

Stimulation of Maxi-K Channels in Trabecular Meshwork by Tyrosine Kinase Inhibitors

Friederike Stumpff, Yang Que, Marianne Boxberger, Olaf Strauss, and Michael Wiederholt

PURPOSE. Muscarinic agonists contract and tyrosine kinase inhibitors relax precontracted trabecular meshwork, a smooth muscle-like tissue involved in the regulation of aqueous humor outflow. The effect of tyrosine kinase inhibitors on membrane currents of cells stimulated by acetylcholine was examined.

METHODS. Cells from bovine trabecular meshwork were studied using both the perforated patch-clamp technique with nystatin and the single-channel technique.

RESULTS. Application of the tyrosine kinase inhibitor genistein (5×10^{-5} M) on trabecular meshwork cells stimulated with acetylcholine resulted in a reversible increase in outward current to $578\% \pm 154\%$ ($n = 16$) of the initial current level. The effect of genistein was dose dependent. Reversal potential was hyperpolarized by 15 ± 3 mV ($n = 9$). Tyrphostin 51, a synthetic inhibitor of tyrosine kinases, had the same effect ($433\% \pm 46\%$; $n = 7$). Daidzein, a nonactive structural analogue of genistein, had no effect ($n = 4$). The stimulation of outward current by tyrosine kinase inhibitors was blocked by substitution of tetraethylammonium (TEA^+) for potassium, whereas the potassium channel blockers glibenclamide (K-ATP) and apamin (low-conductance calcium-activated potassium channel) had no effect. Blockage of the high-conductance calcium-activated potassium channel (maxi-K) by charybdotoxin or iberiotoxin (10^{-7} M) suppressed $86\% \pm 18\%$ ($n = 4$) of the response. Depleting the cells of calcium did not have an effect on the current stimulated by genistein. In the excised inside-out configuration, open probability increased to $417\% \pm 39\%$ ($n = 3$) after exposure to genistein.

CONCLUSIONS. In trabecular meshwork, tyrosine kinase inhibitors activate maxi-K (K_{Ca}) channels. Hyperpolarization caused by efflux of potassium could lead to the relaxation of trabecular meshwork by tyrosine kinase inhibitors. (*Invest Ophthalmol Vis Sci.* 1999;40:1404-1417)

In recent years, increasing attention has been paid to the active contribution of trabecular meshwork in regulating aqueous humor outflow and thus intraocular pressure.¹ Located in the ocular outflow pathway between the anterior chamber and Schlemm's canal, this tissue is not merely passively distended by the ciliary muscle but exhibits active contractile properties of its own with an impact on outflow resistance.^{2,3} In this study, we investigated the effect of tyrosine kinase inhibitors on the membrane currents of trabecular meshwork cells, measured by the patch-clamp technique.

Tyrosine kinase inhibitors are a group of substances of increasing interest as new therapeutic agents for a variety of diseases such as cancer, psoriasis, atherosclerosis, and septic

shock.⁴⁻⁷ In a previous study, we showed that tyrosine kinase inhibitors relax precontracted strips of trabecular meshwork.⁸ Interestingly, strips of ciliary muscle were not relaxed to the same extent. Thus, tyrosine kinase inhibitors seem to be promising candidates for the treatment of glaucoma. Ideally, they should lead to the reduction of intraocular pressure (through relaxation of the trabecular meshwork^{3,8}) while simultaneously improving retinal circulation (through vasodilation^{9,10}). However, much more research is needed to validate these hypotheses.

Apart from their therapeutic potential, tyrosine kinase inhibitors are also intriguing tools for the study of signal transduction pathways.^{6,11} To date, most of the research investigating the physiological role of tyrosine kinases has revolved around their participation in the regulation of mitogenesis and cell migration.¹² By comparison, interest in tyrosine kinases as mediators of smooth muscle contractility is fairly recent.^{10,13} Thus, receptor tyrosine kinases such as the receptor for epidermal growth factor (EGF) have an influence on smooth muscle contractility. In addition, tyrosine kinase inhibitors have been shown to suppress the contractile response of visceral and vascular smooth muscle after stimulation by agents such as acetylcholine.^{10,13} It appears that receptor-mediated responses of smooth muscle cells involve the phosphorylation of proteins on tyrosine residues and that this induces both the release of Ca^{2+} from the sarcoplasmic reticulum, and influx of Ca^{2+} from the outside of the cell. Very little is known about the nature of the proteins that are phosphorylated. However, evi-

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dence is emerging that various ionic channels are among the targets of tyrosine kinases.^{9,10,14-16}

Using the perforated-patch and single-channel configurations of the patch-clamp technique, we were able to show that tyrosine kinase inhibitors enhance outward current of trabecular meshwork cells in a dose-dependent manner, and that this effect is directly mediated by high-conductance calcium-activated potassium channels (maxi-K channels, BK_{Ca}). Interestingly, the stimulation is not caused by the calcium- and voltage-dependence of this channel and implies the regulation by tyrosine kinases.

MATERIALS AND METHODS

Tissue Culture

All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Primary bovine trabecular meshwork cell cultures were prepared as described previously.¹⁷⁻²⁰ In summary, freshly enucleated bovine eyes were obtained from a local abattoir. Small pieces of trabecular meshwork were obtained from the eyes, placed in a sterile petri dish under a glass coverslip, and fed twice weekly with Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (all cell culture material from Biochrom, Berlin, Germany). Cultures were maintained in a 95% air/5% CO₂ atmosphere at 37°C. The cultures were passaged using the trypsin-EDTA method. Only cells from the third passage were used for experiments.

Patch-Clamp Experiments

All experiments were performed essentially as in a previous study.²¹ Nystatin (150 µg/ml) in the patch pipette was used to obtain perforated patches.²² To obtain single cells from confluent cell layers, cells were dispersed by enzymatic treatment with trypsin for 3 minutes. Subsequently, trypsin was inactivated by resuspension in calcium-containing cell culture medium, and cells were allowed to settle on glass coverslips for half an hour at 37°C. The coverslips were then introduced into a perfusion chamber on the stage of an inverted microscope (Axiovert 35; Zeiss, Oberkochen, Germany) and were superfused with Ringer's solution. The chamber itself was heated (LN Temperature Controller, Ratingen, Germany) and the perfusate prewarmed. All experiments were performed at 37°C. Fluid exchange was complete within less than 5 seconds. Borosilicate glass patch pipettes (wall thickness, 0.3 mm; Clark Electromedical Instruments, Reading, UK) were pulled and polished using a DMZ Universal Puller (Zeitz, Augsburg, Germany). The input resistance of the pipettes filled with the standard intracellular solution (see later description) was 3 MΩ to 4 MΩ. Potentials were referenced to the bath using an Ag-AgCl electrode connected to the bath solution by an agar bridge electrode so that a negative potential corresponded to a negative pipette potential. Liquid junction potentials were determined and corrected according to the method described by Neher.²³ Unless indicated otherwise, positive ions flowing into the pipette correspond to a negative current and are depicted in figures as going downward, whereas positive ions flowing out of the pipette are designated by a positive current in the

upward direction. For inside-out patches, the pipette potential corresponds to the negative membrane potential.

As in previous contractility measurements,⁸ cells were superfused with Ringer's solution containing acetylcholine (5×10^{-5} M) before seal formation and throughout the entire recording. Care was taken to ensure stabilization of currents before application of substances.

Data Acquisition

Currents were recorded using an EPC 9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Pulse generation, data collection, and analysis were performed using computer software (TIDA for Windows; HEKA) and filtered with a 2.9-kHz Bessel filter. Records were corrected for capacitance. Whole-cell measurements were not leak subtracted, because it was difficult to distinguish between a nonspecific leak and a physiological non-voltage-dependent current. According to TIDA software, access resistance was below 8 MΩ and automatically corrected. Several types of pulse protocols were used.²¹ In whole-cell experiments, two types of protocols were used. Changes in external solution were monitored using a protocol that generated steps of 200-msec duration to various voltages between -80 mV and 100 mV. After each step, the voltage returned to a holding potential of -40 mV for 200 msec. Currents were continuously sampled at 100 Hz throughout the duration of the protocol (see Fig. 1B; the sequence depicted was repeated hundreds of times to produce the trace seen in Fig. 1C). After completion of the solution changes and a stabilization of current levels, a second, conventional pulse protocol was used to record currents. In this protocol, data were first acquired at the holding potential for 20 msec, then the potential was stepped to a value between -80 mV and 100 mV for 50 msec, followed by a 30-msec return to the holding potential. Throughout this recording, data were sampled at 5 kHz. For single-channel experiments, voltage was also automatically stepped to various levels. Data acquisition started 0.5 seconds after the voltage step at a sampling rate of 10 kHz for a duration of 6 seconds before the next voltage step took place.

