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Inhibitory effects of genistein on ATP-sensitive K^+ channels in rabbit portal vein smooth muscle

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1 Effects on the pinacidil-induced outward current of inhibitors of tyrosine kinases and phosphatases were investigated by use of a patch-clamp method in smooth muscle cells of the rabbit portal vein.

2 A specific tyrosine kinase inhibitor, genistein, inhibited the pinacidil-induced current in a concentration-dependent manner with an IC_{50} of 5.5 μM. Superfusion of Ca^{2+}-free solution did not affect this inhibitory effect of genistein. At higher concentrations, genistein inhibited the voltage-dependent Ba^{2+} and K^+ currents with IC_{50} values of >100 μM and 75 μM respectively. Tyrphostin B46 (30 μM), a tyrosine kinase inhibitor, also inhibited the pinacidil-induced current by 70 % of the control.

3 Sodium orthovanadate (100 μM), an inhibitor of tyrosine phosphatase, slightly but significantly enhanced both the pinacidil-induced and delayed rectifier K^+ currents. Daidzein (100 μM), an inactive analogue of genistein, did not inhibit these currents.

4 Neither herbimycin A (1 μM), lavendustin A (30 μM), tyrphostin 23 (10 μM), which are also tyrosine kinase inhibitors, nor wortmannin (10 μM), a phosphatidylinositol 3-kinase inhibitor, had an effect on either the pinacidil-induced or delayed rectifier K^+ currents. Epidermal growth factor (EGF; 1 μg ml^{-1}) did not induce an outward current or enhance the pinacidil-induced current.

5 Pinacidil alone, in the cell-attached configuration, or pinacidil with GDP, in the inside-out configuration, activated a 42 pS channel in the smooth muscle cells of the rabbit portal vein. Genistein (30 μM) reduced the channel's open probability without inducing a change in unitary conductance at any holding potential (-30 to $+20$ mV).

6 In the inside-out configuration, genistein at 30 μM did not change the mean channel open time, but reduced the burst duration. At 100 μM genistein abolished channel opening. The inhibitory potencies with which 30 and 100 μM genistein acted on the unitary current of the ATP-sensitive K^+ channel were similar to those seen in the whole-cell voltage-clamp configuration.

7 Although direct inhibitory actions of genistein on the ATP-sensitive K^+ channels are not ruled out, our results suggest that a protein tyrosine kinase may play a role in the regulation of ATP-sensitive K^+ channel activity in the rabbit portal vein.

Keywords: ATP-sensitive K^+ channel; K^+ channel; Ca^{2+} current; tyrosine kinase; genistein; vascular smooth muscle

Introduction

Tyrosine kinases play pivotal roles in the control of cell growth and differentiation (Van der Geer *et al*., 1994), and are key enzymes for the regulation of membrane excitability and ion channel function in the central nervous system (Lev *et al*., 1995). Evidence has been accumulating for some years now that tyrosine kinases are involved in receptor-mediated current inhibition, and in contraction and cell-signalling (Williams, 1989; Tsuda. *et al*., 1991; Wijetunge *et al*., 1992; Huang *et al*., 1993; Hollenberg, 1994; Felder, 1995; Wang & Salter, 1994; Moss *et al*., 1995; Wijetunge & Hughes, 1995; Hatakeyama *et al*., 1996). These studies suggest tyrosine phosphorylation of tyrosine residues might be an important mechanism for channel regulation, in addition to the well-attested protein phosphorylation that occurs through serine/threonine kinases. It has also been found that various channels can be phosphorylated by tyrosine kinase and the effect inhibited by tyrosine kinase inhibitors (Huang *et al*., 1993; Moss *et al*., 1995; Lev *et al*., 1995; Swope *et al*., 1995).

With regard to the ATP-sensitive K^+ current, the participation of a tyrosine kinase in the muscarinic inhibition of this current has been obtained in rabbit oesophageal smooth muscle cells and in RAK-K^+ channels co-expressed with muscarinic receptors in *Xenopus* oocytes (Huang *et al*., 1993; Hatakeyama *et al*., 1995). These authors showed that tyrosine kinase enhanced the muscarinic inhibition of the ATP-sensitive K^+ current through modulation of protein kinase C. However, there has been no report of a direct effect of tyrosine kinase on ATP-sensitive K^+ channel activity. The present experiments were designed to elucidate the role of tyrosine kinase in the regulation of the ATP-sensitive (pinacidil-induced) K^+ channels in the rabbit portal vein, by making use of specific tyrosine kinase inhibitors, especially genistein.

Methods

Cell isolation

Male albino rabbits (Nippon White; 1.7–2.0 kg) were anaesthetized with sodium pentobarbitone by intravenous injection (40 mg kg^{-1}; Tokyo Kasei, Tokyo) and exsanguinated. The portal vein was dissected out and isolated by removing the surrounding connective tissue and adipose tissue. The isolated vein was cut into thin strips 1 mm in width and these were incubated for 15 min at 35°C in Ca^{2+}-free solution containing 0.16% collagenase (Wako Pure Chem., Osaka), 0.1% trypsin inhibitor (type-IIS, Sigma Chem., St. Louis, MO), 0.1% bovine serum albumin (fraction V, essentially fatty acid free; Sigma Chem.) and 0.1% dithiothreitol (DTT; Sigma Chem.). After this enzyme treatment, the tissue was transferred to fresh

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Ca^{2+}-free solution (35°C) and single cells were dispersed by gentle agitation with blunt-tipped pipettes. Dispersed cells were stored at 10°C in a fresh solution containing 0.5 mM Ca^{2+} and 0.5 mM Mg^{2+}. Experiments were performed at room temperature (25–28°C).

Signal recording and data analysis

For whole-cell recording of K^+ channel currents, either a physiological salt solution (PSS) or a nominally Ca^{2+}-free physiological salt solution (Ca^{2+}-free PSS) was superfused in the bath (2 ml min^{-1}) and the overflowing solution was siphoned off by a water pump. The pipette was filled with a high-K^+ solution. For the whole-cell recordings of Ca^{2+} channel currents, 10 mM Ba^{2+} solution was in the bath and high-Cs^+ solution in the pipette. Leak current was subtracted for measurements of the Ba^{2+} current amplitude. For the single-channel recording of K^+ channel currents, high-K^+ solution was superfused (2 ml min^{-1}) in the bath and the over flowing solution was siphoned off by a water pump. The pipette was filled with a high-K^+ solution. Membrane currents were recorded by use of whole-cell, cell-attached and inside-out patch voltage-clamp methods (Hamill *et al*., 1981). Patch electrodes (3–5 MΩ) were prepared with a Flaming-Brown type electrode puller (P-98: Sutter Instruments, Novato, CA) and manipulated with a three-dimensional water-driven manipulator (MHW-3, Narishige Sci. Inst. Lab.) or an electric manipulator (Manipulator E: Leitz, Wetzler). Data acquisition was carried out by use of an Axopatch-200 or Axopatch-1D amplifier (Axon Instruments, Foster City, CA) and an IBM-compatible PC (IMC-P4100; Inter Medical Co., Nagoya) and analysis performed by means of pCLAMP 6.0 software (Axon Inst.). A hard copy was obtained with a laser printer (Laser Wind 1040PS; Star, Tokyo).

Curves in Figures 1d and 2c were fitted by the following equation:

$$I_{[\text{genistein}]} = (1 + (IC_{50}/[\text{genistein}]))^{-1},$$

where [genistein], $I_{[\text{genistein}]}$ and IC_{50} are concentration of genistein, the amplitude of the current in the presence of a particular concentration of genistein and the genistein concentration needed to produce half-inhibition of the current, respectively.

