Tyrosine kinases modulate K^+ channel gating in mouse Schwann cells

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(Received 1 April 1999; accepted after revision 28 May 1999)

1. The whole-cell configuration of the patch-clamp technique and immunoprecipitation experiments were used to investigate the effects of tyrosine kinases on voltage-dependent K^+ channel gating in cultured mouse Schwann cells.

2. Genistein, a broad-spectrum tyrosine kinase inhibitor, markedly reduced the amplitude of a slowly inactivating delayed-rectifier current (I_K) and, to a lesser extent, that of a transient K^+ current (I_A). Similar results were obtained on I_K with another tyrosine kinase inhibitor, herbimycin A. Daidzein, the inactive analogue of genistein, was without effect.

3. Unlike herbimycin A, genistein produced additional effects on I_A by profoundly affecting its gating properties. These changes consisted of slower activation kinetics with an increased time to peak, a positive shift in the voltage dependence of activation (by +30mV), a decrease in the steepness of activation gating (9mV per e-fold change) and an acceleration of channel deactivation.

4. The steepness of the steady-state inactivation was increased by genistein treatment, while the recovery from inactivation was not significantly altered.

5. The action of genistein on voltage-dependent K^+ (Kv) currents was accompanied by a decrease in tyrosine phosphorylation of Kv1.4 as well as Kv1.5 and Kv2.1 encoding transient and slowly inactivating delayed-rectifier K^+ channel α subunits, respectively.

6. In conclusion, the present study shows that tyrosine kinases markedly affect the amplitude of voltage-dependent K^+ currents in Schwann cells and finely tune the gating properties of the transient K^+ current component I_A. These modulations may be functionally relevant in the control of K^+ channel activity during Schwann cell development and peripheral myelinogenesis.

Phosphorylation and dephosphorylation are important processes through which the cell modulates ionic channel activity (for reviews see Levitan, 1994; Siegelbaum, 1994; Jonas & Kaczmarek, 1996). Because K^+ channels play crucial roles in shaping action potentials and controlling membrane excitability, neuronal firing patterns, neurotransmitter release, volume regulation and cell proliferation, the modulation of their gating, conductance or kinetics is expected to have an impact on a wide spectrum of cellular functions. Thus, K^+ channels are substrates for protein kinases and phosphatases, and their gating properties were found to be modulated by serine/threonine and/or tyrosine phosphorylation (Levitan, 1994; Siegelbaum, 1994; Jonas & Kaczmarek, 1996). It was shown that phosphorylation by protein kinase C (PKC) of a human A-type K^+ channel (hKv3.4) at the amino-terminal domain specifically eliminates rapid N-type inactivation (Covarrubias *et al.* 1994). Protein kinase A (PKA) and Ca^{2+}–calmodulin-dependent protein kinase (CaMK) were also found to modulate the inactivation of *Shaker* and Kv1.4 K^+ channels, respectively (Drain *et al.* 1994; Roeper *et al.* 1997). Likewise, we recently showed that inhibition of CaMK accelerates the C-type inactivation of *Drosophila* photoreceptor K^+ channels and produces a leftward shift in the voltage dependence of activation (Peretz *et al.* 1998). Serine phosphorylation was also shown to shift the voltage dependence of activation of Kv2.1 channels (Murakoshi *et al.* 1997).

Recently, it has been demonstrated that tyrosine phosphorylation via receptor and non-receptor tyrosine kinases could also play an important role in modulating K^+ channel properties (Jonas & Kaczmarek, 1996). For example, activated insulin receptors increased delayed-rectifier K^+ currents in *Aplysia* bag cell neurons (Jonas *et al.* 1996). In leech neurons, tyrosine dephosphorylation led to a marked increase in cationic channel activity (Aniksztejn *et al.* 1997). In the Jurkat T cell line, the $p56^{lck}$-mediated phosphorylation of the Kv1.3 channel was correlated with an inhibition of voltage-sensitive K^+ currents upon Fas stimulation (Szabo *et al.* 1996). Emerging evidence from heterologous expression studies indicates that the activity

of several Kv1 channels is downregulated following phosphorylation by non-receptor and receptor tyrosine kinases. In human embryonic kidney (HEK) 293 cells, co-expression of Kv1.3 with activated v-src led to a decrease in current amplitude, a slowing of the C-type inactivation and an acceleration of the deactivation; these effects were accompanied by an increased tyrosine phosphorylation of the channel protein (Holmes *et al.* 1996*a*; Fadool *et al.* 1997). Kv1.3 channel is also modulated by epidermal growth factor (EGF) and insulin receptor tyrosine kinases. In HEK 293 transfected cells, EGF treatment inhibited the current amplitude and speeded the C-type inactivation, while insulin application did not affect the inactivation kinetics (Bowlby *et al.* 1997). Modulation of Kv1.3 current amplitude by insulin receptor has also been characterized in native olfactory bulb neurons (Fadool & Levitan, 1998). Other voltage-dependent K^+ (Kv) channels such as Kv1.2 and Kv1.5 were found to be modulated by tyrosine kinases with a strong current suppression but without apparent alterations in channel gating (Huang *et al.* 1993; Timpe & Fantl, 1994; Holmes *et al.* 1996*b*; Tsai *et al.* 1997).

Recently, we showed that the current amplitude of the delayed-rectifier I_K is markedly upregulated following intracellular application of recombinant Fyn tyrosine kinase to mouse Schwann cells and downregulated upon exposure to tyrosine kinase inhibitors (Sobko *et al.* 1998*a*). In the present study, we examined whether tyrosine kinases could also modulate the remaining transient K^+ current (I_A) in cultured mouse Schwann cells. We report that the broad-spectrum tyrosine kinase inhibitor genistein modified the activation and deactivation gating of I_A. Genistein caused a positive shift in the voltage dependence of activation, a slower activation and an acceleration of channel deactivation. The action of genistein on Schwann cell K^+ currents was accompanied by a decrease in tyrosine phosphorylation of the Kv1.4, Kv1.5 and Kv2.1 channel α subunits. The modulation of K^+ channel activity by tyrosine kinases may be functionally relevant in the context of Schwann cell growth and peripheral myelinogenesis.

METHODS

Materials

Herbimycin A and protein A–G sepharose were obtained from Sigma (St Louis, MO, USA). Monoclonal and polyclonal antibodies against Kv1.4, Kv1.5, Kv2.1 and anti-phosphotyrosine 4G10 were from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit anti-S-100 was from Sigma. Genistein and Daidzein were purchased from Calbiochem (San Diego, CA, USA).

Schwann cell primary cultures

Four-day-old (P4) CD-1 mice were killed by decapitation in accordance with national guidelines and sciatic nerves were removed aseptically. Primary Schwann cell cultures were prepared according to Brockes *et al.* (1979). Cells were plated on poly-D-lysine-coated Petri dishes or glass coverslips and grown in Dulbecco's modified Eagle's medium and F12 medium supplemented with 2 mM glutamine, 10% fetal calf serum (FCS) and penicillin and streptomycin (100 units ml^{-1} each). Under these conditions, which allowed proliferation and inhibited the expression of the myelin phenotype, we obtained 90–95% pure Schwann cell cultures, as controlled by S-100 immunofluorescence.

