by PTK. Time course and mean current density data in figures 1D and E show that externally applied daidzein (50 μ M, *n* = 5) did not activate whole cell $I_{K_{ATP}}$.

Genistein activation of $I_{K_{ATP}}$ was concentration-dependent (1–200 μ M) with maximal current activation at 50 μ M. Less activation at 100 and 200 μ M genistein resulted in a bell-shaped concentration–response relation. Fitting the ascending part of a normalized concentration–response relation to a Hill equation gave a concentration of genistein required for half-maximal activation (EC_{50}) of 32.3 ± 1.4 μ M and a Hill coefficient of 11.7 ± 5.1 (fig. 2). Tyrphostin B42, a specific inhibitor of EGF receptor-associated tyrosine kinases but structurally unrelated to genistein compound, had a similar effect and activated $I_{K_{ATP}}$ with an EC_{50} of 30.2 ± 0.2 μ M and a Hill coefficient of 4.9 ± 0.3 (*n* = 4).

Whether the effects of genistein on $I_{K_{ATP}}$ could, in part, be explained by PTP activity to reverse kinase effects was tested by using sodium orthovanadate (1 mM), the inhibitor of PTPs that prevents tyrosine dephosphorylation and thus stabilizes the phosphorylated state. Orthovanadate alone had no effect on $I_{K_{ATP}}$. However, applied prior to genistein, orthovanadate antagonized $I_{K_{ATP}}$ activation, and in its continued presence genistein failed to activate $I_{K_{ATP}}$ in all tested cells (*n* = 5). An inhibitor of serine–threonine phosphatases, 0.1 μ M okadaic acid was used in the pipette solution to assess the specificity of orthovanadate effects (*n* = 3). In contrast to orthovanadate, okadaic acid did not prevent activa-

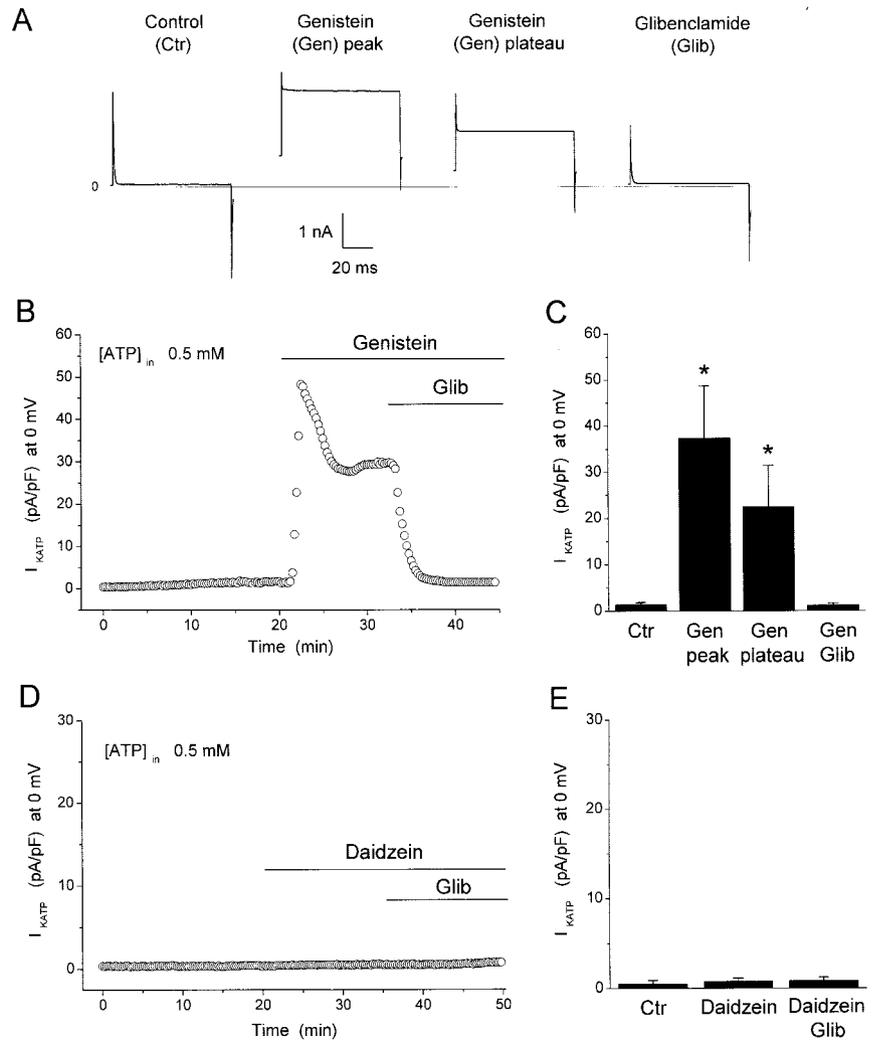


Fig. 1. Effects of genistein (Gen) and daidzein on whole cell K_{ATP} current (I_{KATP}) in guinea pig ventricular myocytes. (A) Traces of whole cell I_{KATP} recorded in control (Ctr) during bath application of 50 μ M genistein and during coapplication of 1 μ M glibenclamide (Glib). Dotted line indicates zero current level. (B) Time course of whole cell I_{KATP} activated by 50 μ M genistein. In control, the myocyte was dialyzed for 20 min with pipette solution containing 0.5 mM ATP before application of genistein. (C) Mean data for I_{KATP} density determined in the absence and presence of 50 μ M genistein. * P < 0.05 versus control, n = 10 cells. (D) Time course of I_{KATP} during exposure to 50 μ M daidzein. (E) Mean current density in the presence and absence of daidzein, n = 5 cells. $[ATP]_{in}$ = intracellular adenosine triphosphate concentration.

tion of whole cell I_{KATP} by genistein (data not shown), suggesting lack of involvement of serine-threonine phosphatases in genistein effects.

Isoflurane Enhances Whole Cell K_{ATP} Current Activated by Genistein

A possible role of the PTK-PTP pathway for isoflurane actions on the sarcolemmal K_{ATP} channel was assessed by first determining whether isoflurane modulates genistein-activated I_{KATP} . Isoflurane (0.5 mM) alone did not activate whole cell I_{KATP} either at 5 mM or 0.5 mM internal ATP (n = 5 in each group). A sample time course of current in figure 3A shows that isoflurane alone did not activate whole cell I_{KATP} at 0.5 mM internal ATP. However, activated by 35 μ M genistein, I_{KATP} (14.5 ± 3.1 pA/pF, n = 10) was further increased in the presence of 0.5 mM isoflurane to 32.5 ± 6.6 pA/pF (n = 10) at 0.5 mM internal ATP (figs. 3B and C). Isoflurane also enhanced I_{KATP} activated by tyrphostin B42 (n = 4, data not shown). Furthermore, at high (5 mM) internal ATP and 35 μ M genistein in the bath, isoflurane activated I_{KATP} in three of the five cells examined. Isoflurane,

however, did not activate I_{KATP} when daidzein was substituted for genistein (n = 5, data not shown). The effects of genistein alone and genistein plus isoflurane were prevented by orthovanadate (1 mM, n = 5) applied prior to genistein and present in the bath solution throughout the experiment (fig. 3D), while okadaic acid (0.1 μ M) was without effect (data not shown).