Solutions

PSS of the following ionic composition was used (mM): NaCl 140, KCl 5.4, $MgCl_2$ 1.2, $CaCl_2$ 2.3 and glucose 12. The nominally Ca^{2+}-free solution used for cell dispersion and for the bath solution was prepared by omission of 2.3 mM $CaCl_2$ from the PSS. High-K^+ solution of the following ionic composition was in the pipette for recording outward currents (mM): KCl 120, pyruvate 20, glucose 20, $MgCl_2$ 3, adenosine

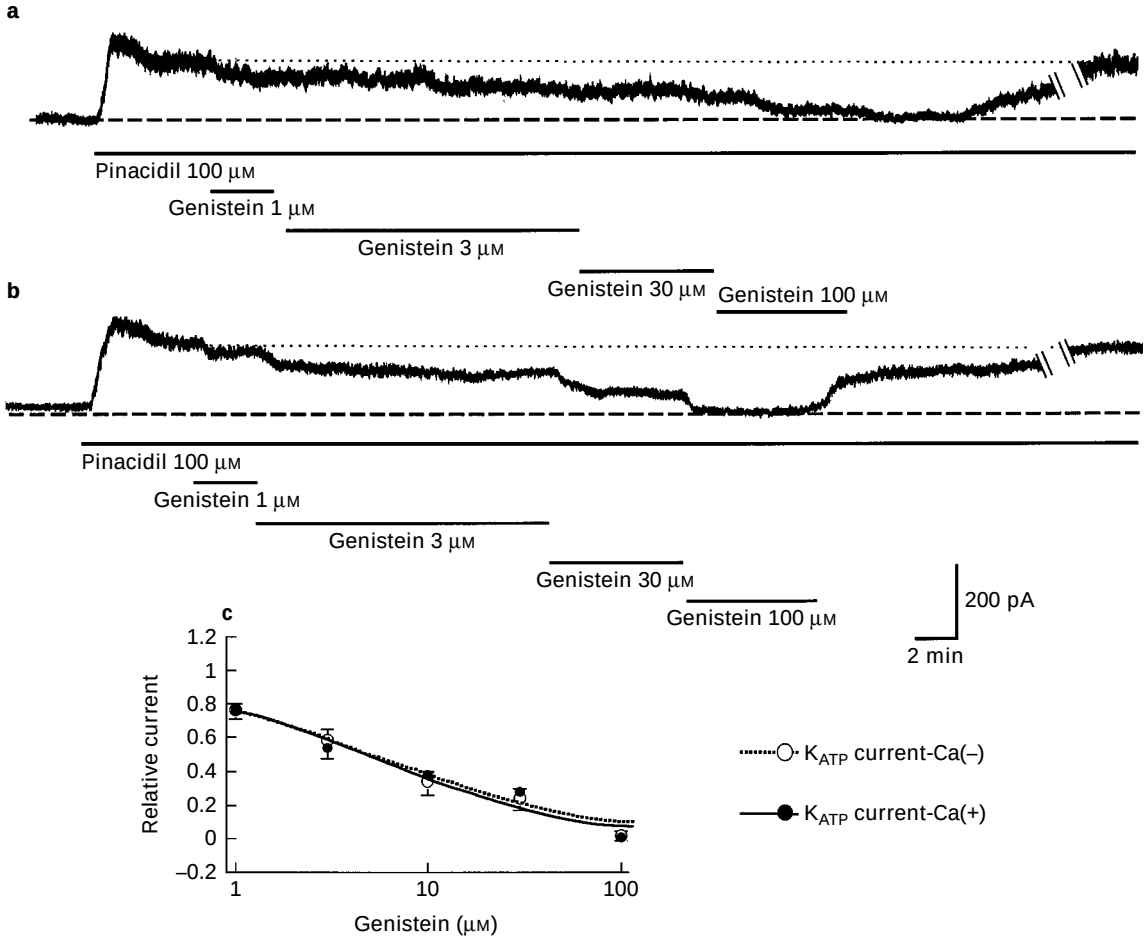

Figure 1 Effects of genistein on pinacidil (100 μM)-induced K^+ currents recorded in PSS (a) and nominally Ca^{2+}-free solution (b). The currents were recorded at a holding potential of −40 mV. Pinacidil (100 μM) and genistein (1–100 μM; cumulatively) were applied as indicated by the bars under each record. High-K^+ solution with 0.1 mM (a) or 4 mM EGTA (b) was in the pipette. Dashed lines indicate the zero-current level and dotted lines indicate the sustained level of the pinacidil induced K^+ currents. (c) Relationship between concentration of genistein (1–100 μM) and relative amplitude of the pinacidil-induced current recorded in PSS (Ca(+)) or in nominally Ca^{2+}-free solution (Ca(−)) In these experiments, genistein was applied after the pinacidil-induced current had reached a sustained amplitude. The amplitude of the pinacidil-induced K^+ current just before the application of genistein was normalized as 1.0. Each symbol indicates the mean value and vertical lines show s.d.