Electrophysiology

Cultured Schwann cells, plated on poly-D-lysine-coated glass coverslips were placed in a recording chamber (1 ml) mounted on the stage of a Zeiss Axiovert 35 inverted microscope. The whole-cell configuration of the patch-clamp technique (Hamill *et al.* 1981) was used to record the macroscopic whole-cell currents at room temperature (22 ± 1 °C). Signals were amplified using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA, USA), were sampled at 1–5 kHz and filtered below 0·4–2 kHz via a four-pole Bessel low-pass filter, according to the experimental protocol. Data acquisition was performed using pCLAMP 6.0.2 software (Axon Instruments) and an IBM-compatible 486 computer in conjunction with a DigiData 1200 interface (Axon Instruments). The patch pipettes were pulled from borosilicate glass (fibre filled) with resistance of 4–8 MΩ and were filled with (mM): 164 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 10 Hepes, 11 glucose at pH 7·4. The external solution contained (mM): 140 NaCl, 5 KCl, 5 $CaCl_2$, 2 $MgCl_2$, 11 glucose, and 10 Hepes at pH 7·4. Series resistances were within 9–16 MΩ and were compensated by 85–95%. Traces were off-line leak subtracted by the Clampfit program of the pCLAMP 6.0.2 software and further analysed by the Axograph 3.0 software (Axon Instruments). Chord conductance (G) was calculated using the following equation:

$$G = I/(V - V_K),$$

assuming a reversal potential of $V_K = -85$ mV, calculated using the Nernst equation. The transient and the sustained components of the K^+ currents were measured at the peak and at the end of the depolarizing traces, respectively. The chord conductance was estimated at various test voltages, V, and then normalized to a maximal conductance value, G_{max}, calculated at +60 mV. Activation and steady-state inactivation curves were fitted by the Boltzmann distribution:

$$I/I_{max} = a/(1 + \exp((V_{0.5} - V_K)/s)).$$

Where $V_{0.5}$ is the half-maximal activation (for the steady-state activation protocol), or the voltage at which half of the steady-state inactivation was removed, and s is the slope of the curve. For fitting procedures, the Simplex algorithm was used. Genistein and herbimycin A were externally perfused and the whole-cell recordings were taken after the effect of the drug reached a steady state. All data were expressed as means ± S.E.M. Statistically significant differences were assessed using Student's t test.

Immunoprecipitations

The preparation of cell lysates, membrane solubilization and immunoprecipitation were carried out as described previously (Sobko *et al.* 1998*a*, *b*). Briefly, confluent cell cultures (100 mm Petri dishes) were washed twice with cold phosphate-buffered saline (PBS) and scraped on ice in PBS, containing 1 mM phenylmethylsulphonylfluoride (PMSF). Following centrifugation, the cell pellets were homogenized in a buffer containing 50 mM Tris, pH 7·4, 1 mM EDTA, 1 mM PMSF, 10 μg ml^{-1} aprotinin and 10 μg ml^{-1} leupeptin and centrifuged at 21 000*g* for 30 min at 4 °C. The pellet (crude membranal fraction) was sonicated and resuspended in the solubilization buffer (10% glycerol, 50 mM Hepes pH 7·4, 10 mM EDTA, 150 mM NaCl, 1·5 mM $MgCl_2$, 1% Triton X-100, 1 mM PMSF, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin). After centrifugation, the extracts were precleared with 1 : 1 slurry of protein A–G sepharose (30 μl per ml of extract) equilibrated in lysis buffer, for 1 h at 4 °C with shaking. After a

short spin, the supernatant was transferred into a new tube and incubated with the antibodies or the respective preimmune serum at 4 °C for 4 h or overnight with shaking. Immune complexes were precipitated after the addition of 30 μl of protein A–G sepharose for 1 h at 4 °C with shaking. After elution, the samples were electrophoresed on SDS–10% polyacrylamide gel (SDS–PAGE) along with standard molecular weight markers. Proteins were then electro-transferred to nitrocellulose filters. Blots were blocked in PBS containing 10% non-fat milk and 0·05% Tween 20 for 1 h at room temperature with shaking, then incubated with the respective antibody overnight at 4 °C. After three washes, blots were incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody, followed by extensive washes in PBS. Labelled proteins were detected by enhanced chemiluminescence (ECL, Amersham). To estimate and compare the total protein inputs in each lane, blots were stripped and re-probed with the respective anti-Kv antibodies. Band signals corresponding to immunoreactive proteins were measured and scanned by image densitometry, using Adobe Photoshop 4.0 software. Densitometric data were normalized to the respective protein input as assessed by reprobing of the blots.

RESULTS

Genistein inhibits whole-cell K^+ current amplitude in mouse Schwann cells

Schwann cells were purified from P4 mouse sciatic nerves and grown *in vitro* in the presence of serum. Under these conditions, they actively proliferated and expressed the Schwann cell marker S-100 (Sobko *et al.* 1998*a*,*b*). As described previously, cultured mouse Schwann cells displayed mainly two types of voltage-gated K^+ currents (Konishi,

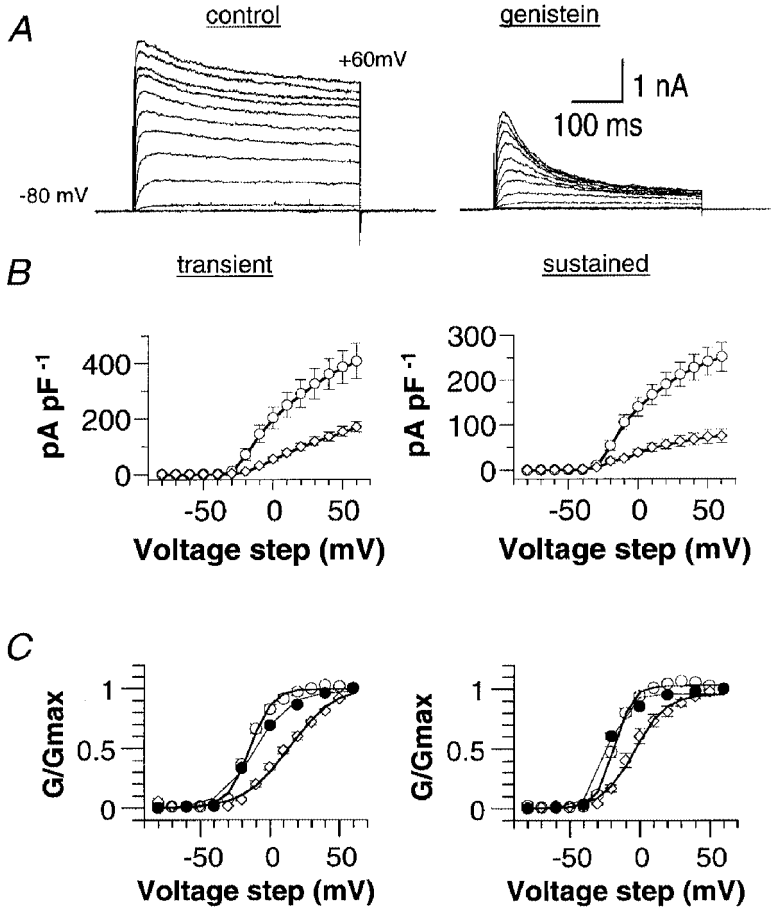

Figure 1. Effects of genistein on the amplitude and voltage dependence of activation of K^+ currents in cultured Schwann cells