Solutions and Chemicals

Control Ringer's solution contained the following ion concentrations (in millimolar): 151 NaCl, 4 KCl, 1.7 CaCl₂, 1 KH₂PO₄, 0.9 MgSO₄, 10 HEPES, and 5 glucose adjusted to pH 7.4 (NaOH). The standard intracellular solution (pipette solution) for whole-cell experiments contained (in millimolar) 119 K-glutamate, 10 NaCl, 1 KH₂PO₄, 0.9 MgSO₄, 3.3 EGTA, 6.6 CaEGTA, and 10 HEPES adjusted to pH 7.2 (NaOH). This solution corresponded to an internal calcium concentration of 2.7×10^{-7} M²⁴ and was referred to as the high-potassium solution. For substitution of potassium in the pipette solution, an equimolar amount of tetraethylammonium hydroxide (TEAOH) was titrated with gluconic acid. For single-channel experiments, the standard bath solution contained (in millimolar) 134 KCl, 11 NaCl, 1 KH₂PO₄, 0.9 MgCl₂, and 10 HEPES adjusted to pH 7.4 (NaOH). Calcium was buffered to 10^{-7} M using EGTA and, for some experiments, to 10^{-5} M using nitrilotriacetic acid according to established procedures.²⁵ The pipette solution for single-channel experiments was the same in principle, but potassium concentration was lowered to 50 mM, sodium was raised to 95 mM, and calcium was raised to 1 mM. All chemicals were of the highest available grade of purity.

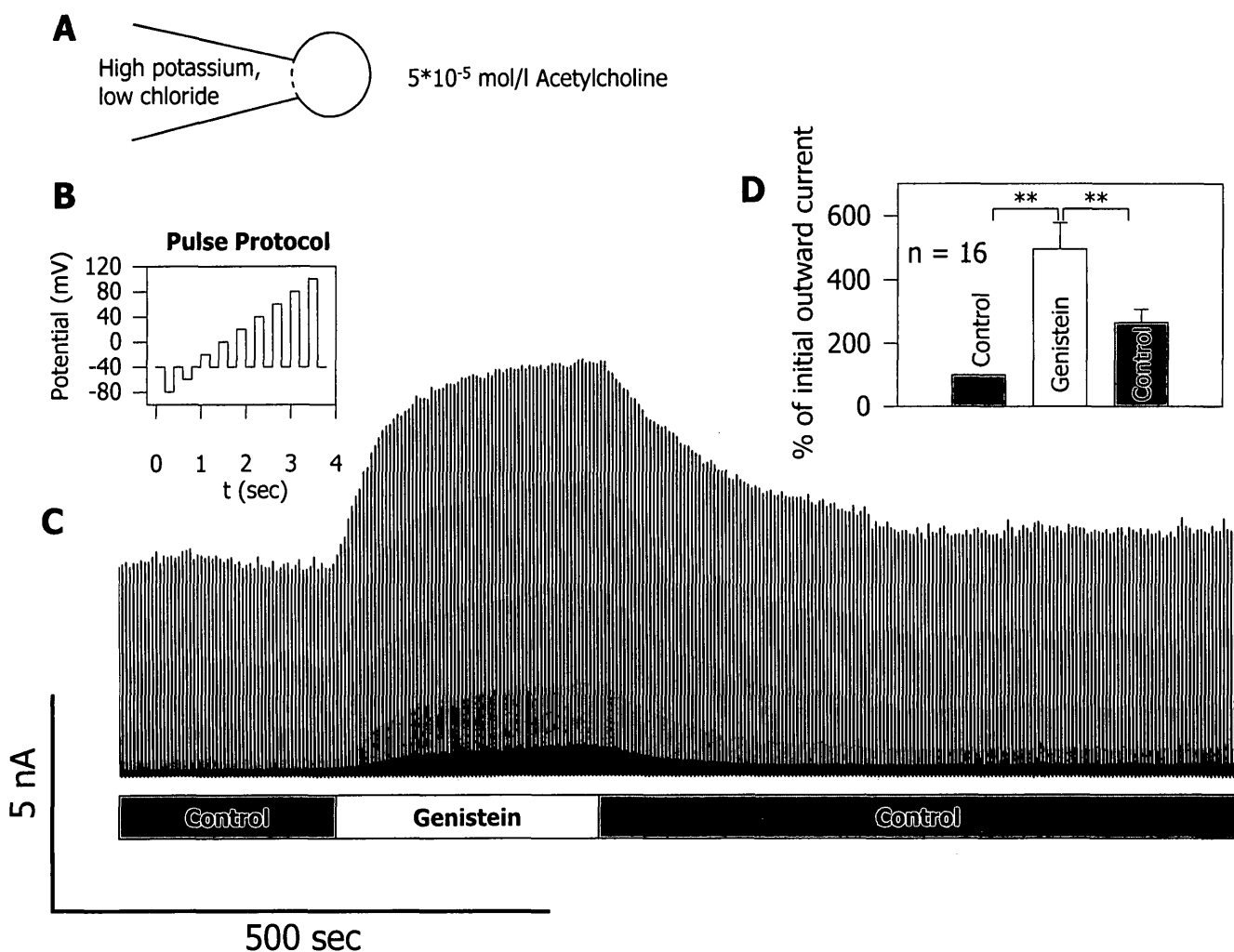


FIGURE 1. (A) Standard patch-clamp configuration with nystatin, using a high-potassium intracellular solution. (B) Standard voltage-pulse protocol. The sequence depicted was repeated hundreds of times to produce the trace seen in (C). (C) Typical recording of a single cell. A strong outward current could be measured, which was reversibly enhanced by genistein (5×10^{-5} M). (D) Summary of results from 16 experiments. $**P < 0.005$.

Charybdotoxin (ChTX) and iberiotoxin (IbTX) were obtained from Bachem (Heidelberg, Germany) and were dissolved in phosphate-buffered saline (PBS) before addition to the solution. Tetraethylammonium chloride (TEACl) and TEOH were from Sigma (Deisenhofen, Germany). Genistein was from Research Biochemical (Cologne, Germany), and tyrphostin 51 and daidzein were from Sigma.

Analysis

In the following, "outward current" refers to current values obtained from pulse protocols as depicted in Figure 1B and at a pipette potential of 80 mV. Because outward current varied greatly from cell to cell, the current level at the beginning of the experiment was taken as 100% and will be referred to as "initial outward current." All changes in outward current were seen in relation to this initial value. All current values were obtained immediately before the subsequent change of solution; in case of recovery, immediately before the end of the experiment (usually caused by rupture of the seal). The changes (in percent) were used to calculate mean values \pm SEM. Significance testing was performed using the paired Stu-

dent's *t*-test and standard software (Sigma Plot Scientific Graph System, ver. 1.02; Jandel, Corte Madera, CA). *N* refers to the number of experiments. Each experiment was performed using one cell. Cells were derived from different explants.