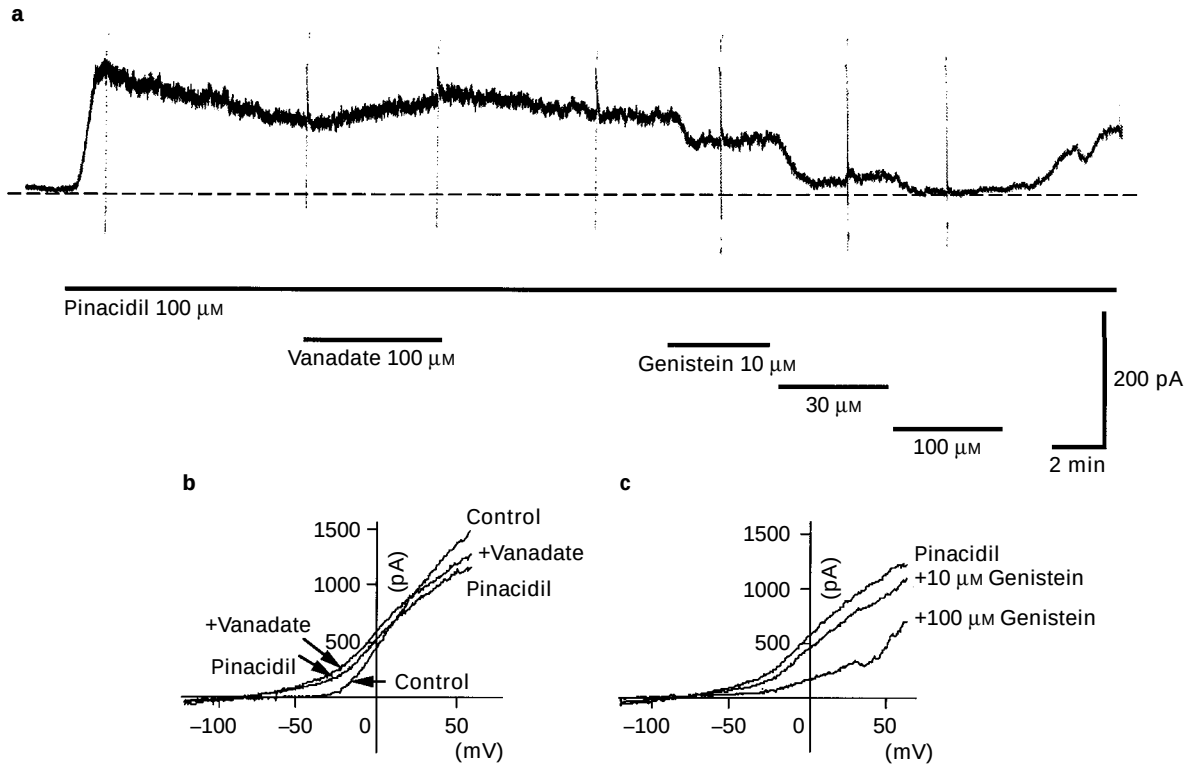

Figure 2 Effects of sodium orthovanadate (100 μM) and genistein (10–100 μM) on pinacidil-induced K^+ current. High-K^+ solution with 4 mM EGTA was in the pipette and a nominally Ca^{2+}-free solution was in the bath. (a) The holding potential was −40 mV and drugs were applied as indicated by the bars. Dashed line indicates the zero-current line. During the current recording, a ramp voltage pulse was applied (from −120 mV to 60 mV; 300 ms in duration). (b–c) Membrane currents evoked by the ramp pulses are superimposed. (b, control) recorded before application of pinacidil; (b, pinacidil), recorded just before application of sodium orthovanadate (4.5 min after application of pinacidil); (b, +vanadate), recorded in the presence of sodium orthovanadate. (c, pinacidil) recorded 6 min after removal of sodium orthovanadate; (c, +10 μM genistein), recorded 2 min after application of 10 μM genistein. (c, +100 μM genistein), recorded 2 min after application of 100 μM genistein.

5′-triphosphate (ATP) 1 and ethyleneglycol-bis[β-aminoethylether]-N,N,N′,N′-tetraacetic acid (EGTA) 0.1 or 4. For recording the Ba^{2+} current, 10 mM Ba^{2+} solution and high-Cs^+ solution (see below) were used in the bath and pipette, respectively. Ba^{2+} solution (mM): $BaCl_2$ 10, NaCl 128, KCl 5.4 and glucose 11. High-Cs^+ solution (mM): CsCl 120, pyruvate 20, EGTA 4, $MgCl_2$ 5, ATP 5 and glucose 20. High-K^+ solution of the following ionic composition was in the bath for the cell-attached and inside-out patch recordings (mM): KCl 120, pyruvate 20, glucose 20 and EGTA 1. High-K^+ solution of the following ionic composition was in the pipette for the cell-attached and inside-out patch recordings (mM): KCl 120, pyruvate 20 and glucose 20. The pH of the solutions was adjusted to 7.37 ± 0.02 with 10 mM 4-[2-hydroxyethyl]-1-piperazine ethansulphonic acid (HEPES; Dojin Kagaku, Kumamoto) titrated with NaOH (PSS, Ba^{2+} solution and Ca^{2+}-free solution), KOH (high-K^+ solution) or tris [hydroxymethyl] aminomethane (high-Cs^+ solution).

Drugs

Drugs used in the present experiments were pinacidil (Shionogi, Osaka), glibenclamide (Sigma Chem., St. Louis, MO), genistein, herbimycin A, tyrphostin 23, tyrphostin B46, wortmannin, epidermal growth factor (EGF; human recombinant) (Wako Pure Chem., Osaka), lavendustin A (Calbiochem, La Jolla, CA), sodium orthovanadate (Sigma Chem.), daidzein (Biomol, Plymouth Meeting, PA) and guanosine 5'-diphosphate (GDP; Sigma Chem.). These drugs, except EGF and GDP, were dissolved in dimethyl sulphoxide (DMSO) and diluted over 2000 times into the bath solution, the final concentration of DMSO being kept under 0.1% even when a combination of more than two drugs was used, except in the experiment with 10 μM herbimycin A. We confirmed that 0.1% DMSO had no effect on any of the membrane currents. EGF was dissolved in deionized water with 1 mg ml^{-1} DTT and stored at −20°C.

Statistics

The results are expressed as mean values with standard deviation. Statistical significance was assessed by use of Student's *t* test and *P* values less than 0.05 were considered to be significant.

Results

Effect of genistein on the pinacidil-induced K^+ current

Application of pinacidil (100 μM) produced an outward current at a holding potential of −40 mV. This current was inhibited by 10 μM glibenclamide (see Figure 3b) and did not decrease in amplitude on the removal of Ca^{2+} ions from the superfusate (Figure 1). As shown in Figure 1a and b, the pinacidil-induced outward current gradually decreased, but reached a plateau several min after the administration of pinacidil. In the present experiments, to show up the effects of genistein, a selective inhibitor of protein tyrosine kinase (Akiyama *et al*., 1987), on this current, we cumulatively applied various concentrations of genistein after the pinacidil-induced current had stabilized. Figure 1 shows an examples of such effects recorded in PSS (Figure 1a) and nominally Ca^{2+}-free solution (Figure 1b). Genistein slightly, at 1 μM and clearly, at 3 μM inhibited the pinacidil-induced outward current in either solution. A high concentration of genistein (100 μM) inhibited this current completely. Figure 1c shows the relationships between the concentration of genistein and the

relative amplitude of the pinacidil-induced K^+ current. There was no difference between the curves obtained in the presence or absence of Ca^{2+}, indicating that neither the pinacidil-induced outward current nor its inhibition by genistein were affected by the extracellular Ca^{2+} concentration. The IC_{50} for the effect of genistein on the pinacidil-induced K^+ current was 5.5 μM in PSS and 5.7 μM in Ca^{2+}-free solution.

Effect of daidzein and sodium orthovanadate

To confirm that the actions of genistein on the pinacidil-induced K^+ associated with its inhibitory effect on tyrosine kinase activity, we next examined the effects of daidzein, an inactive analogue of genistein (Akiyama & Ogawara, 1991), and orthovanadate, a protein tyrosine phosphatase inhibitor. Daidzein (100 μM) did not inhibit the pinacidil-induced K^+ current (0.97 ± 0.05, $n=5$). On the other hand, sodium orthovanadate (100 μM) slightly potentiated the pinacidil-induced K^+ current (1.18 ± 0.10, $n=5$; Figure 2a). After removal of sodium orthovanadate, the amplitude of the pinacidil-induced K^+ current declined to its control level, and subsequent application of genistein (10, 30 and 100 μM) inhibited the current (Figure 2a). Ramp voltage-changes (from -120 to $+60$ mV) were applied during the application of pinacidil alone, sodium orthovanadate and genistein. As shown in Figure 2b, the voltage-dependent K^+ current was evoked at the potential more positive than -30 mV and pinacidil initially increased the outward current over the entire voltage range. However, 4 min or so later, the outward current evoked at positive potentials was inhibited, although the current evoked at negative potentials was still enhanced (Figure 2b, control, pinacidil). Application of sodium orthovanadate (100 μM) then increased somewhat the current evoked by the ramp pulse (Figure 2b, + vanadate). Removal of the vanadate restored the current to the control level (i.e. that recorded before the application of vanadate). When ramp pulses were subsequently applied during the pinacidil-induced effect, it was clear that genistein, at any of the concentrations used (10–100 μM), had an inhibitory effect over the entire potential range (Figure 2c). These results indicate that sodium orthovanadate enhanced and genistein inhibited the pinacidil-induced K^+ current.