A, left, representative traces of whole-cell macroscopic K^+ currents in control mouse Schwann cells. Voltage-dependent outward K^+ currents were evoked by depolarizing cells for 400ms from a holding potential of −80 mV to +60 mV in 10 mV increments. Right, representative traces of K^+ currents recorded from cells first subjected to a 10 min application of 100 μM genistein, using the same activation protocol. *B*, current density (in pA pF^{-1}) is plotted against the voltage step (in mV) for control (○) and genistein-treated cells (◇). The amplitude of the transient (left) and sustained (right) components of the K^+ currents were measured at the peak and at the end of the depolarizing pulses, respectively. Following 100 μM genistein treatment, the transient and sustained I_{max} were reduced by 56 ± 5 and 69 ± 7%, respectively (number of independent experiments, $n = 11–19$). *C*, the normalized conductances (G/G_{max}) of the transient (left) and sustained (right) components of the K^+ currents are plotted against the voltage step (in mV) for control (○), herbimycin A- (●) and genistein-treated cells (◇). The curves were fitted using a Boltzmann distribution ($n = 11–19$).

1989; Amedee *et al.* 1991; Sobko *et al.* 1998*a*, *b*). Depolarizing voltage steps from a holding potential of −80 mV to +60 mV evoked an inactivating transient K^+ current I_A, followed by a slowly inactivating delayed-rectifier current I_K (Fig. 1*A*, left panel). The voltage-dependent K^+ current density (in pA pF^{-1}) was dependent on the age of the culture. For example, at 4–7 days of culture the K^+ current amplitudes were 190 ± 28 and 131 ± 21 pA pF^{-1} for the transient and the sustained components, respectively ($n = 15$), while after about 9–15 days of culture they were significantly upregulated and reached their respective maximal values of 397 ± 47 and 241 ± 24 pA pF^{-1} ($n = 11$; $P < 0{\cdot}01$). These values are comparable to those previously described for cultured mouse Schwann cells prepared from newborn sciatic nerves (Konishi, 1989). Interestingly, the gating properties were qualitatively similar at all stages of culture (Konishi, 1989; Sobko *et al.* 1998*a*, *b* and see below). In the present study, we used 9–15 day cultured Schwann cells.

To investigate the modulatory effects of tyrosine kinases on the Schwann cell K^+ currents, we used the broad-spectrum tyrosine kinase inhibitor, genistein. The K^+ current amplitude was markedly depressed in the presence of genistein (100 μM). I_{max} was inhibited by 56 ± 5 and 69 ± 7 % for the transient and sustained components, respectively ($n = 11$; Fig. 1*A* and *B*). Upon genistein treatment, the maximal current densities were reduced from 397·0 ± 47·4 to 173·8 ± 19·1 pA pF^{-1} ($n = 11$) and from 240·9 ± 24·2 to 73·9 ± 16·8 pA pF^{-1} ($n = 11$) for the transient and sustained components, respectively. The genistein effects reached steady state within 6–8 min of external application and could be reversed following a 10 min washout (Fig. 2*A*). As shown in Fig. 2*B*, daidzein (100 μM), the structurally related but inactive analogue of genistein, was totally ineffective, even after prolonged incubation ($n = 5$), indicating the specificity of genistein inhibitory action. At first appearance, genistein led to an acceleration of the whole-cell inactivation kinetics. However, the decreased

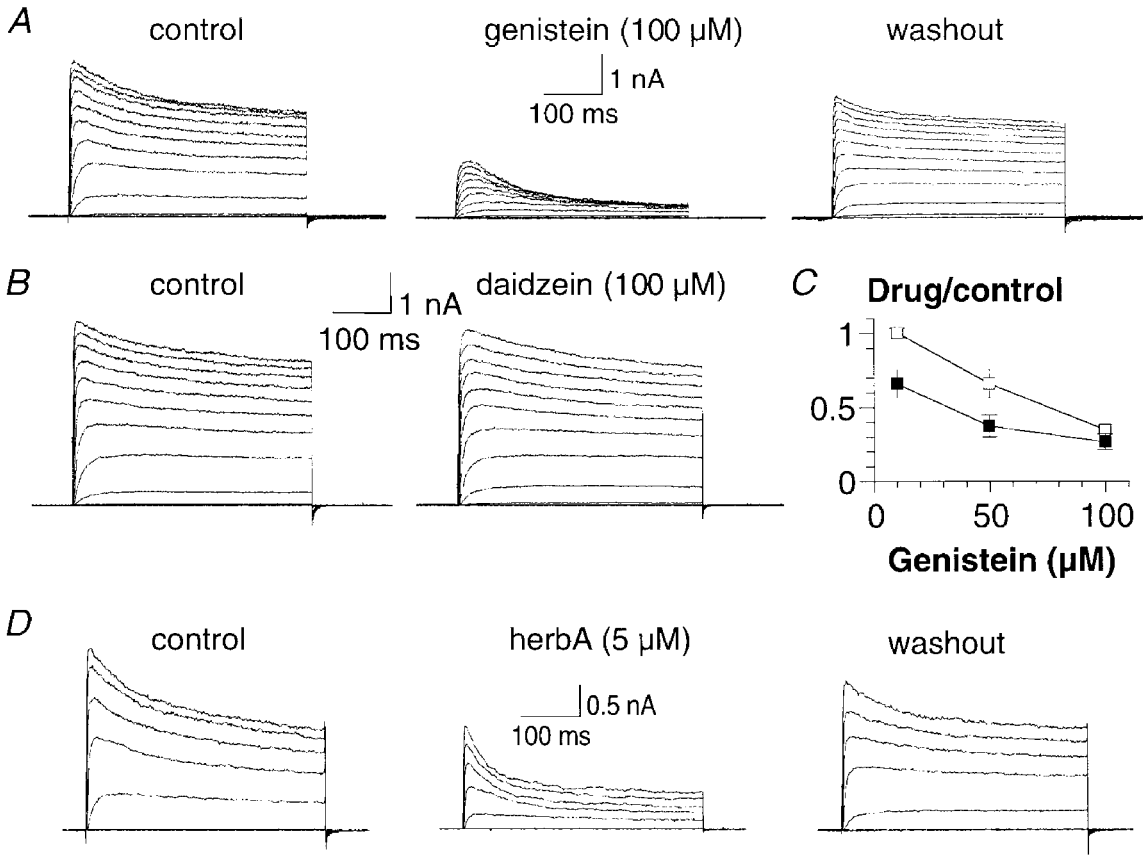

Figure 2. Dose dependence and reversibility of genistein action

A, the same cell was recorded from as in Fig. 1*A*, before (left), following a 10 min incubation with 100 μM genistein (middle) and after a 10 min washout (right). *B*, the same cell was recorded from as in Fig. 1*A*, before and after a 10 min incubation with 100 μM daidzein. *C*, the dose dependence of genistein action was expressed by the ratio of the K^+ currents recorded at +60 mV from cells in the presence and the absence of the drug ($n = 5$–11). This ratio was calculated from the currents measured at the peak (□) and at the end (■) of the depolarizing pulses (400 ms). *D*, the same cell was recorded before (left), following a 10 min incubation with 5 μM herbimycin A (middle) and after a 20 min washout (right). Here, the K^+ currents were evoked by depolarizing the cells for 400 ms from a holding potential of −80 mV to +60 mV in 20 mV increments.