In single-channel experiments, channel conductance was determined by fitting current-voltage relationships using the Goldman-Hodgkin-Katz equation. All fits were performed using commercially available software (Sigma Plot Scientific Graph System, Jandel). Because it was not possible to determine the exact number of channels per patch, channel activity was estimated from the product NP_o , where *N* is the number of channels and P_o is single-channel open probability. The time integral of current divided by the product of mean single-channel current and time of recording is equal to NP_o .²⁶

RESULTS

Stimulation of Outward Current by Tyrosine Kinase Inhibitors

After seal formation and formation of the perforated-patch configuration with nystatin, cells were superfused with a con-

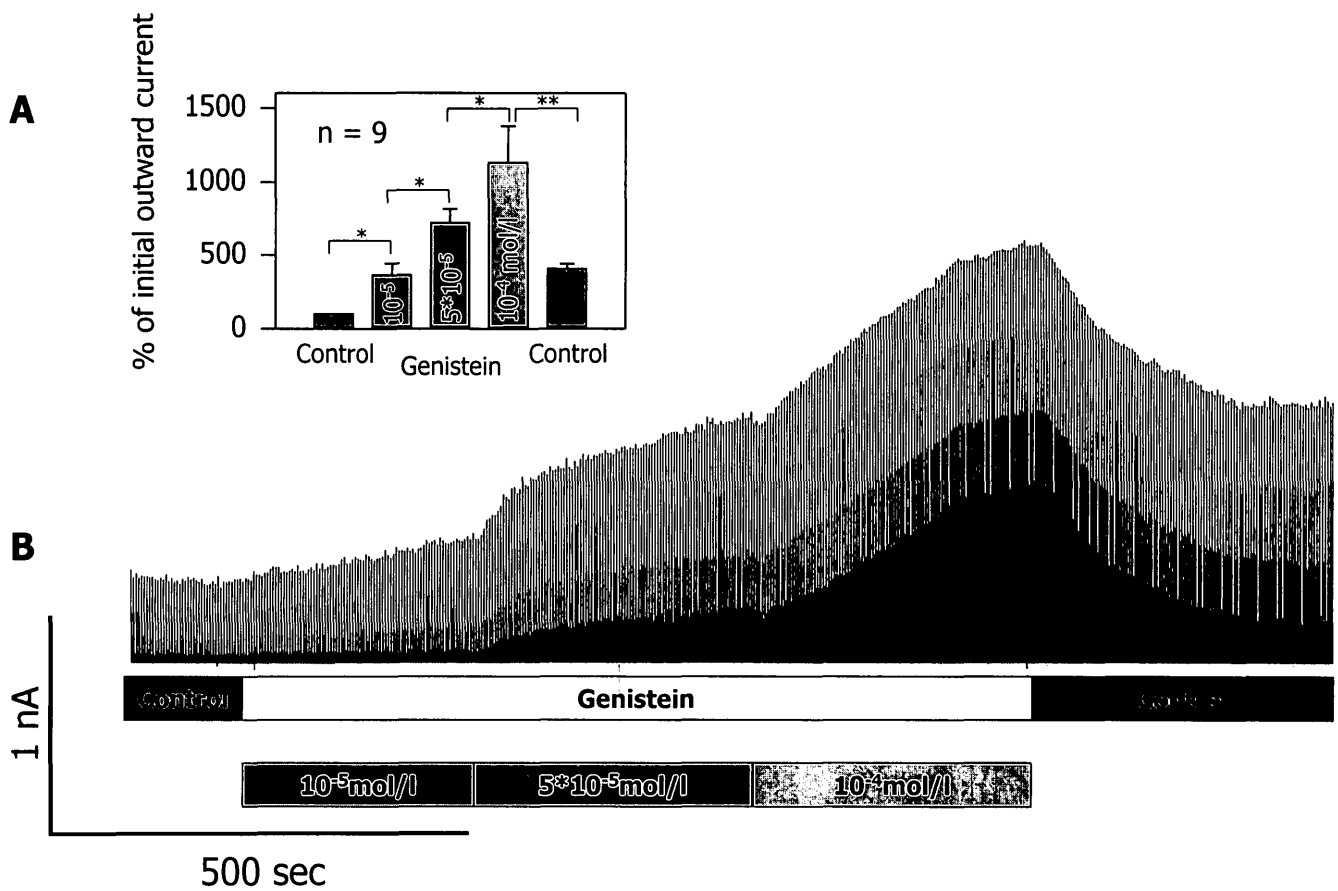


FIGURE 2. Dose dependence of the effect of genistein. (A) Summary of nine experiments. Initial outward current at 80 mV was taken as 100%. * $P < 0.05$; ** $P < 0.005$. (B) Individual trace.

control solution (Ringer's) until current levels had stabilized. For comparison with experiments on trabecular meshwork strips precontracted by muscarinic agonists,^{2,27} acetylcholine (5×10^{-5} M) was routinely added to the external solution in the patch-clamp experiments.

Application of the tyrosine kinase inhibitor genistein in a dose of 5×10^{-5} M resulted in a strong, sustained increase in outward current. Degree of stimulation varied greatly among individual cells, but some degree of stimulation could be observed in each cell, yielding an average stimulation of outward

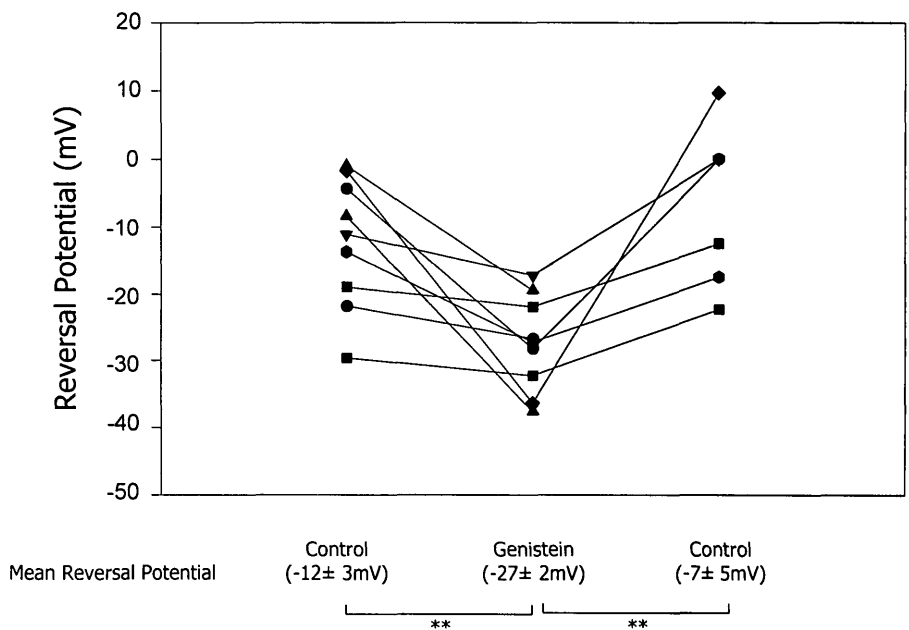


FIGURE 3. In nine cells tested, application of genistein (5×10^{-5} M) significantly lowered reversal potential by 5 ± 3 mV ($n = 9$). ** $P < 0.005$. Each symbol represents a different cell.