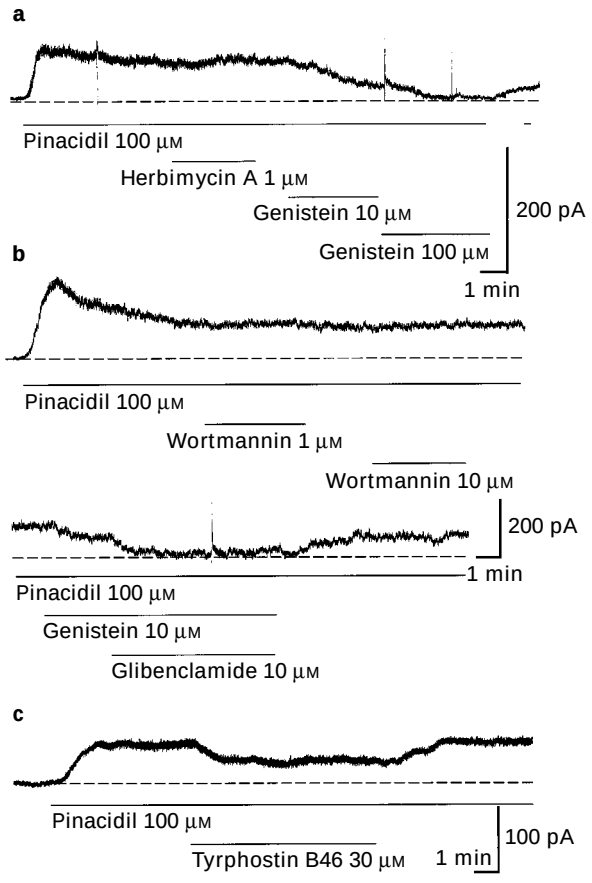

Figure 3 Effects of 1 μM herbimycin A (a), 1 μM wortmannin (b) and 30 μM tyrphostin B46 (c) on the pinacidil-induced K^+ current. The holding potential was kept at -40 mV. PSS was superfused in the bath and high-K^+ solution with 0.1 mM (a and b) or 4 mM (c) EGTA was in the pipette. In (a–c), herbimycin A, wortmannin, genistein, glibenclamide and tyrphostin B46 were added to the bath as indicated by the bars under each record. Dashed lines indicate the zero-current line.

Effect of other tyrosine kinase inhibitors on the pinacidil-induced K^+ current

Herbimycin A (1 μM), another protein tyrosine kinase inhibitor, did not inhibit the amplitude of neither the voltage-dependent K^+ current (data not shown) nor the pinacidil-induced K^+ current (Figure 3a). Subsequent application of genistein (10 and 100 μM; Figure 3a) inhibited the latter current in the same cell, confirming that herbimycin A (1 μM) really was inactive in this respect. Even a higher concentration of herbimycin A (10 μM) had no significant effect on the pinacidil-induced K^+ current (0.84 ± 0.11, $n=4$), although 0.5% DMSO as solvent did have slight inhibitory effects on the pinacidil-induced current (0.91 ± 0.09, $n=3$).

As phosphorylation by many tyrosine kinases leads to activation of PI_3 kinase, we next examined whether or not the inhibitory effects of genistein on pinacidil-induced K^+ current might be related to an effect on PI_3 kinase. To this end, the effects of wortmannin, a PI_3 and myosin light chain kinases inhibitor ($IC_{50}=3.0$ nM for PI_3 kinase, Yano *et al*., 1993; $IC_{50}=0.2$ μM for myosin light chain kinase, Nakanishi *et al*., 1992), were investigated. In fact, wortmannin (1–10 μM) did not affect the pinacidil-induced K^+ current (1 μM; 0.98 ± 0.09, 10 μM; 0.98 ± 0.03, $n=5$). Subsequent application of genistein (10 μM) inhibited the pinacidil-induced K^+ current by about one-third and the cumulative application of glibenclamide (10 μM) then completely inhibited the current (Figure 3b), suggesting that PI_3 kinase is not involved in their effect on the pinacidil-induced K^+ channel.

Application of lavendustin A (30 μM) and tyrphostin 23 (10 μM), inhibitors of the EGF receptor tyrosine kinase, did not suppress the currents induced by 100 μM pinacidil (lavendustin A, 1.13 ± 0.06, $n=3$; tyrphostin 23, 0.86 ± 0.10, $n=5$). EGF (1 μg ml^{-1}) neither produced an outward current nor enhanced the pinacidil-induced K^+ current ($n=3$). On the other hand, tyrphostin B46 (30 μM), another tyrosine kinase inhibitor, reduced the pinacidil-induced K^+ current (0.70 ± 0.20, $n=5$; Figure 3c).

Effect of genistein on the unitary currents induced by pinacidil

In the cell-attached configuration, pinacidil (100 μM) opened a channel with a unitary conductance of 42 pS (Figure 4a and c). In this experiment, in which we used high K^+ solution both in the bath and in the pipette, the unitary current reversed at nearly 0 mV. When the open probability (NPo) was estimated from the all-points amplitude histogram, we could not see a voltage-dependent activation of channel activity (-10 mV, 0.06; -20 mV, 0.112; -30 mV, 0.104). Application of genistein via the bath reduced the NPo with no change in the amplitude of the unitary conductance (Figure 4b and c). The NPo of the pinacidil-induced current in the presence of genistein (30 μM) was 0.02 at -10 mV, 0.03 at -20 mV and 0.04 at -30 mV.

In the inside-out configuration, the 42 pS channel current was not present even in the presence of pinacidil, but intracellular application of GDP reactivated the ATP-sensitive channel (Kajioka *et al*., 1991; Kamouchi & Kitamura, 1994).

Figure 5 shows an example of the recording of the GDP/pinacidil-induced unitary current in a membrane patch. In this experiment, pinacidil (100 μM) was present in the bathing solution throughout the experiment, and only a large amplitude outward unitary current (maxi-K^+ current; Kajioka *et al*., 1991) could be recorded (1st trace). Addition of GDP (300 μM) then opened a unitary current which had a very long channel opening and a small amplitude (unitary conductance of 42 pS; Figure 5, 1st trace). We confirmed that glibenclamide (10 μM) completely abolished the 42 pS current without a significant effect on the maxi-K^+ channel activity (data not shown; cf. Kajioka *et al*., 1991; Kamouchi & Kitamura, 1994). Simultaneous application of genistein (30 μM) inhibited the 42 pS channel and shortened the long channel opening (burst duration); removal of genistein restored the burst duration (2nd and 3rd traces). A high