contribution of I_K unmasks the inactivation kinetics of I_A, which are probably contaminated with residual I_K. This effect is well illustrated in Fig. 6*A* (right panel), where the inactivation of the control whole-cell K^+ current could be fitted by two exponentials (Fig. 6*A*, left panel; $\tau_1 = 37{\cdot}1 \pm 3{\cdot}5$ ms and $\tau_2 = 240{\cdot}3 \pm 33{\cdot}3$ ms; $n = 15$), while that of genistein-treated cells was well fitted by a single exponential ($\tau = 106{\cdot}3 \pm 7{\cdot}8$ ms; $n = 11$).

The effects of genistein were dose dependent (Fig. 2*C*). At 10 μM genistein, the amplitude of the sustained K^+ current (I_K) was reduced by 35%, while that of the transient I_A remained unaffected. At 100 μM genistein, both I_A and I_K amplitudes were depressed (Fig. 2*C*) as shown by a careful examination of the subtracted (control − drug) traces (Fig. 3*A*). Essentially the same results were obtained with herbimycin A, another tyrosine kinase inhibitor (Fig. 2*D* and 3*B*; Sobko *et al.* 1998*a*). I_K was the main component to be downregulated by 2·5 μM herbimycin A, with very little effect, if any on I_A, as indicated by the subtracted traces (Fig. 3*B*). Although less readily than genistein, herbimycin A action could also be reversed following a 20 min washout (Fig. 2*D*). To exclude the possibility that genistein could act as an open-channel blocker, we employed a train protocol. Genistein was applied before channel opening (i.e. before membrane depolarization). If the genistein effect is dependent on channel opening (i.e. open channel block) and if drug on-rate binding is slow relative to channel opening, then one would expect the initial current trace following genistein application to be superimposable on the control trace. In fact, K^+ current inhibition was attained at the first pulse (+30 mV) following an 8 min exposure to genistein and the effect did not increase further upon subsequent stimulations (Fig. 3*C*). Similar results were obtained with herbimycin A (not shown).

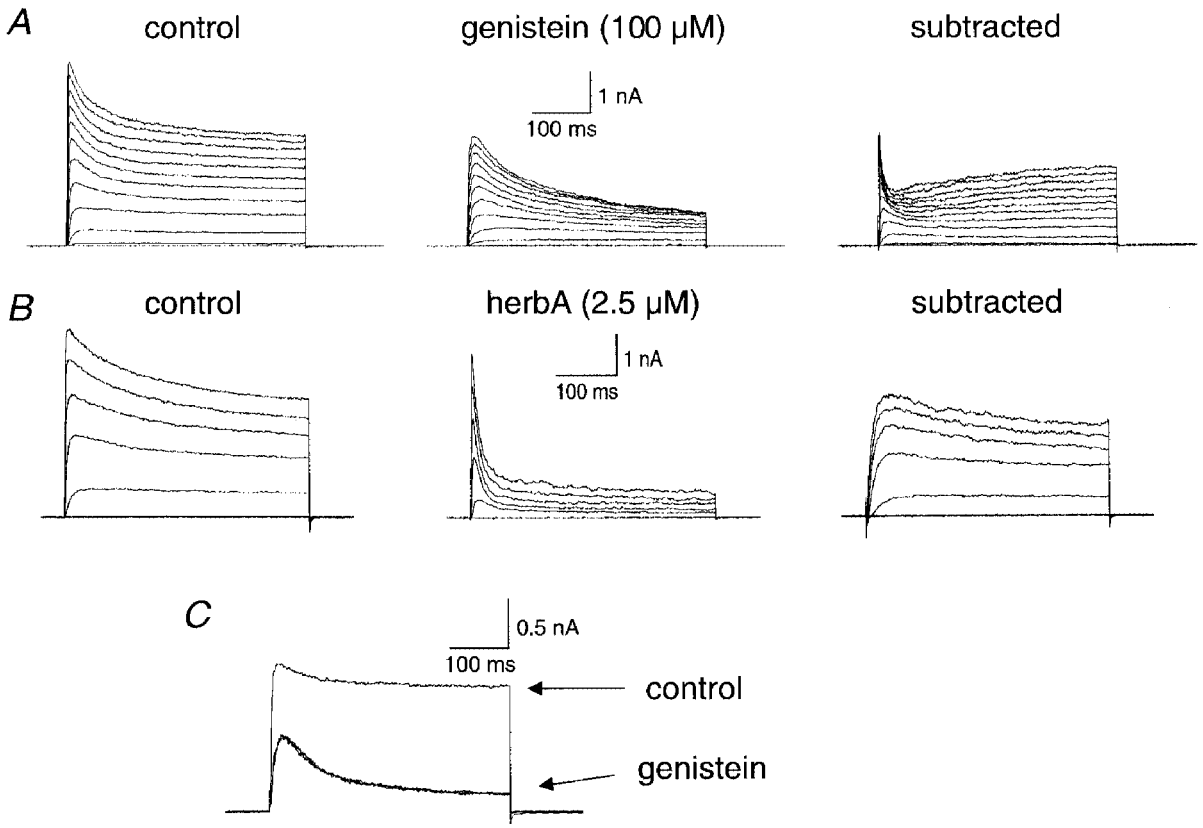

Figure 3. Effects of genistein and herbimycin A on the transient and sustained K^+ current components

A, currents were recorded from the same cell before (left) and after a 10 min application of 100 μM genistein (middle). Subtracted (control − drug) traces are shown (right). Voltage-dependent K^+ currents were evoked by depolarizing cells for 400 ms from a holding potential of −80 mV to +60 mV in 10 mV increments. *B*, currents were recorded from the same cell before (left) and after a 15 min application of 2·5 μM herbimycin A (middle). Subtracted traces are shown (right). The K^+ currents were evoked by depolarizing the cells for 400 ms from a holding potential of −80 mV to +60 mV in 20 mV increments. *C*, the same cell was subjected to a train protocol. After stabilization of the whole-cell recording, a pulse was evoked before (control) and subsequent to a 10 min application of 100 μM genistein (genistein, 3 overlapping pulses are shown). Pulses were evoked by 400 ms steps from a −80 mV holding potential to +40 mV.