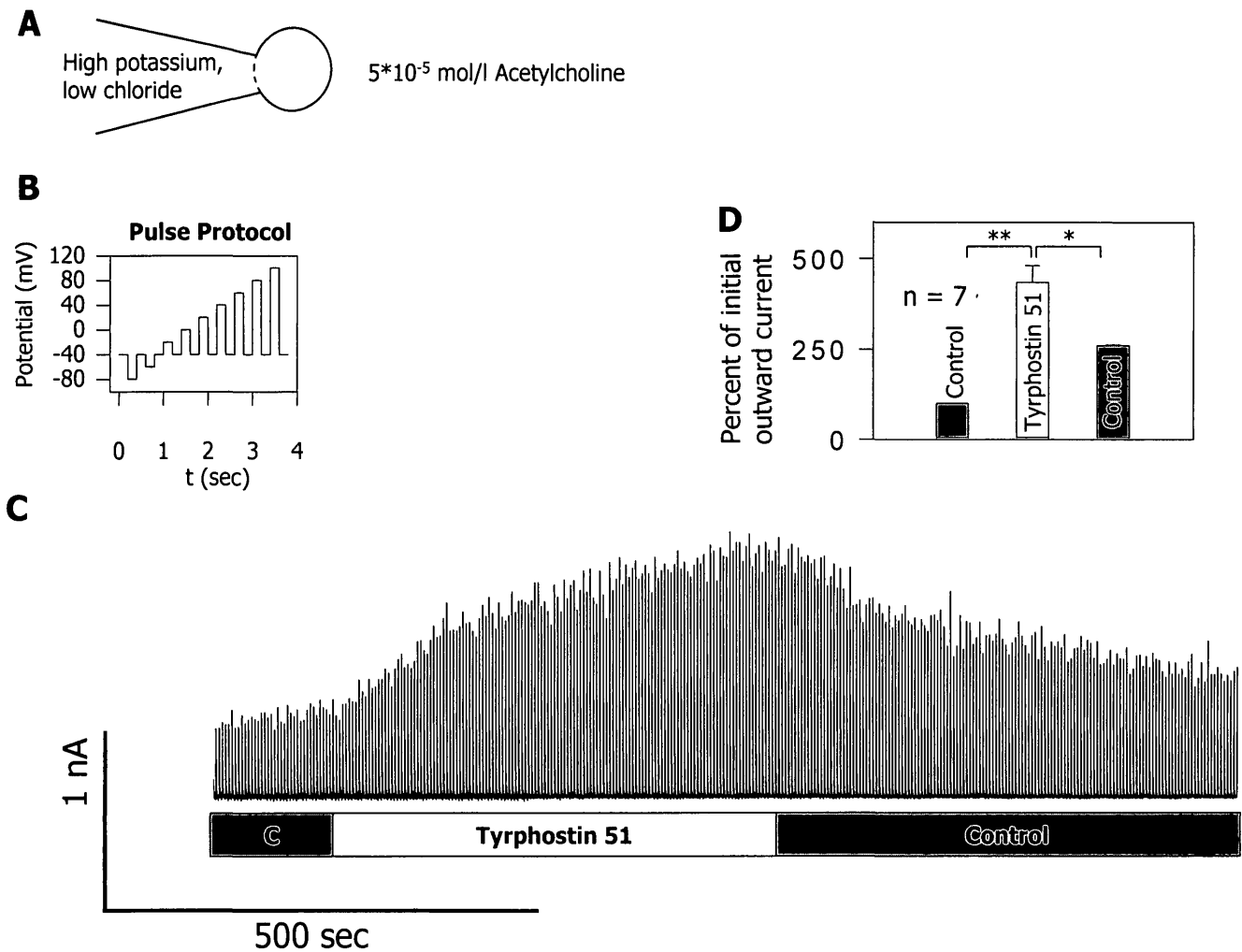


FIGURE 4. (A) Standard patch-clamp configuration. (B) Standard voltage-pulse protocol. (C) Outward current was enhanced by tyrphostin 51 (5×10^{-5} M). (D) Summary of results from seven experiments $**P < 0.005$.

current at 80 mV to $495\% \pm 83\%$ of the initial level ($n = 16$; $P < 0005$; Figs. 1C, 1D). After withdrawal of genistein, recovery occurred to a level of $264\% \pm 43\%$ ($P < 0.05$) as measured at the end of the experiment. Usually, seals ruptured at this point, so that recovery was incomplete because of incomplete washout. When seals remained stable, recovery was usually complete (compare Fig. 1D). In another series of experiments on nine individual cells, different concentrations of genistein were applied to the same cell in succession, showing that the effect was dose dependent. (Fig. 2).

In a second step, we determined membrane potential as the potential at which membrane current equals zero (reversal potential). After seal formation, this value was similar to values obtained by conventional membrane voltage measurements and to values reported in an earlier patch-clamp study (42 ± 4 mV; $n = 22$).²¹ As membrane current stabilized, this value increased to higher levels, perhaps as a result of the diffusion of ionic constituents out of the patch pipette into the cell and vice versa. Genistein reversibly hyperpolarized this value by 15 ± 3 mV ($n = 9$; $P < 0.005$; Fig. 3).

To assess if this effect was attributable to nonspecific actions of genistein, we applied tyrphostin 51, a tyrosine kinase inhibitor with a different mode of action⁷ and a relatively high specificity for the EGF receptor. In these experiments, a

similar elevation of outward current could be evoked ($433\% \pm 46\%$; $n = 7$), with recovery to $259\% \pm 13\%$ (Fig. 4). When genistein and tyrphostin 51 (both in a concentration of 5×10^{-5} M) were applied to the same cell in succession, no significant differences could be observed. Apparently, the stimulation of outward current was not dependent on the type of tyrosine kinase inhibitor used ($n = 4$). In contrast, daidzein (5×10^{-5} M), an inactive structural analogue of genistein,⁷ had no effect ($n = 4$; Fig. 5).

Identification of the Current Stimulated by Tyrosine Kinase Inhibition

To find out which channels were involved in the response of trabecular meshwork cells to genistein, we used the unspecific, but potent blocker of potassium channels TEACl (5 mM; Fig. 6), which blocked the induction of current by genistein completely (more than 99%; $n = 5$). In a second series of experiments, TEACl was substituted for KCl was substituted in the external solution and TEA-gluconate for K-glutamate in the pipette solution. Outward current level was greatly reduced from 0.5 ± 0.1 nA ($n = 9$) in solutions containing potassium to 0.05 ± 0.01 nA ($n = 4$) in potassium-free solutions. In four cells tested in the absence of potassium, genistein (5×10^{-5} M) evoked no significant changes in inward or outward cur-

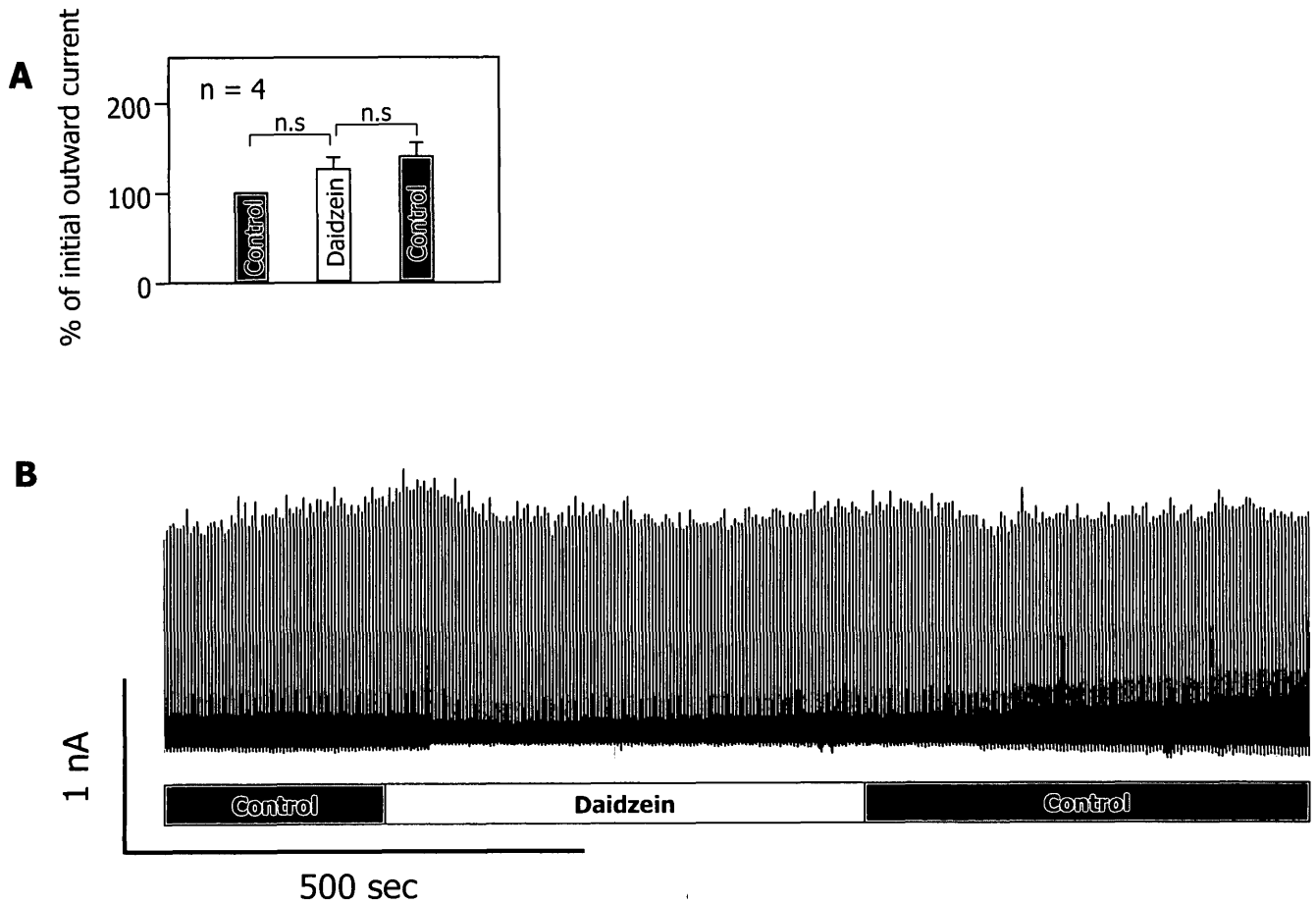


FIGURE 5. In four cells tested, daidzein (5×10^{-5} M) did not induce a significant increase in outward current. (A) Summary of four experiments. (B) Individual experiment.