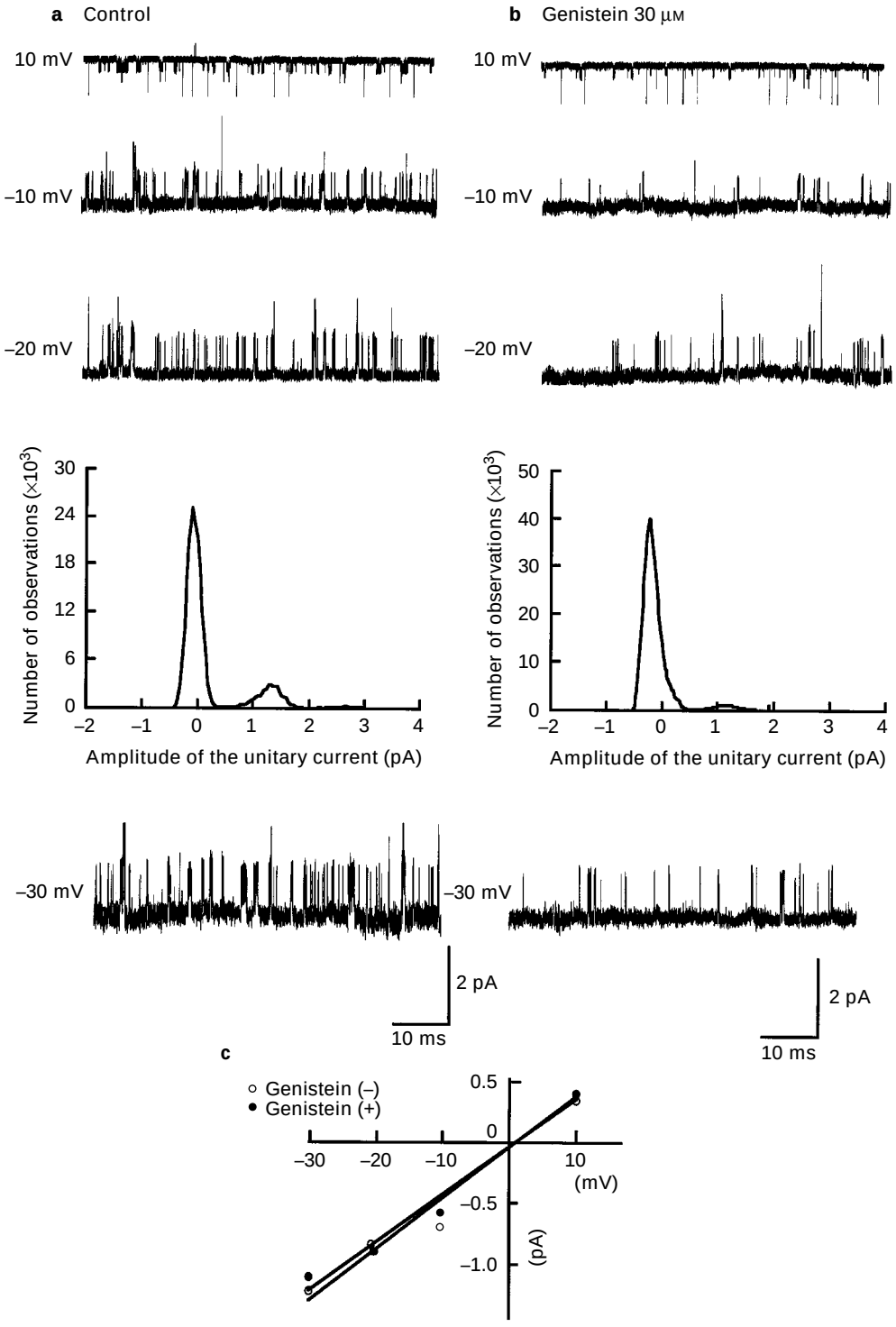

Figure 4 Effect of genistein (30 μM) on the pinacidil-induced unitary current recorded in the cell-attached configuration of the patch-clamp method. High-K^+ solution with 1 mM EGTA was superfused in the bath and was also in the pipette. (a) In the absence of genistein. Unitary currents were recorded at membrane potentials of +10, −10, −20 and −30 mV (a part of each trace is shown). (b) In the presence of 30 μM genistein. Traces were recorded 7 min after application of 30 μM genistein. All-points amplitude histograms recorded at holding potential of −20 mV are also shown (2 min (a) or 3 min (b) recording period with sampling rate of 300 Hz, respectively). (c) Current-voltage relationships for the pinacidil-induced K^+ current in the presence (+) and absence (−) of genistein. The value of the unitary conductance was 42 pS.

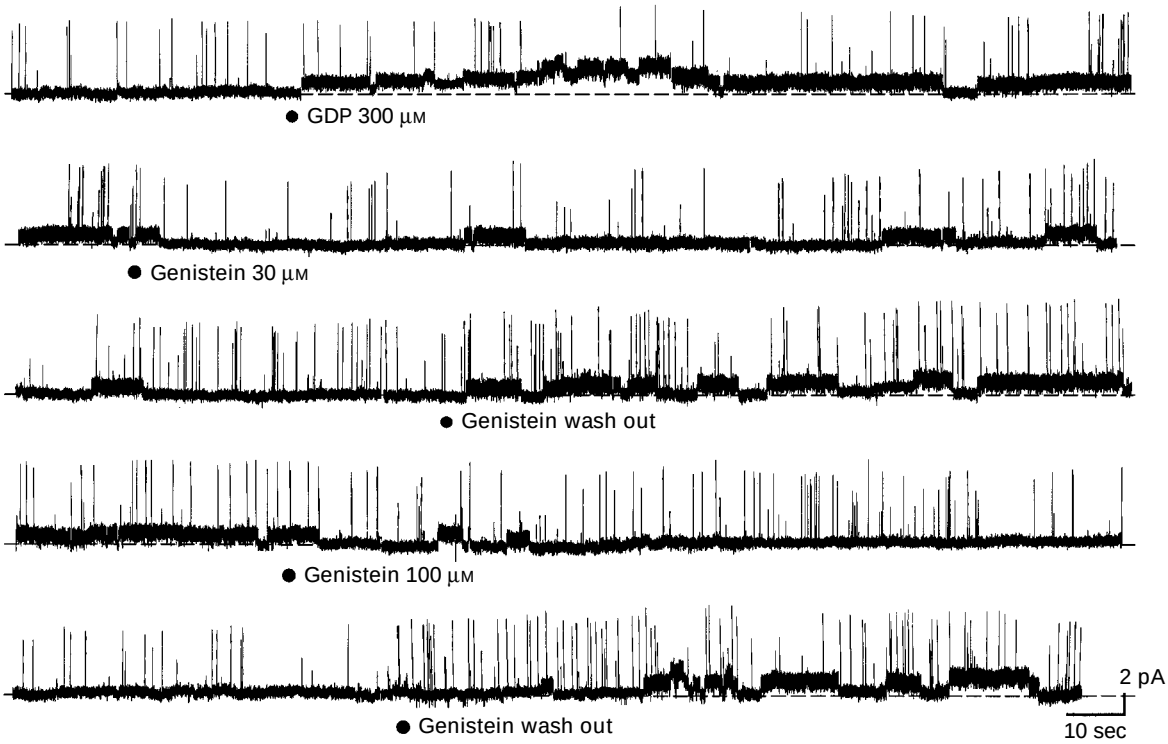

Figure 5 Effect of genistein (30 μM) on the pinacidil/GDP-induced unitary K^+ current recorded in the inside-out membrane patch configuration. The traces shown formed a continuous record from the same membrane patch. Pinacidil (100 μM) was applied throughout the experiment and GDP (300 μM) was present beginning at the time indicated in the top trace and then throughout. Genistein (30 μM) was applied at the time indicated in the second trace and washed out as indicated in the third trace, and 100 μM was applied in the fourth trace and washed out in the bottom trace.