Stimulation of Receptor Protein Tyrosine Kinases Attenuates Genistein and Isoflurane Effects on K_{ATP} Current

If genistein effects are PTK-mediated and PTKs negatively modulate K_{ATP} channel, then stimulation of receptor-associated PTKs should alter or prevent the effects of genistein and isoflurane on I_{KATP} . To test this hypothesis, the growth factor receptor PTKs and insulin receptor PTKs were stimulated with EGF (10 ng/ml), NGF (0.5 μ g/ml), and insulin (5 μ M), respectively. A sample time course in figure 4A shows that, when present in the bath solution throughout the experiment, EGF (10 ng/ml) suppressed the effects of genistein and isoflurane on whole cell I_{KATP} . Not only EGF, but also NGF and insulin

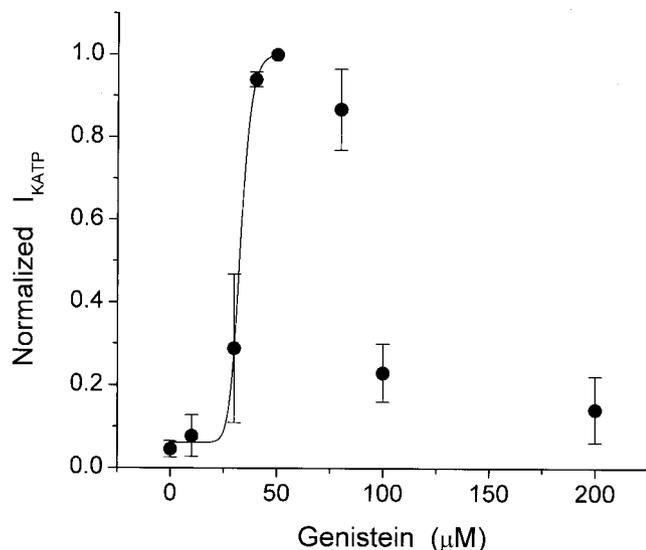


Fig. 2. Concentration–response curve for genistein activation of whole cell K_{ATP} current (I_{KATP}). Currents were normalized to the maximal current at $50 \mu\text{M}$ genistein. Data points are means \pm SEM of measurements from 3–16 cells. A Hill fit to the ascending part of the curve gave an EC_{50} of $32.3 \pm 1.4 \mu\text{M}$ and a Hill coefficient (n_H) of 11.7 ± 5.1 .

attenuated activation of I_{KATP} by genistein (fig. 4B). However, potentiating effect of isoflurane was attenuated by EGF and NGF but was not altered by insulin ($n = 3$ in each group). A gradual decrease in current was also

observed when EGF (20 ng/ml) was applied in the external solution at the plateau of genistein-activated and isoflurane-enhanced whole cell I_{KATP} (fig. 4C).

Genistein Increases Activity of Single K_{ATP} Channel, But Isoflurane Fails to Potentiate Genistein Effect

To determine whether genistein and isoflurane may directly modulate activity of K_{ATP} channels, we tested their effects under cell-free conditions in excised inside-out membrane patches. Single channel activity was monitored at patch potential of $+40 \text{ mV}$ in the presence of 0.2 mM internal ATP. As shown in figure 5, applied to the inside-out patches ($n = 4$), isoflurane alone did not change unitary current amplitude but tended to reversibly decrease single channel activity (fig. 5A). With isoflurane, P_o decreased from control 0.032 ± 0.01 to 0.017 ± 0.006 ($n = 4$; fig. 5B). This effect, however, was not statistically significant.

Genistein applied alone at 30 – $50 \mu\text{M}$ facilitated K_{ATP} channel opening. Figure 6 shows recordings of single K_{ATP} channel activity and the corresponding all-points histograms from a representative inside-out patch. While unitary current amplitude was not altered (control, $2.06 \pm 0.04 \text{ pA}$; genistein, $2.1 \pm 0.06 \text{ pA}$; $n = 10$), P_o was increased from 0.053 ± 0.016 to 0.183 ± 0.039 in the presence of genistein (fig. 6A; $n = 10$). When coapplied with genistein, isoflurane did not alter unitary cur-