rent. Thus, the genistein-induced current was caused by an outflow of potassium, excluding participation of other ions such as chloride. In subsequent experiments, various potassium channel blockers were tested. For this purpose, outward current was measured in control solution (Ringer's). Then, the blocker was added to assess the contribution of the channel in question to the outward current of the resting cell. Subsequently, genistein (or tyrphostin 51) was added in the presence of the blocker. Withdrawal of the blocker in the presence of genistein was followed by a return to the control solution. This made it possible to determine the genistein-induced increases in outward current in the presence and absence of the blocker and to determine the relative difference between them using data obtained from one cell. Glibenclamide (10^{-5} M), which blocks ATP-dependent potassium channels (K-ATP) had no significant effect, either on resting current or on the current enhanced by genistein. This was not dependent on whether glibenclamide was applied before genistein ($n = 4$) or afterward ($n = 7$; Fig. 7). Apamin (10^{-6} M), a blocker of the low-conductance calcium-dependent potassium channel (SK), also did not block the genistein-induced increase in outward current (Fig. 8), regardless of whether it was applied before ($n = 4$) or after ($n = 3$) genistein. Because trabecular meshwork cells express maxi-K channels,²¹ we tried blocking these channels with the specific blockers IbTX (10^{-7} M; Fig. 9) and ChTX (10^{-7} M; Fig. 10). Both blockers reduced resting outward current. Whereas IbTX blocked resting outward current

to $22\% \pm 6\%$ ($n = 4$; $P < 0.001$) of the initial level at 80 mV, ChTX blocked it to $71\% \pm 16\%$ ($n = 4$; $P < 0.001$), reflecting the greater potency of IbTX for blocking this channel.²⁸ Both agents significantly blocked the major part of the genistein-induced increase in outward current in all cells tested. In the presence of ChTX, the response to genistein was only $38\% \pm 6\%$ ($n = 4$; $P < 0.05$) of the response observed in the absence of ChTX. Iberitoxin reduced the response to genistein to $22\% \pm 5\%$ ($n = 4$; $P < 0.05$) of the response in the absence of this blocker (Fig. 9). When the order of application was reversed and genistein was applied before IbTX, IbTX reduced the response to $9\% \pm 3\%$ ($n = 4$; $P < 0.05$). The order in which the substances were applied did not have a significant effect on the outcome of the experiment. The outward current enhanced by tyrphostin 51 was also sensitive to blockage of the maxi-K channel. In the presence of ChTX, the response was $47\% \pm 14\%$ ($n = 4$; $P < 0.05$) of the unblocked response (Fig. 10), while in the presence of IbTX, only $12.78\% \pm 2\%$ ($n = 6$; $P < 0.001$) of the response remained.

Variation of Cytosolic Calcium on Genistein-Induced Outward Current

To assess a possible contribution of calcium to this response, cells were preincubated in calcium-free solution containing 10 mM EGTA to prevent influx of intracellular calcium from the extracellular space. To prevent influx of calcium from cytosolic stores, they were depleted by a thapsigargin pulse (10^{-6} M)

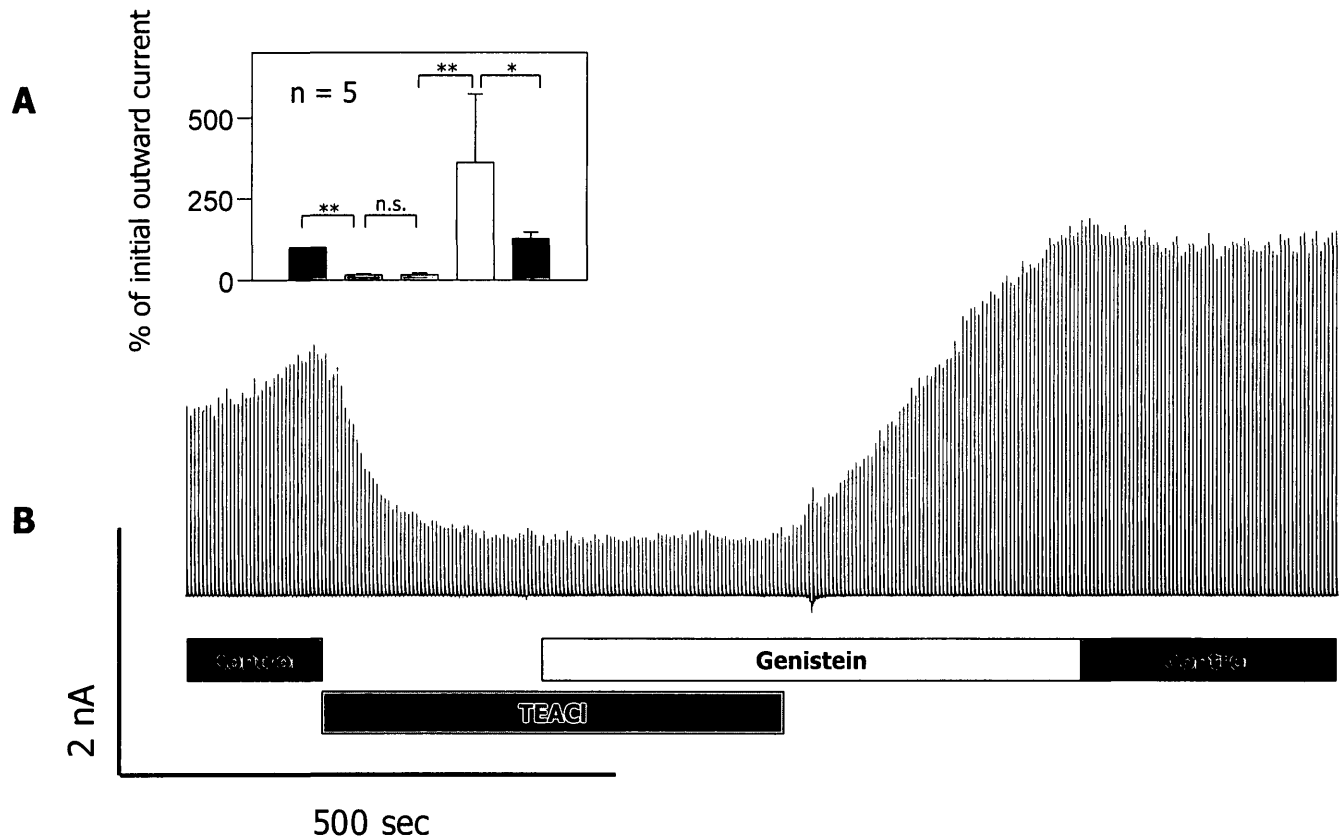


FIGURE 6. In five cells tested, TEACl (5 mM) completely suppressed the response to genistein (5×10^{-5} M). (A) Summary of five experiments. (B) Individual experiment.

of 400 seconds' duration.²⁹ Under these conditions, application of genistein (5×10^{-5} M) in a calcium-free solution had the same effect as in a solution with normal calcium. Reapplication of calcium in the continued presence of genistein had no effect on outward current (Fig. 11).