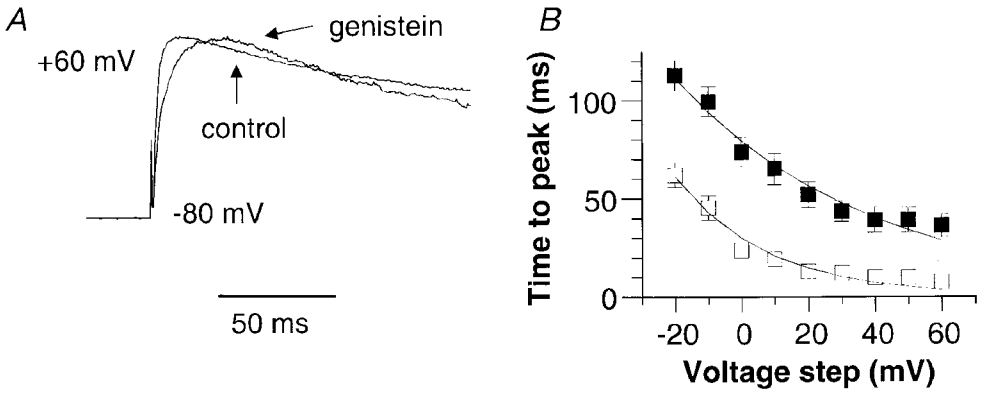

Figure 4. Activation kinetics as measured by the time to peak in control and genistein-treated cells

A, normalized current traces in control and genistein-treated cells as measured by a depolarizing step to +60 mV from a holding potential of −80 mV. *B*, time to peak parameter values ($n = 11$) were plotted against the voltage steps in control (□) and genistein-treated cells (■). Note that in some cases the error bars are smaller than the symbols.

Genistein acts on I_A to slow down the activation kinetics and shifts the voltage dependence of activation

Genistein and herbimycin A similarly suppressed K^+ current amplitude. However, in contrast to herbimycin A, genistein produced additional effects on the I_A currents by profoundly affecting the gating properties. Following genistein application, there was a pronounced positive shift in the voltage dependence of activation (by +30 mV and +16 mV for the transient and the sustained components, respectively) as well as a decrease in the steepness of activation gating (Fig. 1*C*). For the transient component of the K^+ current, the parameters of activation varied from $V_{0.5} = -14.9 \pm 0.9$ mV and $s = -7.3 \pm 0.5$ in control cells to $V_{0.5} = +14.8 \pm 2.4$ mV and $s = -15.8 \pm 0.6$ in genistein-

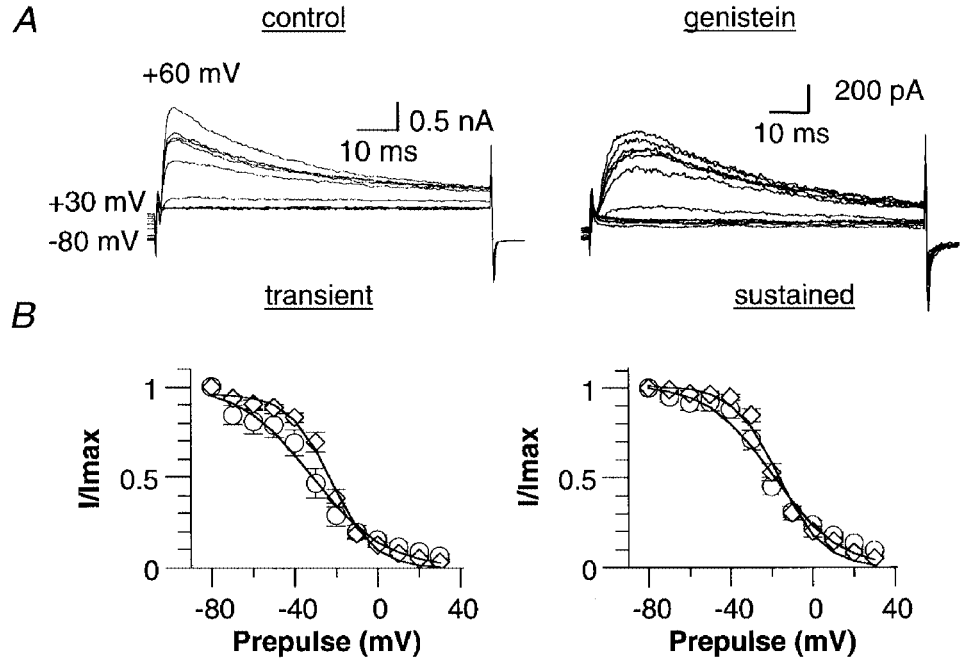

Figure 5. K^+ current steady-state inactivation in control and genistein-treated cells

A, representative traces of control (left) and genistein-treated cells (right), using a steady-state inactivation protocol. Cells were subjected to 1 s inactivating prepulse from −80 to +30 mV in 10 mV increments and stepped to a +60 mV test pulse of 80 ms duration. *B*, steady-state inactivation curves of the transient (left) and sustained (right) K^+ current components of control (○) and genistein-treated cells (◇) were fitted using a Boltzmann distribution ($n = 6$).

treated cells ($n = 11$–19). For the sustained component of the K^+ current, the Boltzmann distribution values varied from $V_{0.5} = -18.1 \pm 0.9$ mV and $s = -6.3 \pm 0.4$ in control cells to $V_{0.5} = -1.8 \pm 3.3$ mV and $s = -10.8 \pm 1.1$ in genistein-treated cells ($n = 11$–19). The decrease in the steepness of activation gating was reflected by a change of about 9 mV per e-fold change in the slope of the normalized conductance curve (as measured for the transient component) (Fig. 1*C*). This change in channel gating was observed during recording of the same cell before and after genistein application (Fig. 2*A* and 3*A*) as well as by direct recording subsequent to prior incubation with genistein (8 min preincubation) (Fig. 1*A*). This excludes problems of stability of the recordings (i.e. disappearance of liquid junction potentials during prolonged whole-cell recording) as an artefactual source of gating changes. Furthermore, prolonged whole-cell recording of control untreated cells did not change the voltage-dependent gating properties of the K^+ channels (not shown). The activation parameters of herbimycin A-treated cells were not significantly different from those of control Schwann cells with a $V_{0.5} = -10.3 \pm 1.2$ mV, $s = -11.3 \pm 0.5$ and a $V_{0.5} = -20.8 \pm 1.2$ mV, $s = -7.8 \pm 0.8$ ($n = 13$) for the transient and the sustained components, respectively.

Figure 4 shows that genistein markedly slowed the activation kinetics of the remaining I_A. The time to peak is voltage dependent and its value decreased with progressive depolarizing test pulses from 62 ms at −20 mV to 7 ms at +60 mV ($n = 25$) (Fig. 4*A* and *B*). In the presence of genistein, the time to peak was significantly increased at all