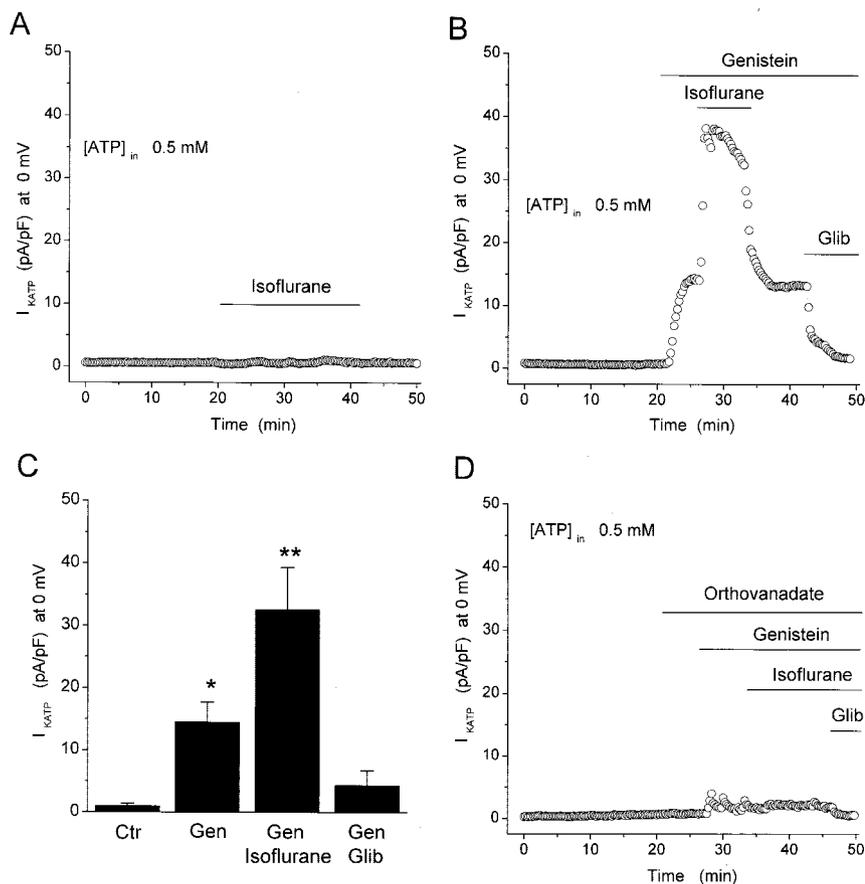


Fig. 3. Potentiation by isoflurane of genistein-activated whole cell K_{ATP} current (I_{KATP}). (A) Time course of I_{KATP} in the presence of isoflurane alone. The cell was dialyzed for 20 min with the pipette solution containing 0.5 mM ATP before isoflurane was applied in the bath solution at 0.5 mM . Isoflurane alone did not activate whole cell I_{KATP} . (B) Time course of I_{KATP} activated by $35 \mu\text{M}$ genistein and 0.5 mM isoflurane, at 0.5 mM internal ATP. Genistein activated current was further increased by isoflurane. (C) Mean data for I_{KATP} density in control (Ctr), in the presence of genistein (Gen), genistein plus isoflurane, and genistein plus glibenclamide (Glib). $*P < 0.05$ versus control, $**P < 0.05$ versus genistein alone, $n = 10$ cells. (D) Time course of I_{KATP} during continued bath presence of 1 mM orthovanadate. Orthovanadate prevented activation of I_{KATP} by genistein and subsequent current potentiation by isoflurane. $[ATP]_{in}$ = intracellular adenosine triphosphate concentration.

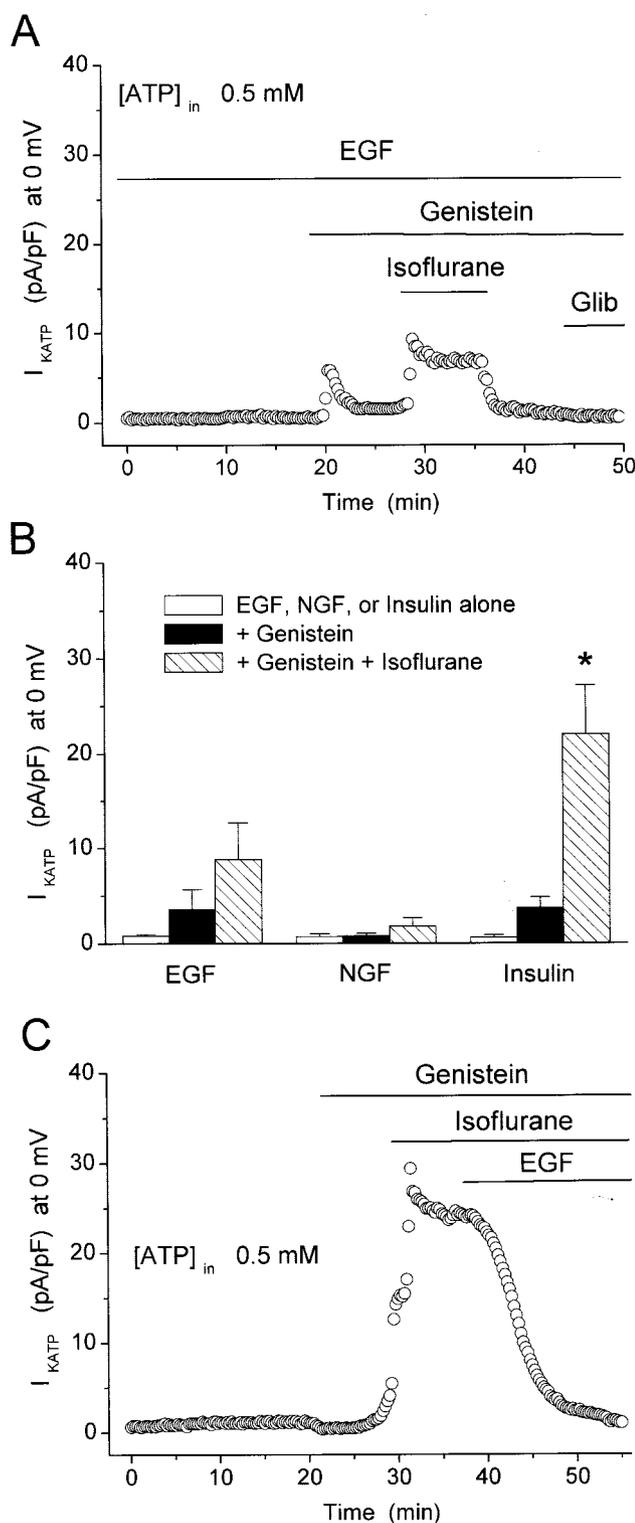


Fig. 4. Stimulation of receptor associated protein tyrosine kinases (PTKs) with epidermal growth factor (EGF), nerve growth factor (NGF), or insulin attenuates the effects of genistein and isoflurane on whole cell K_{ATP} current (I_{KATP}). (A) Time course of I_{KATP} in the presence of EGF (10 ng/ml) shows a marked attenuation of current activation by 50 μ M genistein and potentiation by 0.5 mM isoflurane (compare with fig. 3A). (B) Summary of the effects of EGF (10 ng/ml), NGF (0.5 μ g/ml), and insulin (5 μ M) on I_{KATP} activation by genistein, and potentiation by isoflurane (compare with fig. 3B). Insulin did not prevent isoflurane po-

tent amplitude (2.11 ± 0.04 pA), and unlike during whole cell conditions, failed to increase channel activity. With genistein plus isoflurane, P_o was 0.162 ± 0.051 ($n = 10$; figs. 6A and B). Daidzein at 50 μ M ($n = 3$) did not alter P_o in the absence or presence of isoflurane, as shown in figure 6C. Since receptor-associated PTKs and PTPs may exist in excised membrane patches as channel regulatory proteins, we also tested whether orthovanadate affects single K_{ATP} channel activity. At 0.2 mM internal ATP, orthovanadate (1 mM) prevented P_o increase by genistein, and the effect was reversible ($n = 3$, data not shown).