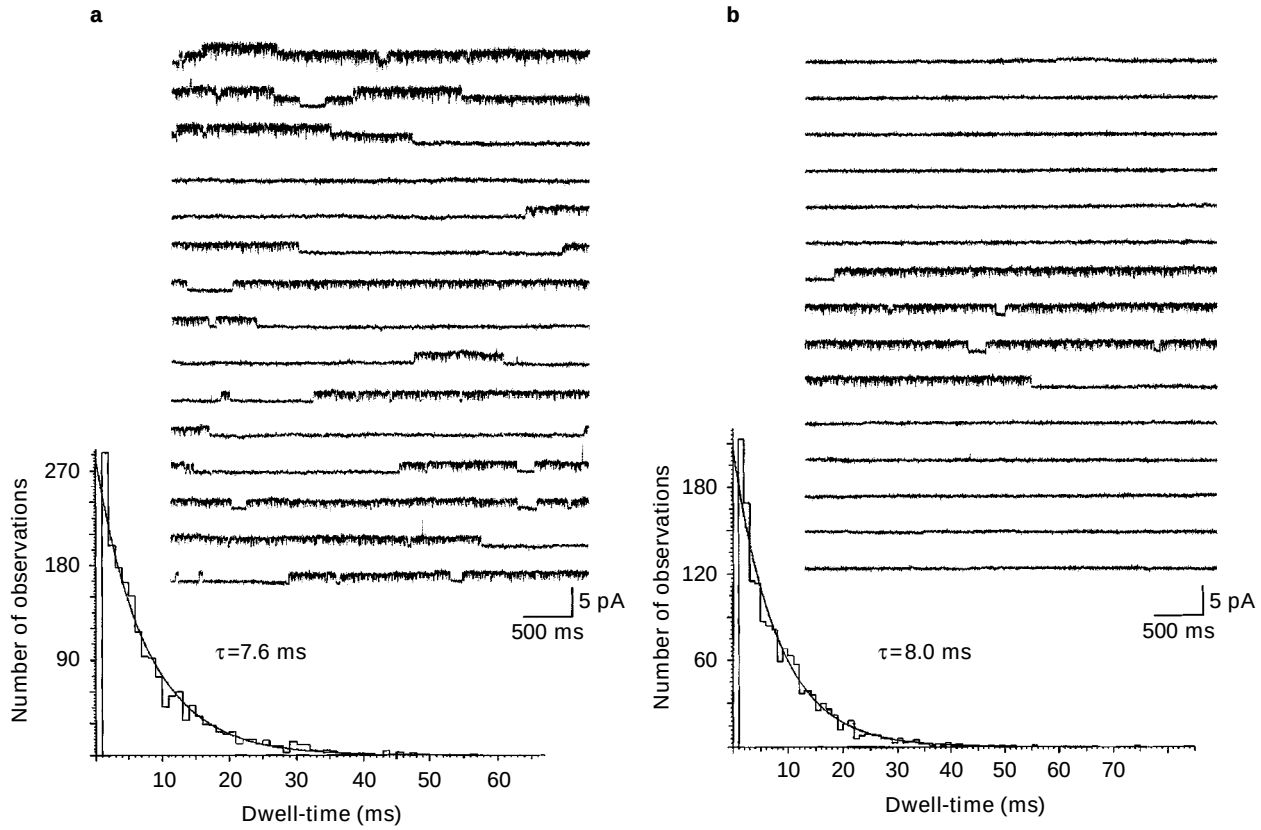

Figure 6 Open-time histograms and examples of traces recorded in the absence (a) and presence (b) of 30 μM genistein. Unitary currents were recorded from an inside-out membrane patch with 100 μM pinacidil and 300 μM GDP in the bath. Histograms were drawn from the data shown (over 30 s for control and over 42.75 s for genistein). Data in the presence of genistein were obtained 3 min after its administration. As can be seen in the figure, the number of channels in this membrane patch was more than 2, and in control the first 3 traces were disregarded for the estimation of mean open time. Curves were fitted with a single exponential.

concentration of genistein (100 μM) abolished the unitary current of the 42 pS channel, but the maxi-K^+ channels were still seen to be open (4th trace). Removal of 100 μM genistein re-opened the 42 pS channel current (last trace). Figure 6 indicates the mean open time of the 42 pS channel current in the absence and presence of 30 μ μM genistein. Although the number of channel openings was reduced, the mean open time was not modulated by 30 μM genistein (control, 7.6 ms; genistein, 8.0 ms).

Effect of genistein on voltage-dependent K^+ and Ba^{2+} currents

Genistein (>30 μM) inhibited the voltage-dependent K^+ current evoked by a ramp voltage pulse from −120 mV to 60 mV, when high K^+ solution with 0.1 or 4 mM EGTA was in the pipette. The amplitude of the outward current seen in the absence of genistein (control) was always larger when PSS was in the bath and high K^+ solution with 0.1 mM EGTA was in the pipette than when nominally Ca^{2+}-free solution was in the bath and high-K^+ solution with 4 mM EGTA was in the pipette. This suggests that Ca^{2+}-dependent and -independent K^+ outward currents were evoked under the former ionic conditions. The inhibitory effects of genistein on the voltage-dependent K^+ current could be recorded under both sets of ionic conditions (Figure 7a and b). The final removal of genistein (100 μM) restored the outward current to the control level. Figure 7d shows the relationships between genistein concentration and the relative amplitude of the outward K^+ currents measured at +40 mV, when the amplitude seen in the absence of genistein was normalized as 1.0. There was no difference between the relationships observed in the two sets of ionic conditions (in the absence of Ca^{2+} and in the presence of Ca^{2+}).

When Cs^+ solution was placed in the pipette and 10 mM Ba^{2+} solution was superfused in the bath, genistein (>30 μM) attenuated the amplitude of the voltage-dependent Ba^{2+} current evoked by a ramp pulse from −100 mV to 80 mV (Figure 7c). On application of genistein (30 and 100 μM), both the activation potential of voltage dependent Ba^{2+} current and the peak of the current-voltage relationship were shifted towards more positive potentials. As shown in Figure 7d (solid triangles), the peak amplitude of the voltage dependent Ba^{2+} current was inhibited in a concentration-dependent manner by genistein. The IC_{50} values for the effect of genistein on the voltage-dependent K^+ current recorded in the presence of Ca^{2+} or absence of Ca^{2+} were 75.3 μM and 65.3 μM, respectively. The IC_{50} for its effect on voltage dependent Ba^{2+} current was 112.6 μM.

Daidzein (100 μM) did not inhibit either the voltage-dependent K^+ current (0.99 ± 0.02, $n=5$) nor the voltage dependent Ba^{2+} current (1.00 ± 0.02, $n=6$), while sodium orthovanadate (100 μM) slightly potentiated both the voltage-dependent K^+ current (1.08 ± 0.07, $n=6$). Herbimycin A (10 μM) had no significant effect on the voltage-dependent K^+ current (0.97 ± 0.04, $n=4$) and wortmannin did not affect the voltage-dependent K^+ current either (1 μM; 1.00 ± 0.01, 10 μM; 0.99 ± 0.02, $n=5$).