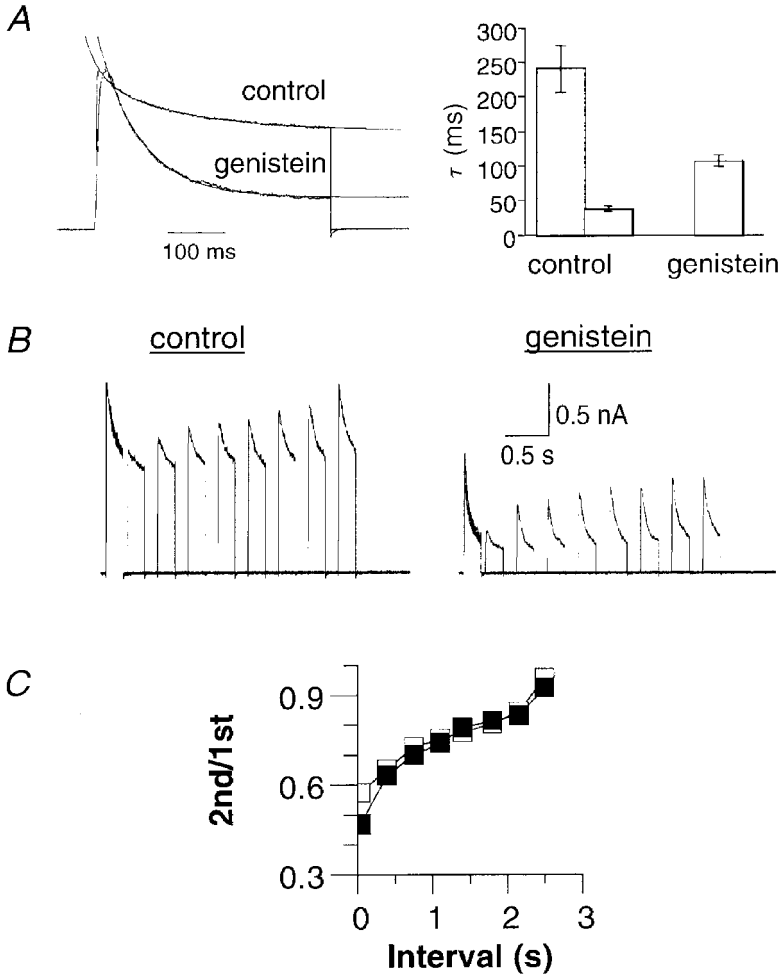

Figure 6. Effects of genistein on K^+ current inactivation and recovery from inactivation

A, left, normalized current traces of control and genistein-treated cells, following a +60mV test pulse (400 ms duration) from a −80 mV holding potential. The exponential fits are shown and superimposed on the current traces. Right, inactivation time constants (τ, in ms) measured in control($n = 11$) and genistein-treated cells ($n = 15$) using the same protocol as in *A*. *B*, recovery from inactivation measured in control (left) and genistein-treated cells (right) using a twin pulse protocol. Cells were subjected to a first depolarizing test pulse to +60 mV of 200 ms duration followed 2·5 s later by an identical second test pulse, with 350 ms decrement interpulse intervals. *C*, the recovery from inactivation in control (□) and genistein-treated cells (■) was measured using the ratio of the peak amplitudes of the second *versus* the first pulse as a function of the time interval ($n = 6$–12). Note that the error bars are smaller than the symbols.

voltages, when compared with control ($n = 11$; $P < 0{\cdot}01$, Fig. 4*B*). For example, at +40 mV the time to peak was increased from 10 ms in control cells to 40 ms in genistein-treated cells. The slowing of the activation kinetics was also obvious in the steady-state inactivation protocol (Fig. 5*A*).

Genistein increases the steepness of the voltage dependence for steady-state inactivation but does not alter the recovery from inactivation

To check whether tyrosine kinase inhibition could affect the inactivation process we examined the steady-state inactivation and the recovery from inactivation in control and genistein-treated Schwann cells. Cells were stepped for 1 s to inactivating prepulse potentials before a voltage jump to +60 mV (Fig. 5). Following genistein application, there was a slight (though non-significant) rightward shift and a significant increase in the steepness ($n = 6$, $P < 0{\cdot}05$) of the voltage dependence of the steady-state inactivation curve. The Boltzmann distribution parameters of the transient component were $V_{0{\cdot}5} = -28{\cdot}27 \pm 3{\cdot}55$ mV, $s = 15{\cdot}84 \pm 1{\cdot}27$ ($n = 6$) and $V_{0{\cdot}5} = -23{\cdot}17 \pm 1{\cdot}72$ mV, $s = 9{\cdot}92 \pm 1{\cdot}51$ ($n = 6$), for control and genistein, respectively. The sustained component parameters were $V_{0{\cdot}5} = -19{\cdot}41 \pm 2{\cdot}44$ mV, $s = 16{\cdot}12 \pm 1{\cdot}43$ ($n = 6$) and $V_{0{\cdot}5} = -17{\cdot}67 \pm 1{\cdot}53$ mV, $s = 10{\cdot}52 \pm 1{\cdot}30$ ($n = 6$), for control and genistein, respectively (Fig. 5*B*).

As mentioned above, inactivation of the control whole-cell K^+ current could be fitted by two exponentials, while in genistein-treated cells the inactivation kinetics were well fitted by a single exponential (Fig. 6*A*). This leads to an apparent acceleration of the whole-cell inactivation kinetics, but it probably reflects the increasing contribution of I_A inactivation kinetics. In this respect, genistein did not significantly affect the recovery from inactivation. The recovery from inactivation was investigated using a twin pulse protocol. Recovery was quantified by measuring the ratio of the peak amplitudes of the second *versus* the first pulse as a function of the time interval (Fig. 6*B* and *C*). There was no significant difference in the time course of recovery from inactivation between control and genistein-treated cells.

Genistein accelerates channel deactivation

In the deactivation protocol, cells were stepped from a holding potential of −80 mV to +40 mV for different pulse durations and repolarized to −40 mV before and after genistein application (Fig. 7*A*). The decay phase of the tail currents was best fitted by two exponentials (Fig. 7*B*). The

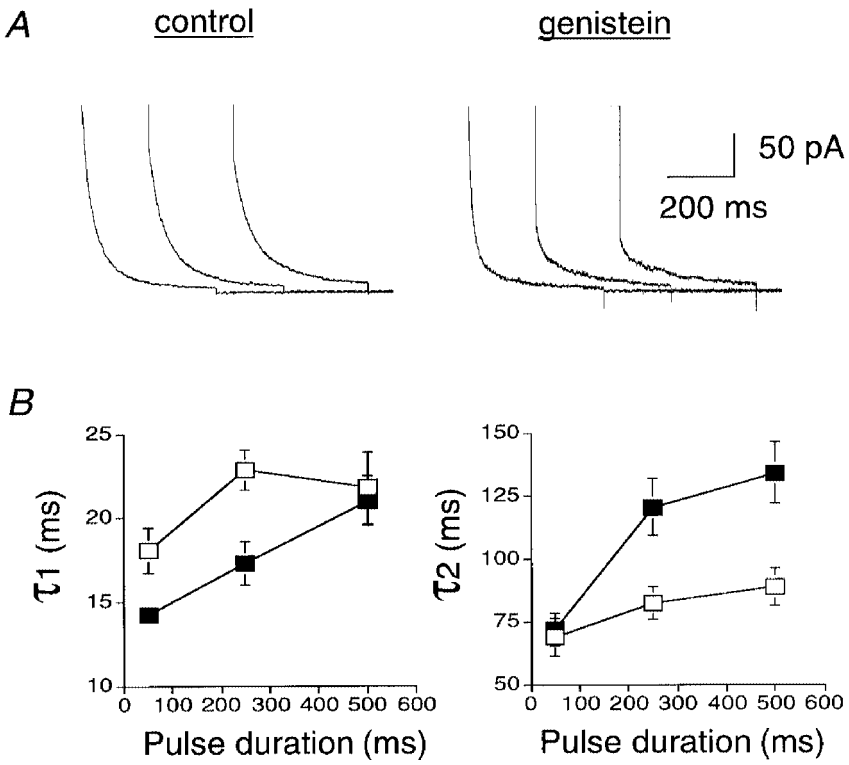

Figure 7. Genistein affects channel deactivation

A, representative current traces showing deactivating tails from control (left) and genistein-treated cells (right). Cells were stepped from a −80 mV holding potential to a +40 mV test pulse of varying durations (50, 250 and 500 ms) and then repolarized to −40 mV to maximize the tail currents. *B*, the decay phase of the tail currents was fitted by a biexponential function $I = A_0 + A_1\exp(-t/\tau_1) + A_2(\exp -t/\tau_2)$, where A_1 and τ_1, A_2 and τ_2 are the amplitudes and the deactivation time constants of the fast and slow components, respectively. The fast (τ_1) and slow (τ_2) deactivation time constants of control (□) and genistein-treated cells (■) were plotted against pulse duration ($n = 11$).