Genistein-activated Single K_{ATP} Channel Is Potentiated by Isoflurane in the Continuous Presence of Protein Tyrosine Phosphatase 1B

Inability of isoflurane to potentiate single, genistein-activated K_{ATP} channel in inside-out patches suggested that another factor, not available during cell-free conditions, might be required for this effect. The excised membrane patches may contain receptor PTKs and membrane-associated PTPs, but will lack soluble PTPs. Since PTP1B has recently been shown to modulate activity of ventricular sarcolemmal K_{ATP} channel,²¹ we tested whether this phosphatase might be needed for isoflurane potentiation of genistein effects. Figure 7 summarizes the results obtained from four single channel experiments in which genistein (50 μ M) increased P_o from control 0.018 ± 0.005 to 0.114 ± 0.040 , and at coapplication of PTP1B (1 μ g/ml), P_o remained at 0.143 ± 0.046 . Notably, isoflurane applied to genistein-activated channels in the presence of PTP1B caused a marked increase in P_o (0.473 ± 0.184 , $n = 4$). These results suggest a possibility of direct modulation of cardiac K_{ATP} channel by genistein and PTP1B, which may, in part, play a role in isoflurane potentiation of channel activity.

Discussion

Possible contribution of the PTK-PTP signaling pathway to modulation of isoflurane effects on the cardiac sarcolemmal K_{ATP} channel was investigated in guinea pig ventricular myocytes using the PTK inhibitor genistein. The results show that extracellularly applied genistein activates the whole cell I_{KATP} with an EC_{50} of 32 μ M, a concentration at which the activity of serine-threonine protein kinases A and C are not affected.³⁴ Furthermore, applied to the cytosolic side of excised membrane

tentation. * $P < 0.05$ versus genistein, $n = 3$ cells in each group. (C) Time course of I_{KATP} activation by genistein and isoflurane. Activated current was suppressed by 20 ng/ml EGF applied to the bath solution at the current plateau. [ATP]_{in} = intracellular adenosine triphosphate concentration.

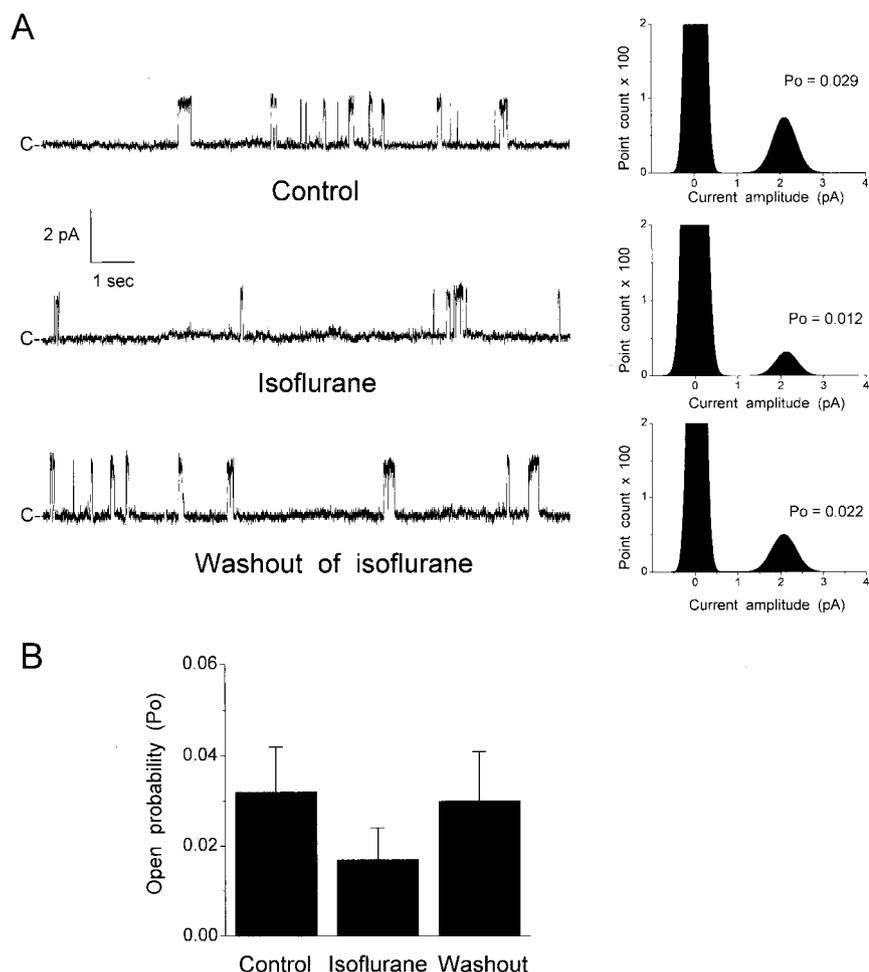


Fig. 5. Effects of isoflurane alone on single K_{ATP} channel activity in the inside-out patch. (A) Channel activity was monitored at +40 mV patch potential, in the presence of 0.2 mM internal ATP. Upward deflection indicates outward current. (C-) indicates closed state. Shown are 13-s recordings of K_{ATP} channel activity (left) and corresponding all-points amplitude histograms (right), where the y-axis representing point counts was expanded to show reversibility of isoflurane effects. (B) Mean open probability (P_o) data determined from four patches in control, during application of 0.5 mM isoflurane, and after anesthetic washout show that inhibition by isoflurane was not statistically significant.

patches, genistein increases single K_{ATP} channel activity by increasing channel P_o . Several lines of evidence suggest a possible involvement of PTK signaling pathway in these actions: (1) genistein activation of the whole cell $I_{K_{ATP}}$ is ATP-dependent; (2) a structurally unrelated inhibitor of PTKs, tyrphostin B42, may also activate whole cell $I_{K_{ATP}}$; (3) the inactive structural analog of genistein, daidzein, does not activate $I_{K_{ATP}}$ at the whole cell or single channel level; (4) a specific inhibitor of PTPs, orthovanadate, prevents genistein activation of whole cell or single K_{ATP} channel currents, whereas the serine-threonine phosphatase inhibitor, okadaic acid, does not alter genistein effects; and (5) activation of the whole cell $I_{K_{ATP}}$ by genistein is markedly attenuated during stimulation of receptor-associated PTKs with EGF, NGF, or insulin. These results taken together suggest a possibility of inhibitory control of cardiac sarcolemmal K_{ATP} channels by basal PTK activity.