Single-Channel Recordings

Next, we investigated the effect of genistein on channels in the excised inside-out configuration of the patch-clamp technique. In inside-out patches, we observed the same 300-pS channel described previously.²¹ We verified the identity of this channel as a maxi-K channel by stimulating it with CaCl_2 (10^{-5} M) and observing the reversal potential in asymmetrical potassium chloride solution. When genistein (10^{-4} M) was added to the cytosolic side of the patch, the open probability of the channel increased by $417\% \pm 39\%$ ($n = 3$; $P < 0.05$; Fig. 12).

DISCUSSION

Using a preparation of bovine trabecular meshwork cells, we were able to show that the application of the tyrosine kinase inhibitors genistein and tyrphostin 51 stimulated an outward current through activation of maxi-K channels previously characterized.²¹ Stimulation could also be observed in excised patches and therefore seems to be the result of a signaling cascade involving only membrane-bound components. In particular, it was not a result of changing levels of cytosolic calcium. Hyperpolarization caused by activation of maxi-K channels seems to be one of the underlying effects explaining

the previously described relaxation of trabecular meshwork strips in response to the application of tyrosine kinase inhibitors.⁸

The stimulation of current by genistein was dose dependent in a range typical of other cell types and ionic channels.^{9,15,30-34} This range of concentration corresponds to the range in which relaxation of smooth muscle in general¹³ and relaxation of trabecular meshwork in particular⁸ has been observed.

The bovine model is a well-established experimental model.^{1-3,17-19,27,35-38} Unlike human eyes, bovine eyes allow macroscopic separation of trabecular meshwork and ciliary muscle, making it possible to measure directly the tonus of trabecular meshwork and to show that this tissue contracts and relaxes similarly to smooth muscle cells in response to various substances.^{8,27,38,39} Other groups have shown that agents that induce contraction elevate cytosolic calcium.^{18,40-42} In addition, both human and bovine trabecular meshwork cells express smooth muscle α -actin and myosin.^{43,44} Although the mechanism resulting in contraction remains unknown,^{45,46} functionally, trabecular meshwork cells contract and relax similarly to smooth muscle cells in response to changes in cytosolic calcium. In another study on the regulation of outflow rate, we were able to show that relaxation of trabecular meshwork is associated with an enhancement of outflow and contraction with a reduction in outflow facility.³ Substances that relax trabecular meshwork more strongly than ciliary muscle may therefore have a beneficial impact on intraocular pressure. Tyrosine kinase inhibitors have that property.⁸

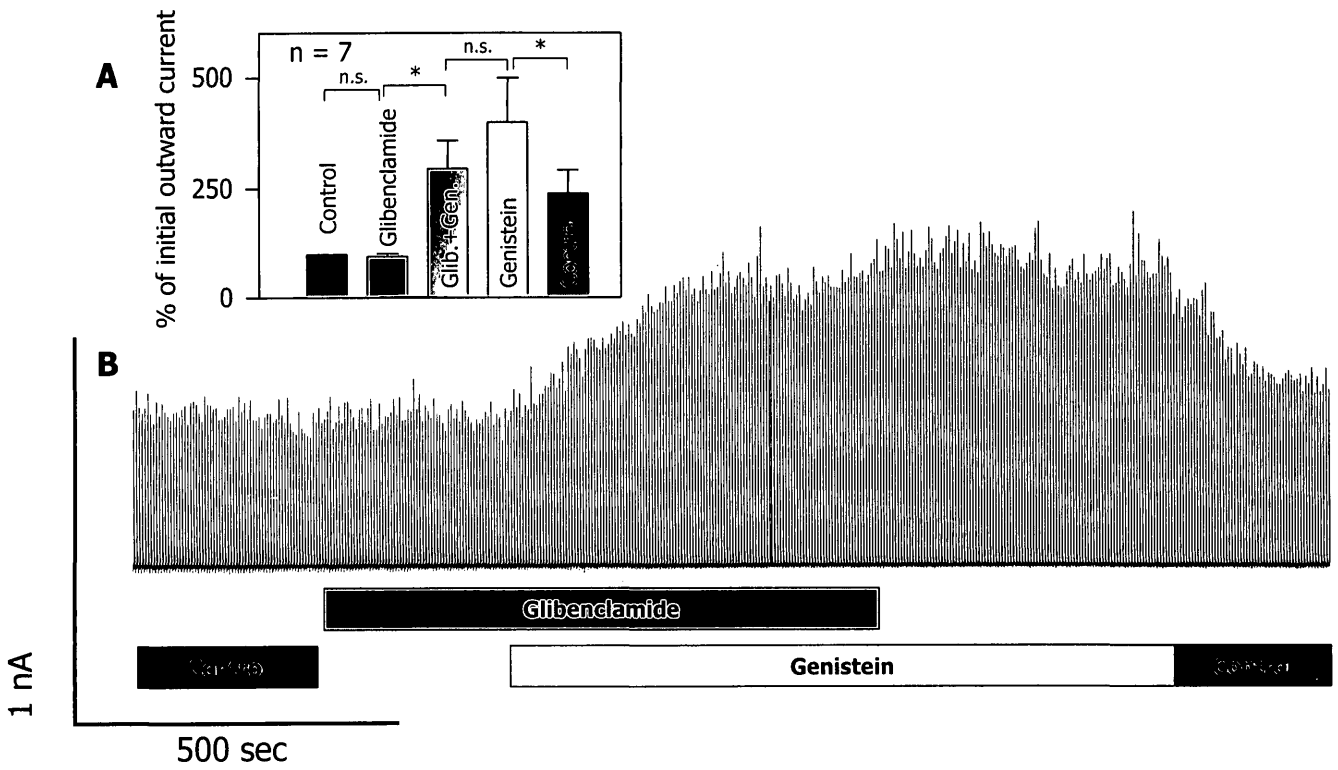


FIGURE 7. Glibenclamide (10^{-5} M), a blocker of ATP-dependent potassium channels, had no significant effect on the induction of current by genistein (5×10^{-5} M). (A) Summary of seven experiments. Initial outward current at 80 mV was taken as 100%. * $P < 0.05$. (B) Individual experiment.