deactivation time constants slightly increased with longer pulse durations; however, the effect was sharper in genistein-treated cells. The main effect of genistein (100 μM) was to accelerate the fast deactivation phase with a decrease of τ_1 at short pulse durations (50 and 250 ms) and a significant increase in the relative amplitude of the fast component (from $0{\cdot}56 \pm 0{\cdot}03$ to $0{\cdot}71 \pm 0{\cdot}03$ at 50 ms; $n = 11$, $P < 0{\cdot}05$). In the presence of genistein, τ_1 decreased from $22{\cdot}9 \pm 1{\cdot}2$ to $17{\cdot}3 \pm 1{\cdot}3$ ms ($n = 11$; $P < 0{\cdot}01$). In addition, genistein caused a significant increase in the slow deactivation time constant τ_2 from $82{\cdot}3 \pm 6{\cdot}4$ to $120{\cdot}4 \pm 11{\cdot}1$ ms ($n = 11$; $P < 0{\cdot}01$) (Fig. 7*B*).

Genistein decreases the tyrosine phosphorylation of the Kv1.4, Kv1.5 and Kv2.1 channel α-subunits

We and others showed that both cultured Schwann cells and sciatic nerve express a large repertoire of K^+ channel α-subunits, including Kv1.2, Kv1.4, Kv1.5 and Kv2.1 (Mi *et al.* 1995; Rasband *et al.* 1998; Sobko *et al.* 1998*a*,*b*). To correlate at the molecular level the suppressing effect of genistein on I_A and I_K, we carried out reciprocal immunoprecipitation-immunoblot analysis with anti-phosphotyrosine and anti-Kv channel antibodies. Kv1.5 and Kv2.1 α-subunits, which encode delayed-rectifier K^+ channels, were found to be strongly tyrosine phosphorylated under basal culture conditions (Fig. 8). Anti-Kv1.5 antibodies specifically immunoprecipitated a major 65 kDa species which was strongly tyrosine phosphorylated (Fig. 8*A*). A doublet of 105 kDa and 115 kDa Kv2.1 immunoreactive proteins was also found to be tyrosine phosphorylated (Fig. 8*C*). The Kv1.4 α-subunit, which encodes a rapidly inactivating K^+ channel, was also found to be constitutively tyrosine phosphorylated. Anti-Kv1.4 antibodies specifically immunoprecipitated a 94 kDa species (Fig. 8*B*). Pretreatment of Schwann cells with 100 μM genistein for 15 min resulted in a significant decrease of 62 ± 13, 69 ± 15 and 40 ± 9% ($n = 4$–6; $P < 0{\cdot}05$) of Kv1.4, Kv1.5 and Kv2.1 tyrosine phosphorylation, respectively, as assessed by band densitometry analysis of immunoprecipitations.

DISCUSSION

The present study shows that in Schwann cells, inhibition of tyrosine kinases by the broad-spectrum tyrosine kinase inhibitor genistein suppresses the amplitude of the slowly inactivating delayed-rectifier current (I_K) and to a lesser extent that of the transient K^+ current (I_A). This effect is mimicked by another tyrosine kinase inhibitor herbimycin A (this study and Sobko *et al.* 1998*a*). Genistein action is accompanied by a marked decrease in the basal tyrosine phosphorylation of the Kv1.4, Kv1.5 and Kv2.1 channel α-subunits. Importantly, genistein exerts additional action on the remaining I_A by modulating activation and deactivation gating through a positive shift in the voltage dependence of activation, a slower activation and an acceleration of channel deactivation. Genistein also leads to a steeper voltage dependence of the steady-state inactivation.

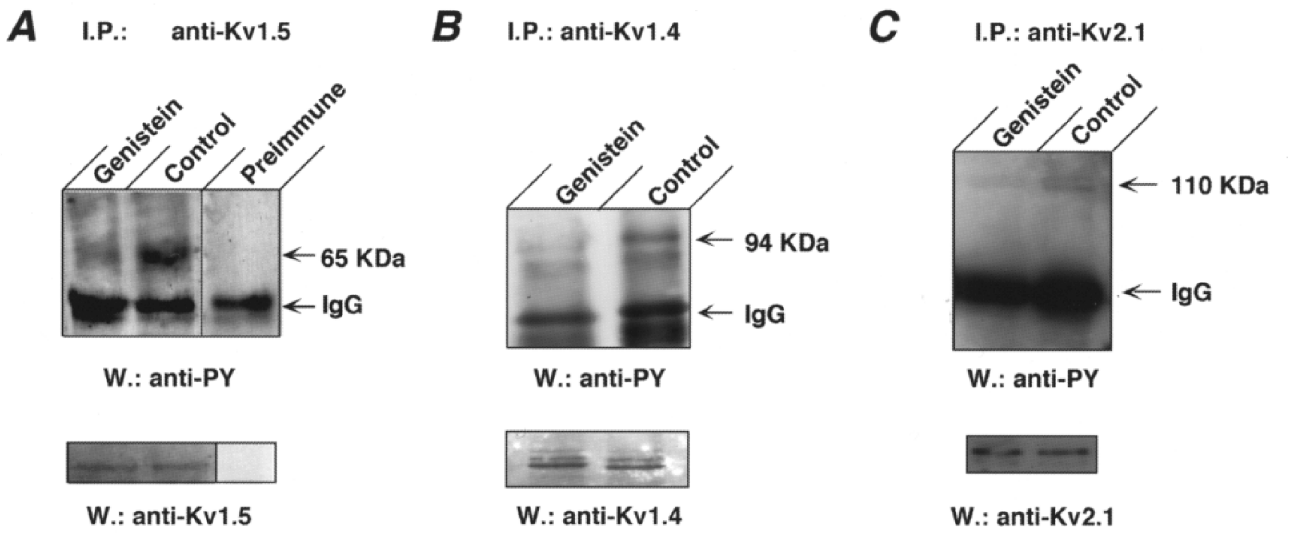

Figure 8. Genistein decreases the tyrosine phosphorylation of the Kv1.4, Kv1.5 and Kv2.1 channel α-subunits

A, Schwann cell membranal fractions from control and genistein-treated cells (100 μM genistein for 15 min) were subjected to immunoprecipitation with anti-Kv1.5 (I.P.: anti-Kv1.5) or preimmune antibodies and blots were probed with anti-phosphotyrosine (W: anti-PY). *B*, Schwann cell membranal fractions from control and genistein-treated cells were subjected to immunoprecipitation with anti-Kv1.4 (I.P.: anti-Kv1.4) and blots were processed as in *A*. *C*, Schwann cell membranal fractions from control and genistein-treated cells were subjected to immunoprecipitation with anti-Kv2.1 (I.P.: anti-Kv2.1) and blots were processed as in *A*. To estimate and compare the total protein inputs in each lane, blots were stripped and re-probed with the respective anti-Kv antibodies.