The finding that genistein increases single K_{ATP} channel activity could be explained either by the presence of closely associated tyrosine kinases and phosphatases in the membrane patches³⁵ or by a direct action of genistein on the channel protein. The former mechanism appears to be supported by the finding that inhibition of PTP prevents the effects of genistein not only

during whole cell conditions but also in excised patches. If membrane-bound PTK and PTP activities are balanced in a patch, genistein inhibition of basal PTK would shift balance toward PTP-dependent dephosphorylation, promoting channel activation. However, orthovanadate inhibition of PTP would produce an opposite effect. As predicted, during cell-free conditions, orthovanadate prevented activation of the K_{ATP} channel by genistein. High variability in the magnitude of genistein-activated whole cell current in this study might reflect different levels of cellular expression of the proteins involved. The patch-to-patch variability in activation of the single channel current could be explained by differences in distribution of kinases and phosphatases in randomly excised membrane patches. Thus, similar to the results obtained in the whole cell configuration, activation of single K_{ATP} channels by genistein could be explained by inhibition of basal PTK activity, and prevention of tyrosine phosphorylation on the channel protein.

The cardiac K_{ATP} channel is an octameric complex of four Kir6.2 subunits of the inward rectifier K channel forming the channel pore, and four SUR2A receptor subunits. A member of the ATP binding cassette superfamily, SUR2A confers channel sensitivity to nucleotides, K channel openers, and sulfonylureas. The amino acid

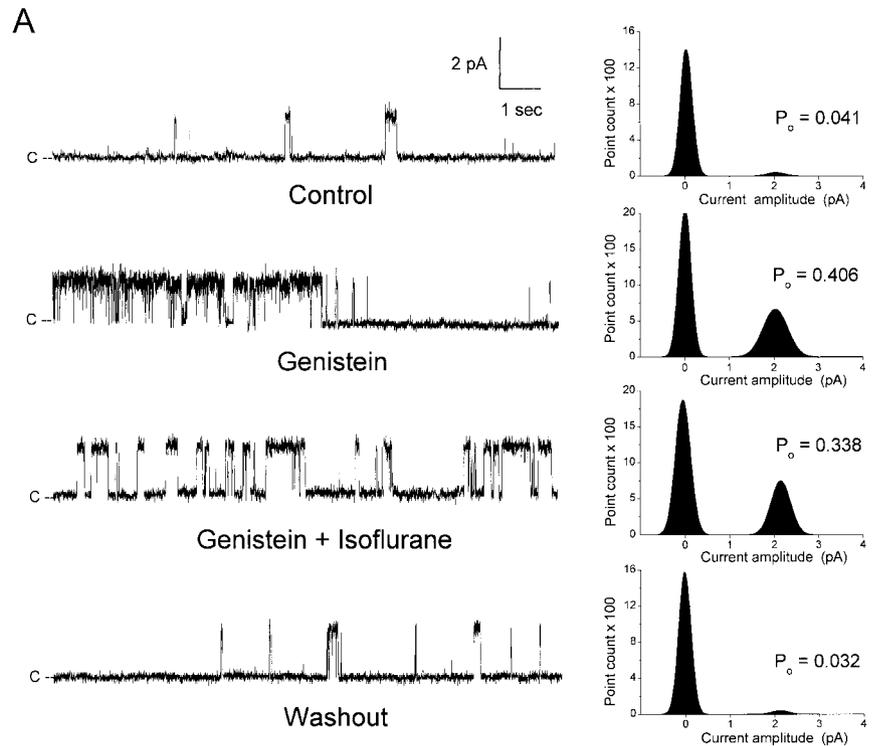
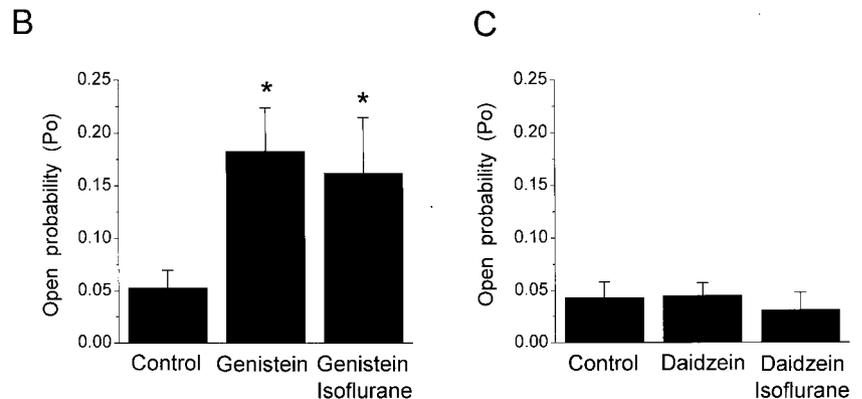


Fig. 6. Effects of genistein and isoflurane on single K_{ATP} channel activity in an inside-out patch. Shown are 10-s recordings of K_{ATP} channel activity at patch potential of +40 mV (*left*) and corresponding all-points amplitude histograms (*right*). Upward deflections indicates outward current. (C-) indicates closed state. (A) At 0.2 mM ATP on the cytosolic side of the patch, genistein activated single channel current and increased channel open probability (P_o). Coapplied with genistein, isoflurane failed to further increase P_o . (B) Summary of genistein effects shows a significant increase in P_o by 50 μ M genistein and no additional effect by isoflurane. * $P < 0.05$ versus control, $n = 10$ patches. (C) Summary of daidzein effects on P_o . During experimental conditions as above, daidzein (50 μ M, $n = 3$ patches) had no effect on P_o , with or without isoflurane present.



sequence of cardiac K_{ATP} channel shows 7 tyrosine residues on each Kir6.2 subunit and approximately 34 tyrosine residues located in various regions of the SUR2A receptor protein.^{36,37} It is possible that PTKs may target some of these residues, and tyrosine phosphorylation attenuates channel activity. By inhibiting PTK and preventing tyrosine phosphorylation in a particular region of the channel, genistein might promote its opening. However, it is difficult to speculate about sites of genistein action, because potential PTK phosphorylation sites on the cardiac sarcolemmal K_{ATP} channel have not been identified.

The concentration-response curves for genistein and tyrphostin B42 were steep, with high Hill coefficients that were greater for genistein (11.7 ± 5.1) than tyrphostin B42 (4.9 ± 0.3). This may partly reflect characteristics of a ligand-gated ion channel where allosteric inter-

action of several molecules of these compounds with the channel may be involved.