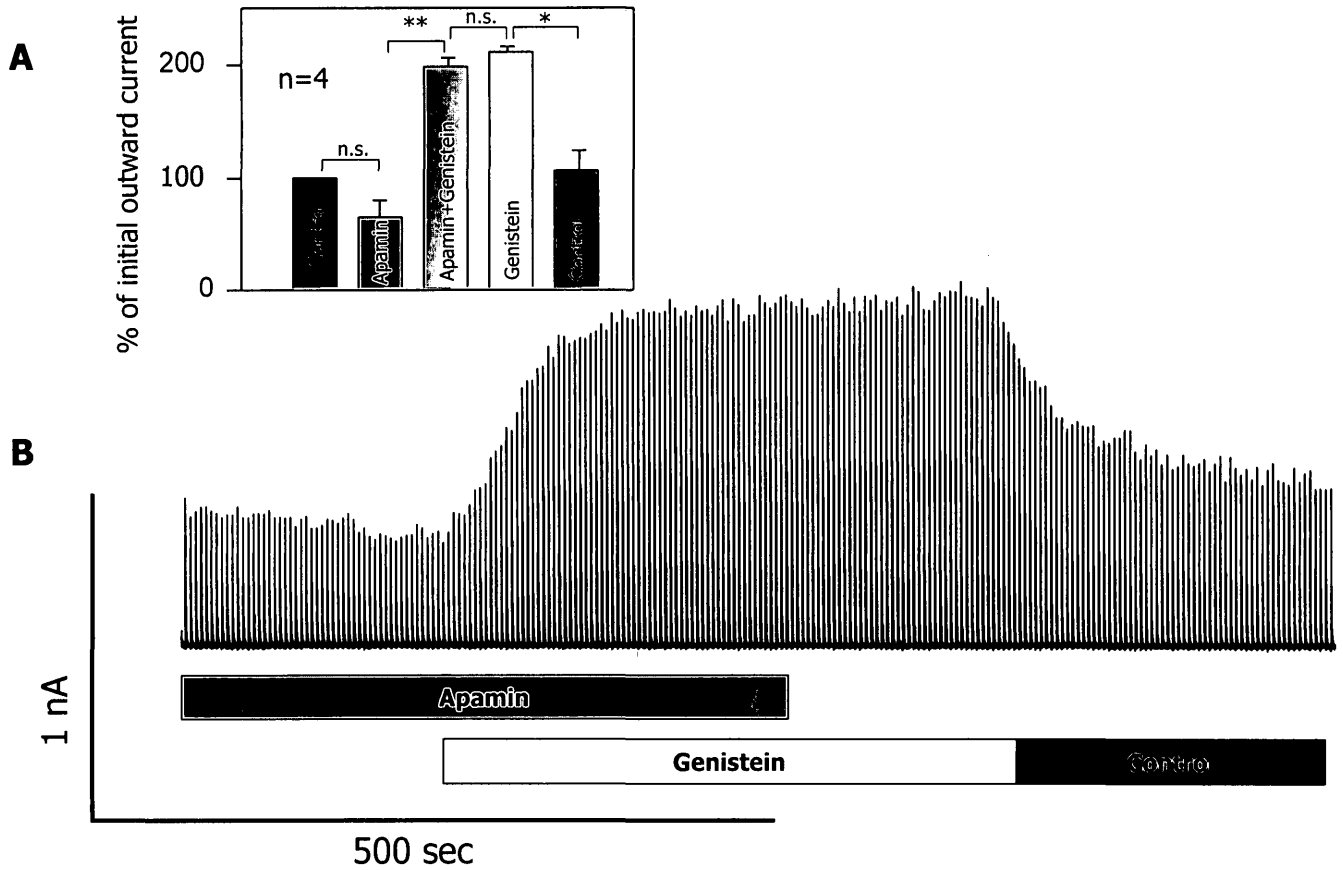


FIGURE 8. Apamin (10^{-6} M), which blocks low-conductance calcium-activated potassium channels, had no effect on the induction of outward current by genistein (5×10^{-5} M). (A) Summary of four experiments. Initial outward current at 80 mV was taken as 100%. ** $P < 0.005$. (B) Individual experiment.

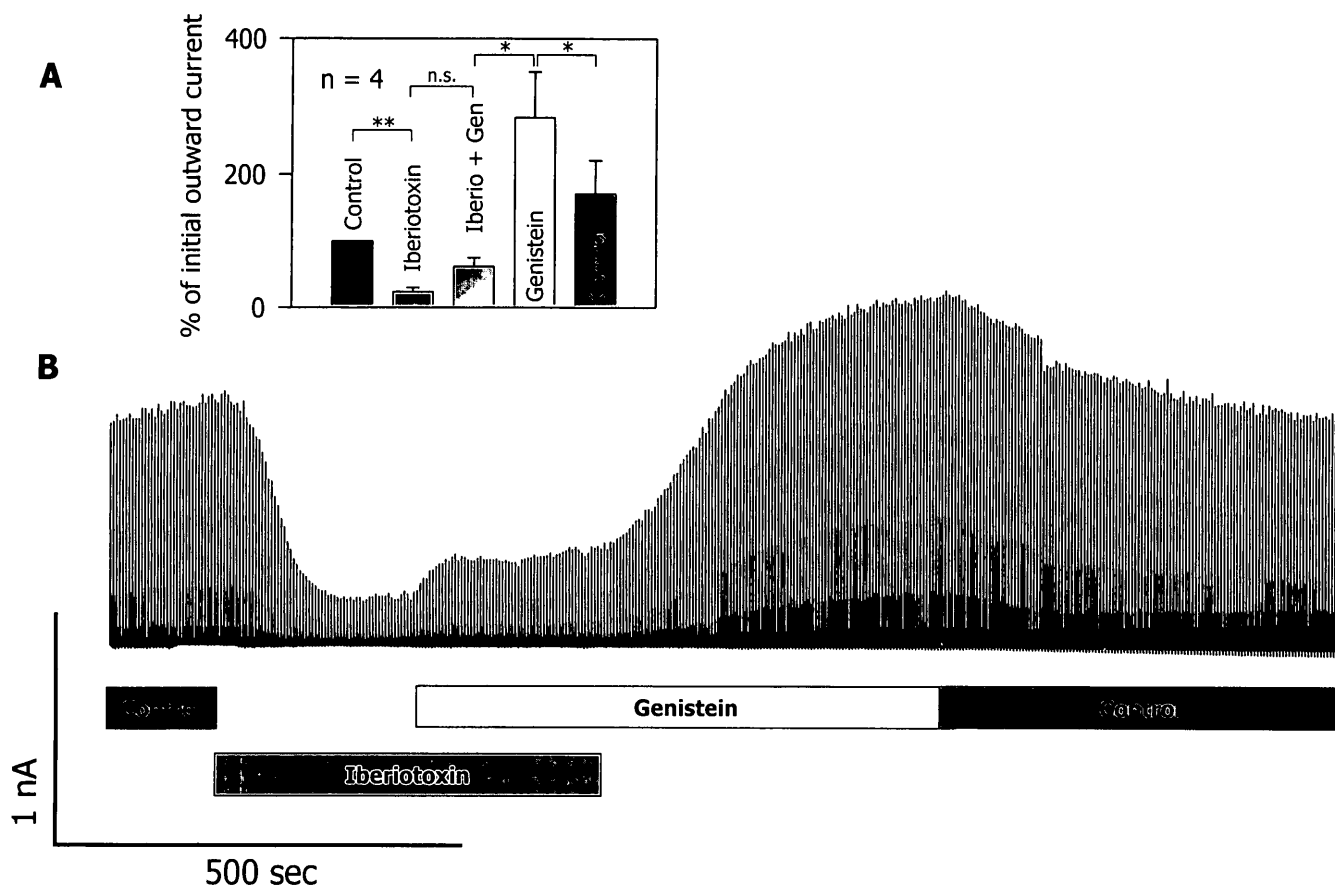


FIGURE 9. Iberiotoxin (10^{-7} M), a specific blocker of high-conductance calcium-activated potassium channels (maxi-K), blocked the major part of the genistein (5×10^{-5} M)-induced current. (A) Summary of four experiments. Initial outward current at 80 mV was taken as 100%. * $P < 0.05$. (B) Individual experiment.