Several lines of evidence indicate that tyrosine kinases exhibit multiple regulatory effects on ionic channels such as channel clustering and desensitization, as well as modulation of channel gating (For review see Siegelbaum, 1994; Jonas & Kaczmarek, 1996; Thomas & Brugge, 1997; Boxall & Lancaster, 1998). Here we show that in Schwann cells, tyrosine kinases exert multiple actions on I_A and I_K. While genistein and herbimycin A suppress the amplitude of I_K and to a lesser extent that of I_A, genistein additionally modulates the gating of the remaining I_A. We show here that the suppression of I_K and I_A produced by genistein is accompanied by a marked decrease in the basal tyrosine phosphorylation of Kv1.5 and Kv2.1 as well as Kv1.4. However, the mechanisms underlying the constitutive activation of I_K and I_A are not yet elucidated. Genistein acts in a dose-dependent manner. At low concentration (10 μM), it appears to selectively affect I_K, while at higher concentration it suppresses both I_A and I_K. Genistein action is specific and does not appear to involve a voltage-dependent channel block either in the open state or in the resting state. This is shown by the ineffectiveness of its inactive analogue daidzein and the lack of voltage dependence of current inhibition. In a train protocol, K^+ current inhibition was attained at the first pulse, subsequent to the exposure of cells to genistein, and the inhibitory effect did not increase further upon subsequent stimulations. In the case of channel block in the open state, one would expect the initial current trace following genistein application to be superimposable on the control trace (if drug on-rate binding is slow relative to channel opening). In the case of channel block in the resting state, one would expect depolarization to reverse the block. Our data do not account for the above features and, thus, do not accommodate with an open-channel block or with a 'reverse use dependence'. The striking changes in channel gating (see below) and the accompanying changes in Kv channel tyrosine phosphorylation point to a tyrosine kinase-mediated process.

Genistein is known to exert a broad inhibitory action on both receptor and non-receptor tyrosine kinases, while herbimycin A at low concentrations appears to affect preferentially, although not exclusively, non-receptor tyrosine kinases (Akiyama & Ogawara, 1991; Levitzki & Gazit, 1995). It is possible that genistein acts on an additional, yet unidentified, tyrosine kinase to modulate I_A gating. The most striking features of genistein action on I_A are a pronounced rightward shift in the voltage dependence of activation (+30 mV), a decrease in the steepness of activation gating (9 mV per e-fold change), and a slowing of the activation kinetics, as well as an acceleration of channel deactivation. These effects are mostly exerted on voltage-dependent conformational changes leading to channel activation and closure. It is worth noting that genistein also exerts a small but significant action on the inactivation process producing a steeper voltage dependence and a non-significant small rightward shift of the steady-state inactivation. This suggests that tyrosine kinases finely tune the operative voltage range at which I_A is active.

To our knowledge, this is the first study describing an action of tyrosine kinases on the activation gating of voltage-dependent K^+ channels. Previous studies reported effects of tyrosine kinases on either current amplitude and/or inactivation and deactivation kinetics, but not on activation gating (Huang *et al.* 1993; Timpe & Fantl, 1994; Holmes *et al.* 1996*b*; Bowlby *et al.* 1997; Fadool *et al.* 1997). Interestingly, a recent work described similar effects on the activation gating of HERG K^+ channels (Barros *et al.* 1998). However, it was shown to involve a PKC-dependent pathway not tyrosine kinases.

The mechanisms underlying the rightward shift in the voltage dependence of activation and the decrease in the steepness of activation gating are unknown. Perozo & Bezanilla (1990) originally observed that phosphorylation of the delayed-rectifier K^+ channel in squid axon, which leads to a rightward shift of the activation curve and a slowing of the activation kinetics, causes an increase in the density of negative surface charges on the cytoplasmic side of the membrane. They suggested that phosphorylation induces a local hyperpolarization responsible for the shift of voltage-dependent gating toward more depolarized potentials (Perozo & Bezanilla, 1990). However, our results describe a shift in the opposite direction. It is possible that inhibition of tyrosine kinases mediated by genistein may either directly or allosterically disrupt the electrostatic interactions existing between the two putative voltage-sensing regions of the channel, S2 and S4, that were shown to be crucial for the gating process (Seoh *et al.* 1996; Tiwari-Woodruff *et al.* 1997; Cha & Bezanilla, 1997).

With respect to the molecular identity of I_A in Schwann cells, we recently showed that Kv1.4 α-subunits are plausible candidates (Sobko *et al.* 1998*b*). Kv1.4 subunits could form heteromultimers with Kv1.5 subunits. Thus, I_A may be accounted for by the channel activity of Kv1.5–Kv1.4 heteromultimers and/or homomultimeric Kv1.4 α-subunits. The Kv1.4 protein sequence exhibits a putative tyrosine kinase phosphorylation site at its C-terminus, and we showed here that genistein reduces the constitutive tyrosine phosphorylation of Kv1.4 α-subunits. To our knowledge, this is the first study demonstrating that Kv1.4 α-subunits can be modulated by tyrosine phosphorylation.

The modulation of I_A and I_K by tyrosine kinases, may be functionally relevant to the control of K^+ channel activity during Schwann cell development and myelination. I_K is thought to be linked to Schwann cell proliferation during development and following Wallerian degeneration of sciatic nerves (Konishi, 1989; for review see Chiu, 1991). More recently, we found that I_K is constitutively activated by a Src family tyrosine kinase in Schwann cells, further suggesting a role in Schwann cell proliferation (Sobko *et al.* 1998*a*). It was also shown that I_K is downregulated in the Schwann cell soma as proliferation ceases and myelination proceeds (for review see Chiu, 1991). In this context, a developmental decrease in tyrosine kinase activity and subsequently in I_K could contribute to the exit of Schwann

cells from the cell cycle and the onset of myelination. With respect to I_A, it was shown that downregulation of its activity is permissive for axonal contact (Despeyroux *et al.* 1994). Thus, modulation of I_A gating by tyrosine kinases may have substantial impact on the ability of Schwann cell processes to contact peripheral axons.

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Acknowledgements

We would like to thank Dr Eitan Reuveny for helpful discussions and Emily Levine for careful reading of the manuscript. This research was supported by grants from the EEC BIO-CT97-2207 and the Minerva Foundation (to B.A). Dr A. Peretz was supported by a Weizmann Institute post-doctoral fellowship of the Koret foundation. Bernard Attali is an incumbent of the Philip Harris and Gerald Ronson Career Development chair.

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