Genistein inhibits PTK *via* competition with ATP at its binding site, the Walker A motif in the nucleotide binding domain (NBD) of the kinase catalytic subunit.⁸ Genistein may interact with NBDs of other proteins, inhibiting, for example, DNA topoisomerase II,³⁸ but increasing activity of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel.³⁷ In the CFTR channel, where ATP hydrolysis at NBD1 is thought essential for channel opening and ATP hydrolysis at NBD2 causes channel closure, genistein increases the single channel activity by prolonging bursting activity. This effect is also a recognized consequence of the CFTR mutation that specifically prevents ATP hydrolysis at NBD2 but not at NBD1. Thus, genistein activation of the

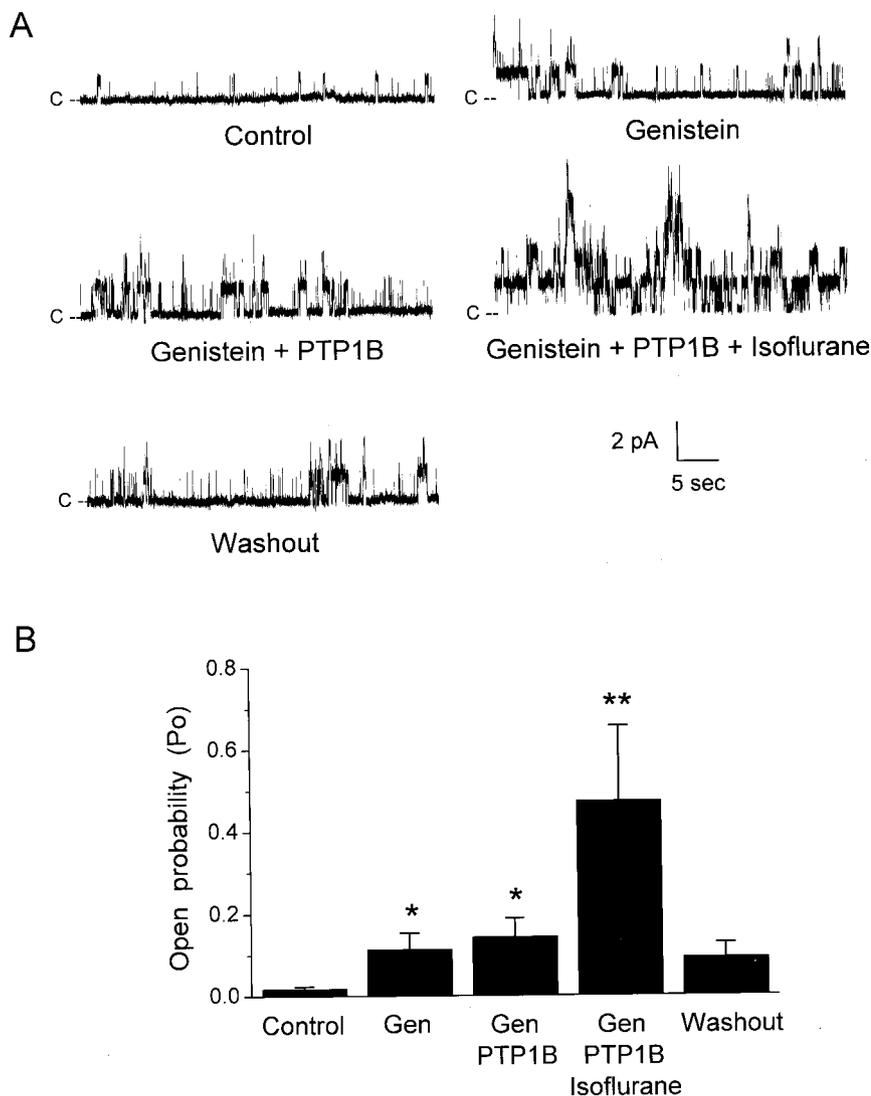


Fig. 7. Isoflurane potentiates activity of single K_{ATP} channel in the presence of genistein and protein tyrosine phosphatase 1B (PTP1B). (A) K_{ATP} channel activity was recorded from an inside-out patch at +40 mV patch potential in the presence of 0.2 mM internal ATP. Upward deflections indicate outward current. C- indicates closed state. Channel activity was increased in the presence of genistein (50 μ M) and was not changed on addition of 1 μ g/ml PTP1B. When coapplied with genistein and PTP1B, isoflurane (0.5 mM) markedly increased channel activity. (B) Summary of P_o data obtained with the protocol described in (A). * $P < 0.05$ versus control, ** $P < 0.05$ versus genistein (Gen), and Gen plus PTP1B, $n = 4$ patches.

CFTR channels is thought to result from inhibition of ATP hydrolysis at NBD2 that delays channel closure.³⁹

In contrast to the CFTR channel, genistein inhibition of the ATPase activity in cardiac K_{ATP} channels would be expected to cause channel closure. Our study, however, shows activation of the K_{ATP} channel in the presence of genistein. This may be explained by differences in the function and modulation of NBDs between these channels.⁴⁰ In the cardiac K_{ATP} channel, binding of ATP at NBD1, and Mg_2ADP at NBD2, and cooperativity between NBDs are essential for channel activation.^{40,41} Local activities of ATPase and creatine kinase modulate cardiac K_{ATP} channel function. A marked inhibition of creatine kinase activity that occurs during metabolic or ischemic stress may increase local adenosine diphosphate concentration and facilitate channel activation.⁴¹ Thus, the mechanism of genistein action could involve modulation of SUR2A activity *via* altering some of these enzymes. At the present, however, it is not known whether genistein modulates creatine kinase or ATPase activity in ventricular myocytes.

During our experimental conditions, isoflurane alone did not induce I_{KATP} in guinea pig ventricular myocytes, but the genistein-activated whole cell I_{KATP} was further increased in the presence of isoflurane at low internal ATP. Furthermore, even at high internal ATP, where genistein alone does not activate I_{KATP} , coapplication of isoflurane may induce whole cell current, suggesting that both genistein modulation of PTK activity and interaction with the channel may be important for isoflurane potentiation. This is further supported by the finding that stimulation of receptor-associated PTKs with EGF and NGF markedly attenuates both genistein activation and isoflurane potentiation of the whole cell I_{KATP} . These results also suggest that EGF or NGF receptor PTKs or their downstream signaling pathways might be involved in isoflurane potentiation, because stimulation of insulin receptor-associated PTKs attenuated only the effects of genistein, but not isoflurane potentiation. Such differential effects of EGF and NGF stimulation *versus* insulin receptor stimulation may imply yet-unknown but distinct mechanisms of isoflurane potentiation of the cardiac K_{ATP} channel.