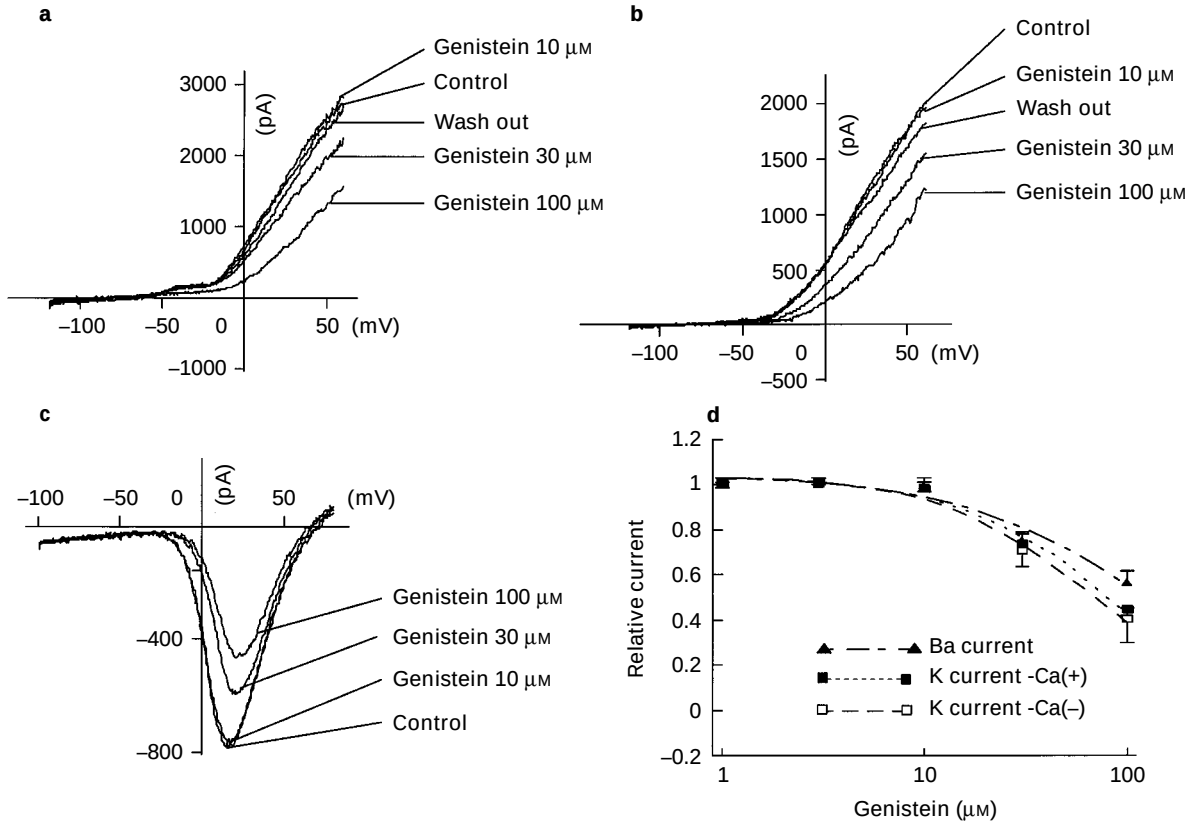

Figure 7 Effects of genistein (10–100 μM) on voltage-dependent K^+ and Ba^{2+} currents. (a and b) Effect of genistein on the voltage-dependent K^+ current recorded in PSS (2.3 mM Ca^{2+}) or nominally Ca^{2+}-free solution. High-K^+ solution with 0.1 mM (a) or 4 mM (b) EGTA was in the pipette. A ramp voltage pulse (from −120 to +60 mV, a holding potential of −70 mV, 300 ms duration) was applied every 3 min and genistein was applied after a repeatable current was recorded at least 3 times. Traces were recorded before (control), and at 6 min after application of either 10 μM, 30 μM or 100 μM genistein, and at 10 min after removal of 100 μM genistein. (c) Effect of genistein on the Ba^{2+} current. The Ba^{2+} current was evoked by a ramp pulse (from −100 to +80 mV; 300 ms duration) at a holding potential of −70 mV. Current traces recorded before (control) and at 6 min after application of either 10 μM, 30 μM or 100 μM genistein are superimposed. (d) Relationships between concentration of genistein (1–100 μM) and the relative amplitude of the voltage-dependent K^+ current and the voltage-dependent Ba^{2+} current. Each symbol indicates the mean value with vertical lines showing s.d. The amplitude of the current measured at +40 mV (voltage-dependent K^+ current) or at the peak of the *I*-V curve (Ba^{2+} current) in the absence of genistein was normalized as 1.0.

Discussion

In the present experiments on the rabbit portal vein, we have demonstrated that genistein reduced the open probability of the ATP-sensitive K^+ channel without inducing a change either in the mean open time or in the unitary conductance. The inhibition exerted by genistein (<100 μM) occurred reversibly and repeatably, and was similar in this regard, to that of glibenclamide. This indicates that the site at which genistein acts might be on the ATP-sensitive K^+ channel/sulphonylurea receptor or on associated proteins. A contribution by cytosolic regulating mechanisms can be ruled out with respect to the channel inhibition induced by genistein, as the inhibitory effects of genistein were observed on the ATP-sensitive K^+ channel activated by GDP in the inside-out membrane. As the ATP-sensitive K^+ channels in the rabbit portal vein are rapidly inactivated by membrane excision (Kajioka *et al*., 1991; Kamouchi & Kitamura, 1994), no effect of cytosolic regulation would be expected to be seen in the inside-out configuration.

Activation of the ATP-sensitive K^+ current by protein kinase A has been reported by Quayle *et al*. (1994). However, it is unlikely that inhibition of the ATP-sensitive K^+ current by genistein involves an effect on the protein kinase A-mediated response, because genistein, at up to 100 μM, has little effect on protein kinases A and C (Akiyama *et al*., 1987) and a specific peptide inhibitor of protein kinase A has been found to have no effect on pinacidil-induced K^+ current (Quayle *et al*., 1994). Hatakeyama *et al*. (1995) demonstrated the involvement of tyrosine kinases in protein kinase C-mediated inhibition of ATP-sensitive K^+ channels, that occurs on activation of muscarinic M_3 receptors in rabbit oesophageal smooth muscle cells. It has been shown that some muscarinic receptor subtypes, including M_3, can simultaneously activate multiple signal transduction pathways, such as those involving phospholipases A_2, C and D, as well as tyrosine kinases (Felder, 1995), and that diacylglycerol directly stimulates intrinsic tyrosine kinase (insulin receptor) (Arnold & Newton, 1996). These results suggest the presence of multiple interacting steps at which tyrosine kinases might be involved in the receptor/ion-channel signal transduction pathway, and that tyrosine kinase can modulate the ATP-sensitive K^+ channels in both an excitatory and an inhibitory manner.

In rat ventricular cells, Kwak et al. (1996) showed that phosphorylation of the tyrosine residue suppressed the ATP-sensitive K^+ channels, estimated from enhancing effects of genistein on the reactivation process of the cardiac ATP-sensitive K^+ channels by ATP. In contrast, in the rabbit portal vein genistein suppressed the ATP-sensitive K^+ currents. For activation of pinacidil-induced K^+ current in the rabbit portal vein, both K^+ channel openers and intracellular channel modulators, such as Mg^{2+}-ATP, GDP or other nucleotides diphosphate, were prerequisite. Indeed, in the absence of a K^+ channel opener, no outward current (both whole-cell and unitary currents) could be induced even in the presence of the above intracellular channel modulators (Kajioka & Kitamura, 1991; Kamouchi *et al*., 1994). On the other hand, K^+ channel opener was not required to activate the cardiac ATP-sensitive K^+ channels (Tsung & Kurachi, 1991). As channel activities of the ATP-sensitive K^+ channels in rat ventricular and rabbit portal vein cells were recorded under different conditions, further experiments will be required to clarify the presence of complicated pathways involving tyrosine phosphorylation.