In contrast to the results obtained in the whole cell configuration, isoflurane had no potentiating effect on K_{ATP} channel in excised membrane patches. One possible explanation is that this effect may require not only inhibition of PTKs, but also input from another signaling pathway, or a cytosolic factor that is missing during cell-free conditions. Since dynamic and opposing activities of both PTKs and PTPs are important for catalyzing phosphorylation-dephosphorylation processes, we focused on cytosolic PTPs that are accessible in intact cells⁴² and during whole cell recording conditions but absent during cell-free conditions. The phosphatase PTP1B was previously reported to prevent single K_{ATP} channel rundown in membrane patches from rat ventricular myocytes.²¹ Our study shows that, in the presence of PTP1B, isoflurane may potentiate genistein-activated channels. These results may suggest possible modulatory role for both PTKs and PTPs for cardiac K_{ATP} channel and enhancement of its activity by isoflurane. However, we cannot exclude a possibility of direct interaction of PTP1B or other tyrosine phosphatases with the channel.

Protein tyrosine kinases are an important component of intracellular signaling in ischemic preconditioning and may also play an important role in cardioprotection mediated by volatile anesthetics. Genistein has been shown to prevent the beneficial action of ischemic preconditioning *via* inhibition of PTKs. However, the role of PTKs for sarcolemmal K_{ATP} channel activation in ischemic preconditioning or anesthetic preconditioning has not been defined. Recent whole-animal studies have demonstrated that PTKs are activated in ischemic preconditioning.⁴³ Activation of both PTKs and protein kinase C during multiple ischemic preconditioning stimuli has been shown to reduce infarct size by opening the K_{ATP} channel. This is in apparent contradiction to the current findings, which suggest inhibitory control of PTKs over sarcolemmal K_{ATP} channel. However, the *in vivo* studies have demonstrated that PTKs enhance the activity of mitochondrial K_{ATP} channels and not necessarily sarcolemmal K_{ATP} channels.⁴⁴ Cardiac K_{ATP} channels are important contributors to ischemic preconditioning, and recent evidence points to a predominant role of mitochondrial K_{ATP} channels in the triggering of preconditioning, while sarcolemmal K_{ATP} channels appear to mediate protection during the reperfusion phase. Although both types of K_{ATP} channel are thought to be involved in cardioprotection by volatile anesthetics,²⁸ their exact roles are not clarified, and the mechanism by which anesthetic enhancement of K_{ATP} channel activity protects the heart has been a focus of intense investigation.

In conclusion, the results from this study suggest a modulatory role of the PTK-PTP signaling pathway in regulation of the cardiac sarcolemmal K_{ATP} channel, possibly *via* inhibitory control by basal PTK activity. At reduced intracellular ATP, inhibition of PTKs could promote channel opening. This further suggests that physi-

ologic-metabolic conditions that modify endogenous PTK and PTP activities could shift the channel into an activatable state. Although our results suggest that inhibition of PTKs may underlie genistein actions on the cardiac sarcolemmal K_{ATP} channel, a direct interaction of this isoflavone and also tyrosine phosphatases with the channel cannot be ruled out. This study also shows that isoflurane may potentiate genistein-activated I_{KATP} during whole cell conditions but not in cell-free membrane patches, implying a possibility of another cytosolic factor requirement for isoflurane effects. The study suggests that a tyrosine phosphatase, such as PTP1B, may be a candidate factor to play a role in isoflurane potentiation of the cardiac sarcolemmal K_{ATP} channel.

References

- Hunter T, Cooper JA: Protein tyrosine kinases. *Annu Rev Biochem* 1985; 54:897-930
- Fantl WJ, Johnson DE, Williams LT: Signaling by receptor tyrosine kinases. *Annu Rev Biochem* 1993; 62:453-81
- Sugden PH, Bogoyevitch MA: Intracellular signaling through protein kinases in the heart. *Cardiovasc Res* 1995; 30:478-92
- Levitan IB: Modulation of ion channels by protein phosphorylation and dephosphorylation. *Annu Rev Physiol* 1994; 56:193-212
- Siegelbaum SA: Ion channel control by tyrosine phosphorylation. *Curr Biol* 1994; 4:242-5
- Davis MJ, Wu X, Nurkiewicz TR, Kawasaki J, Gui P, Hill MA, Wilson E: Regulation of ion channels by protein tyrosine phosphorylation. *Am J Physiol Heart Circ Physiol* 2001; 281:H1835-62
- Fisher EH, Charbonneau H, Tonks NK: Protein tyrosine phosphatases: A diverse family of intracellular and transmembrane enzymes. *Science* 1991; 253:401-6
- Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S-I, Itoh N, Shibuya M, Fukami Y: Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* 1987; 262:5592-5
- Chiang C-E, Chen S-A, Chang M-S, Lin C-I, Luk H-N: Genistein directly inhibits L-type calcium currents but potentiates cAMP-dependent chloride currents in cardiomyocytes. *Biochem Biophys Res Commun* 1996; 223:598-603
- Katsube Y, Yokoshiki H, Nguyen L, Yamamoto M, Sperelakis N: Inhibition of Ca^{2+} current in neonatal and adult rat ventricular myocytes by the tyrosine kinase inhibitor, genistein. *Eur J Pharmacol* 1998; 345:309-1
- Ogura T, Shuba LA, McDonald TF: L-type Ca^{2+} current in guinea pig ventricular myocytes treated with modulators of tyrosine phosphorylation. *Am J Physiol Heart Circ Physiol* 1999; 276:H1724-33
- Wang YG, Lipsius SL: Genistein elicits biphasic effects on L-type Ca^{2+} current in feline atrial myocytes. *Am J Physiol Heart Circ Physiol* 1998; 275:H204-12
- Boixel Ch, Tessier S, Pansard Y, Lang-Lazdunski L, Mercadier JJ, Hatem SN: Tyrosine kinase and protein kinase C regulate L-type Ca^{2+} current cooperatively in human atrial myocytes. *Am J Physiol Heart Circ Physiol* 2000; 278:H670-6
- Wu J-Y, Cohe IS: Tyrosine kinase inhibition reduces I_f in rabbit sinoatrial node myocytes. *Pflügers Arch Eur J Physiol* 1997; 434:509-14
- Sims C, Chiu J, Harvey RD: Tyrosine phosphatase inhibitors selectively antagonize β -adrenergic receptor-dependent regulation of cardiac ion channels. *Mol Pharmacol* 2000; 58:1213-21
- Sorota S: Tyrosine protein kinase inhibitors prevent activation of cardiac swelling-induced chloride current. *Pflügers Arch Eur J Physiol* 1995; 431:178-85
- Shuba LM, Asai T, Peltzer T, McDonald TF: Activation of cardiac chloride conductance by the tyrosine kinase inhibitor genistein. *Br J Pharmacol* 1996; 119:335-45
- Hool LC, Middleton LM, Harvey RD: Genistein increases the sensitivity of cardiac ion channels to β -adrenergic receptor stimulation. *Circ Res* 1998; 83:33-42
- Washizuka T, Horie M, Obayashi K, Sasayama S: Genistein inhibits slow component delayed-rectifier K currents via a tyrosine kinase-independent pathway. *J Mol Cell Cardiol* 1998; 30:2577-90
- Nishio M, Habuchi Y, Tanaka H, Morikawa J, Okanou T, Kashima K: Tyrosine kinase-dependent modulation by interferon- α of the ATP-sensitive K^+ current in rabbit ventricular myocytes. *FEBS Lett* 1999; 445:87-91
- Kwak YG, Park SK, Cho KP, Chae SW: Reciprocal modulation of ATP-sensitive K^+ channel activity in rat ventricular myocytes by phosphorylation of tyrosine and serine/threonine residues. *Life Sci* 1996; 58:897-904
- Townsend C: Nucleotides alter genistein effects on ATP-sensitive K^+ channels (abstract). *Biophys J* 2001; 80:A2819

23. Warltier DC, al-Wathiqui MH, Kampine JP, Schmeling WT: Recovery of contractile function of stunned myocardium in chronically instrumented dogs is enhanced by halothane or isoflurane. *ANESTHESIOLOGY* 1988; 69:552-65
24. Kersten JR, Schmeling TJ, Hettrick DR, Pagel PS, Gross GA, Warltier DC: Mechanism of myocardial protection by isoflurane: Role of adenosine triphosphate-regulated potassium (KATP) channels. *ANESTHESIOLOGY* 1996; 85:794-807
25. Kersten JR, Lowe D, Hettrick DA, Pagel PS, Gross GJ, Warltier DC: Glyburide, a KATP channel antagonist, attenuates the cardioprotective effects of isoflurane in stunned myocardium. *Anesth Analg* 1996; 83:27-33
26. Kersten JR, Schmeling TJ, Pagel PS, Gross GJ, Warltier DC: Isoflurane mimics ischemic preconditioning *via* activation of K_{ATP} channels: Reduction of myocardial infarct size with an acute memory phase. *ANESTHESIOLOGY* 1997; 87:361-70
27. Toller WG, Kersten JR, Gross ER, Pagel PS, Warltier DC: Isoflurane preconditions myocardium against infarction *via* activation of inhibitory guanine nucleotide binding proteins. *ANESTHESIOLOGY* 2000; 92:1400-7
28. Toller WG, Gross ER, Kersten JR, Pagel PS, Gross GJ, Warltier DC: Sarcolemmal and mitochondrial adenosine triphosphate-dependent potassium channels: Mechanism of desflurane-induced cardioprotection. *ANESTHESIOLOGY* 2000; 92:1731-9
29. Rosco AK, Christensen JD, Lynch C III: Isoflurane but not halothane, induces protection of human myocardium *via* adenosine A₁ receptors and adenosine triphosphate-sensitive potassium channels. *ANESTHESIOLOGY* 2000; 92:1692-701
30. Han J, Kim E, Ho WK, Earm YE: Effects of volatile anesthetic isoflurane on ATP-sensitive K⁺ channels in rabbit ventricular myocytes. *Biochem Biophys Res Commun* 1996; 229:852-6
31. Fujimoto K, Bosnjak ZJ, Kwok WM: Isoflurane-induced facilitation of the cardiac sarcolemmal K_{ATP} channel. *ANESTHESIOLOGY* 2002; 97:57-65
32. Kwok WM, Martinelli AT, Fujimoto K, Suzuki A, Stadnicka A, Bosnjak ZJ: Differential modulation of the cardiac ATP-sensitive potassium channel by isoflurane and halothane. *ANESTHESIOLOGY* 2002; 97:50-6
33. Stadnicka A, Bosnjak JZ, Kampine JP, Kwok WM: Modulation of cardiac inward rectifier K⁺ current by halothane and isoflurane. *Anesth Analg* 2000; 90:824-33
34. Akiyama T, Ogawara H: Use and specificity of genistein as inhibitor of protein-tyrosine kinases. *Methods Enzymol* 1991; 201:362-85
35. Tonks NK, Neel BG: From form to function: Signaling by protein tyrosine phosphatases. *Cell* 2000; 87:365-8
36. Inagaki N, Gono T, Clement IV JP, Wang C-Z, Aguilar-Bryan L, Bryan J, Seino S: A family of sulfonylurea receptor determines the pharmacological properties of ATP-sensitive K⁺ channels. *Neuron* 1996; 16:1011-7
37. Aguilar-Bryan L, Clement JP IV, Gonzales G, Kunjilwar K, Babenko A, Bryan J: Toward understanding the assembly and structure of KATP channels. *Physiol Rev* 1998; 78:227-45
38. Markovits J, Linossier C, Fosse P, Couprie J, Pierre J, Jacquemin-Sablon A, Saucier JM, Le Pecq JB, Larsen AK: Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. *Cancer Res* 1989; 49:5111-7
39. French PJ, Bijman J, Bot AG, Boomaars WEM, Scholte BJ, DeJonge HR: Genistein activates CFTR Cl⁻ channels *via* a tyrosine kinase- and protein phosphatase-independent mechanism. *Am J Physiol Cell Physiol* 1997; 273:C747-55
40. Ueda K, Komine J, Masuo M, Seino S, Amachi T: Cooperative binding of ATP and MgADP in the sulfonylurea receptor is modulated by glibenclamide. *Proc Natl Acad Sci U S A* 1999; 96:1268-72
41. Bienengraeber M, Alekseev AE, Abraham MR, Carrasco AJ, Moreau C, Vivaudou M, Dzeja PP, Terzic A: ATPase activity of the sulfonylurea receptor: A catalytic function for the KATP channel complex. *FASEB J* 2000; 14:1943-52
42. Frangioni JV, Beahm PH, Shifrin V, Jost CA, Neel BG: The nontransmembrane tyrosine phosphatase PTP 1B localizes to the endoplasmic reticulum *via* its 35 amino acid C-terminal sequence. *Cell* 1992; 68:545-60
43. Fryer RM, Schultz Jo El J, Hsu AK, Gross GJ: Pretreatment with tyrosine kinase inhibitors partially attenuates ischemic preconditioning in rat hearts. *Am J Physiol Heart Circ Physiol* 1998; 275:H2009-15
44. Tanno M, Tsuchida A, Nozawa Y, Matsumoto T, Hasegawa T, Miura T, Shimamoto K: Roles of tyrosine kinase and protein kinase C in infarct size limitation by repetitive ischemic preconditioning in the rat. *J Cardiovasc Pharm* 2000; 35:345-52