A considerable number of studies have been published on the modulating effects of tyrosine phosphorylation on various ionic channels. These include the NMDA (N-methyl-D-aspartate) receptor-mediated Ca^{2+} influx in neurones (O'Dell *et al*., 1991; Wang & Salter, 1994), a cloned delayed rectifier potassium channel (Huang *et al*., 1993), a reconstituted nicotinic acetylcholine receptor current (Hopfield *et al*., 1988), epithelial sodium transport in an A6 cultured cell-line (Matsumoto *et al*., 1993), non-selective cation channels in ileum smooth muscle cells (Inoue *et al*., 1994), calcium channels in vascular smooth muscle cells (Wijetunge *et al*., 1992; Wijetunge & Hughes, 1995) and swelling-activated chloride channels in atrial myocytes (Sorota, 1995). In the majority of these studies the function of the modulating effect of tyrosine phosphorylation on ionic channels was examined by use of genistein, a specific inhibitor of protein tyrosine kinase. The IC_{50} of genistein was shown to be >100 μM for the NMDA receptor-mediated Ca^{2+} influx (Wang & Salter, 1994), >20 μM for the $GABA_A$ receptor channel (Moss *et al*., 1995), 20–40 μM for the non-selective cation channels (Inoue *et al*., 1994) and 36–50 μM for the voltage-dependent Ca^{2+} channel (rabbit ear artery, Wijetunge *et al*., 1992; colonic muscularis mucosa cells, Hatakeyama *et al*., 1996; rat pregnant myometrium, Kusaka & Sperelakis, 1996). In the present experiments, we confirmed that genistein at similar concentrations could inhibit the voltage-dependent K^+ and Ba^{2+} currents (K^+ current, 75.3 μM; Ba^{2+} current, 112.6 μM). No difference was noted between the genistein concentrations required to inhibit this K^+ current whether the experiment was performed in the Ca^{2+}-containing solution (PSS) or in the Ca^{2+}-free solution. This suggests that genistein inhibited Ca^{2+}-dependent and Ca^{2+}-independent K^+ currents to the same extent. By comparison with these IC_{50} values, the IC_{50} for the pinacidil-induced K^+ current indicated that this current was 5 to 20 times more sensitive to genistein ($IC_{50}=5.5$ μM). The IC_{50} values for the effects of genistein were shown to be 2.6 μM for the EGF receptor and 30 or 26 μM, respectively, for a 60 kDa protein derived from the *Src* oncogene or 110 kDa protein from the *Gag* oncogene (Akiyama *et al*., 1987). The IC_{50} value observed in the present experiments with genistein on the ATP-sensitive K^+ channel corresponds well to those previously obtained. To judge from the present and other experiments on sodium orthovanadate, a tyrosine phosphatase inhibitor as well as a blocker of Na^+-K^+ ATPase and Ca^{2+} ATPase (Searle *et al*., 1983; Raeymaekers *et al*., 1983), current augmentation by vanadate is not specific to the voltage-dependent Ca^{2+} channel, but also affects various K^+ currents, including the pinacidil-induced K^+ current in vascular smooth muscle cells (Wijetunge *et al*., 1992; Xiong & Cheung, 1995; Hatakeyama *et al*., 1996). Augmentation of such ionic currents by orthovanadate and their inhibition by genistein, but not by daidzein, is fully consistent with the effects being produced via tyrosine phosphorylation. Interestingly, direct evidence has been obtained for phosphorylation of a delayed rectifier K^+ channel (Kv 1.2) by tyrosine kinase and its inhibition by a low concentration of genistein (8 μM) (Huang *et al*., 1993; Lev *et al*., 1995).

Recently, Wijetunge & Hughes (1995) and Hatakeyama *et al*. (1996) have shown, in rabbit ear artery and colonic mucosal muscle cells, that the platelet-derived growth factor- and EGF-receptors or its related tyrosine kinases might be involved in the modulatory mechanisms that act on the voltage-dependent Ca^{2+} current. As, in the present experiments, direct application of EGF (1 μg ml^{-1}; 5 times higher concentration than that of Hatakeyama *et al*. (1996)) did not affect the membrane current in the presence or absence of pinacidil, neither the EGF receptor nor EGF-receptor-related tyrosine kinases appear to be involved in the mechanism underlying the action of genistein on the ATP-sensitive K^+ current in the rabbit portal vein.

In the present experiments, we did not find any significant effects of tyrphostin 23, lavendustin A and herbimycin A on the pinacidil-induced current. However, the effects of tyrphostin 23 were marginal (12% inhibition) at 10 μM and a higher concentration of tyrphostin 23 (30 μM) reduced the amplitude of the pinacidil-induced K^+ current by 70% ($n=2$; R. Ogarta & K. Kitamura, unpublished observations). Chronic application of lavendustin A has been shown to inhibit the EGF-receptor-associated tyrosine kinase at very low concentration under *in vitro* condition (IC_{50} of ca. 12 nM; Onoda *et al*., 1989). However, it has been also found that lavendustin A does not inhibit the tyrosine kinase *in situ* (Onoda *et al*., 1990). Similarly, herbimycin A has been shown not to be effective either *in vitro* (Uehara & Fukazawa, 1991) or when directly applied via the bath or pipette (Sorota, 1995).

There might be several reasons for the lack of an inhibitory action of lavendustin A and herbimycin A in the present experiments despite the effectiveness of genistein. These include a failure of lavendustin A and herbimycin A to achieve rapid membrane permeation and the existence of a nonspecific action by genistein. However, these possibilities are very slight as the application of herbimycin A (1 μM) via the pipette solution did not abolish the pinacidil-induced K^+ current, and has no significant effect on the unitary current induced by pinacidil and GDP in the inside-out membrane patch (R. Ogata & K. Kitamura, unpublished observation). Further, Tilly *et al.* (1993) and Sorota (1995) recently found that acute administration of herbimycin A (1 μM) did not exert any effect on the volume-sensitive Cl^- current in a human cultured intestinal cell line, but that it did inhibit the Cl^- current after 1 or 2 days incubation in culture medium. Sorota (1995) speculated that, either reduced sulphydryl groups present in the cytoplasm inactivate the herbimycin A, or that herbimycin A prevents the expression of a gene for a factor that is essential for the function of that channel.

On the other hand, Smirnov and Aaronson (1995) showed that genistein and ST 638, both tyrosine kinase inhibitors, each directly inhibited a delayed K^+ current recorded in rat and rabbit pulmonary arteries. Furthermore, Paillart *et al.* (1997) showed that inhibition of voltage-dependent Na^+ channel by genistein was not mediated by tyrosine kinase inhibition, because other tyrosine kinase inhibitors, including lavendustin A, had no effect on the current. We also failed to show inhibitory effects of some tyrosine kinase inhibitors on pinacidil-induced K^+ current, such as tyrphostin 23, herbimycin A and lavendustin A. In the present experiments, ATP-sensitive K^+ channels could be recorded in the presence of pinacidil and GDP, but in the absence of ATP. These findings indicate that genistein and tyrphostin B46 directly act on the pinacidil-induced K^+ channel and inhibit its activity in the rabbit portal vein.

The involvement of channel phosphorylation has been postulated in the activation of ATP-sensitive K^+ channels, partly because channel activation requires either Mg^{2+}-ATP or nucleotides diphosphate (Tsung & Kurachi, 1991; Kamouchi & Kitamura, 1994). Although no one has yet identified the direct phosphorylation site for tyrosine kinase in the ATP-sensitive K^+ channels (uK_{ATP}, Inagaki *et al.*, 1995), our results suggest that tyrosine phosphorylation might be involved in the activation of ATP-sensitive K^+ channels. As genistein may possibly interact with ATP to achieve its effect on tyrosine kinase (Akiyama *et al.*, 1987), the inhibitory actions of genistein obtained here might be related to the Mg^{2+}-ATP-mediated activation of ATP-sensitive K^+ channels.

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