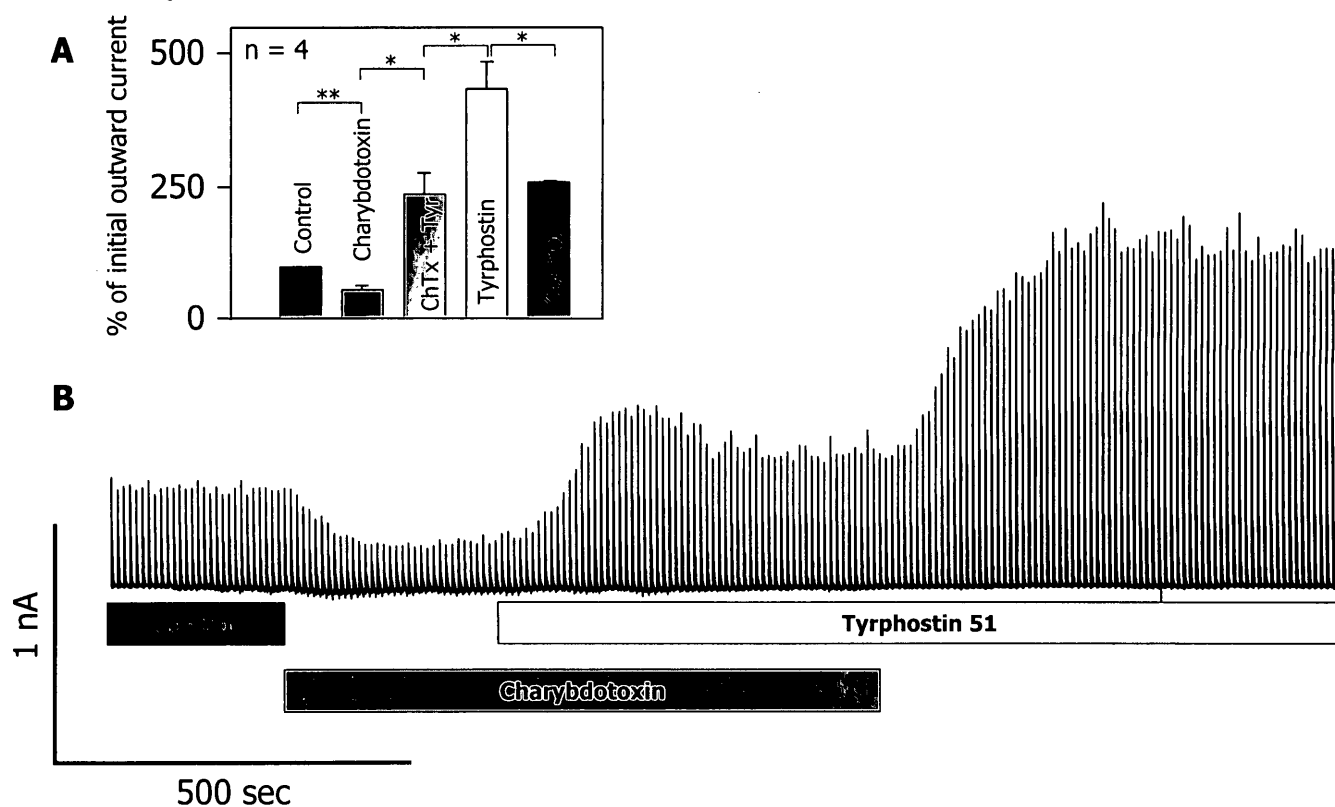


FIGURE 10. Charybdotoxin (10^{-7} M), another blocker of maxi-K channels, blocked the major part of the current induced by tyrphostin 51 (5×10^{-5} M). (A) Summary of four experiments. Initial outward current at 80 mV was taken as 100%. * $P < 0.05$. (B) Individual experiment.

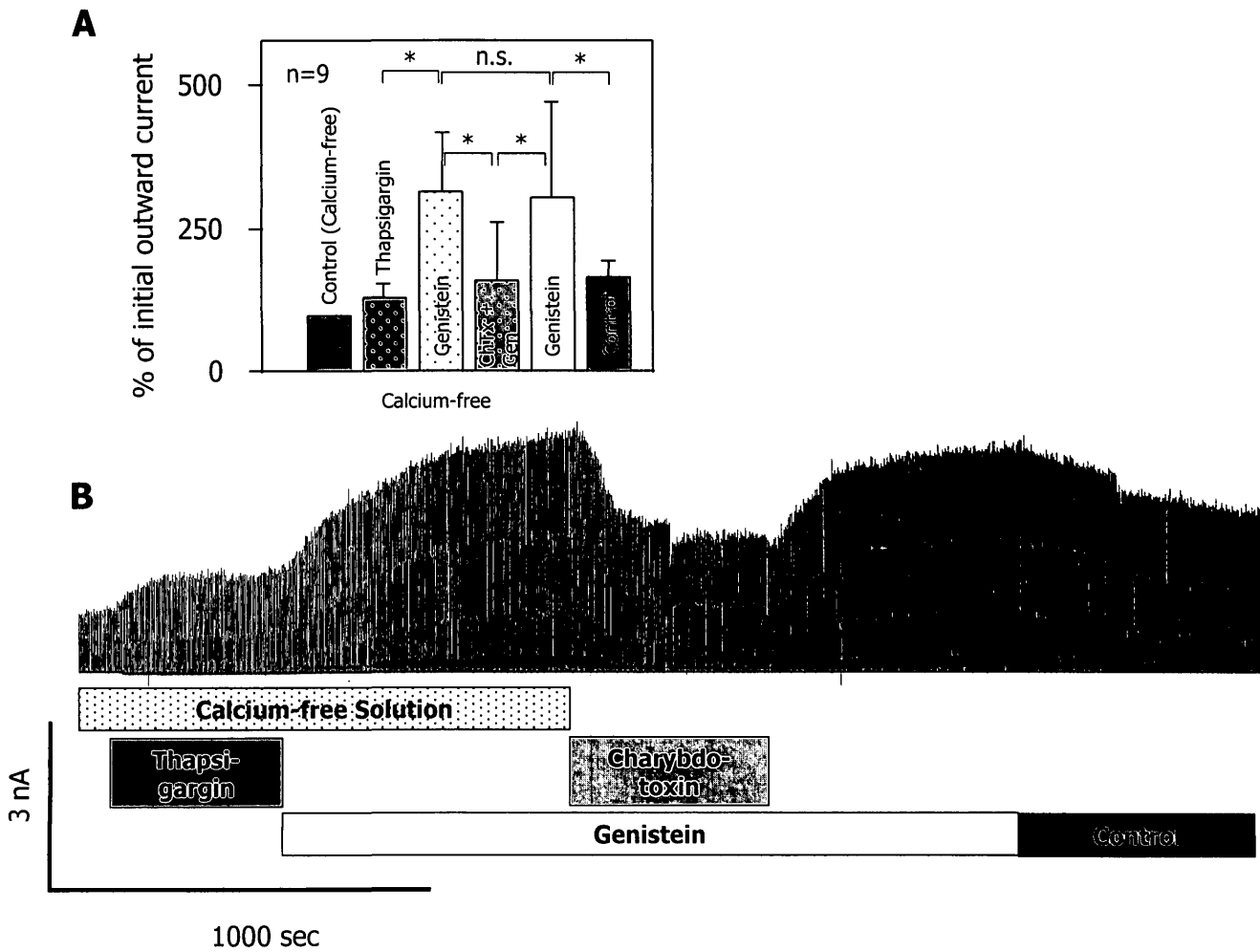


FIGURE 11. The effect of genistein on outward current level in cells depleted of calcium by thapsigargin (10^{-6} M) in a calcium-free solution buffered with 10 mM EGTA. (A) Summary of four experiments. Initial outward current at 80 mV was taken as 100%. Nonsignificant: $P > 0.1$. (B) Individual experiment.

Tyrosine kinase inhibitors are a group of substances whose therapeutic potential for a number of diseases is currently emerging.^{1,6} A vast number of compounds exist with variable specificity for various cellular tyrosine kinases. We were able to show a relaxation of trabecular meshwork in response to genistein and tyrphostin 51.⁸ Although genistein reportedly blocks a number of tyrosine kinases and is thus an excellent scanning tool in searching for a tyrosine kinase-dependent mechanism, tyrphostin 51 shows an affinity for the EGF receptor tyrosine kinase.^{8,47} Because daidzein, a nonactive structural analogue, showed no effect, it is safe to assume that the stimulation of the channel by genistein and tyrphostin is caused by a specific inhibition of tyrosine phosphorylation, although some caution should be used in interpreting the exact nature of the tyrosine kinase involved. More research is needed before it is safe to say that the EGF receptor affects the maxi-K channel, particularly because in other preparations of smooth muscle, the membrane-bound tyrosine kinase pp60^{c-src} seems to be involved in mediating contraction.¹³

Recent reports link tyrosine kinases to the relaxation and contraction of visceral and vascular smooth muscle.^{13,48} Receptor-mediated contraction has been shown to involve the phosphorylation of various proteins on tyrosine residues. These include *rasGAP* and *PLCγ*^{13,49}; many others have yet to

be identified. One of the effects of receptor-activated tyrosine phosphorylation in smooth muscle is an increase both in calcium influx from the outside and in calcium release from the stores.¹³

Although most research has centered on the alteration of cytosolic proteins by tyrosine kinases, the regulation of membrane proteins by protein tyrosine kinases has also been studied to some extent. Using preparations from various tissues, the increase in the open probability of L-type calcium channels by tyrosine phosphorylation has been reported by a number of investigators.^{14,30,32,50} In contrast, there is only scant information on the regulation of other ionic channels by tyrosine phosphorylation. Thus, Chiang et al.⁵⁰ report the inhibition of cAMP-dependent chloride channels, and Minami et al.⁵¹ report activation of nonselective cation channels by genistein. The reports on potassium channels differ. Reports on vascular smooth muscle include stimulation^{9,52} and suppression³¹ of potassium channels by tyrosine phosphorylation. The same confusing situation can be found with cardiomyocytes, in which both stimulation⁵³ and suppression^{54,55} are also reported. Experimental protocols used in these studies varied, and more studies are needed before a serious attempt can be made to compare and interpret the data.

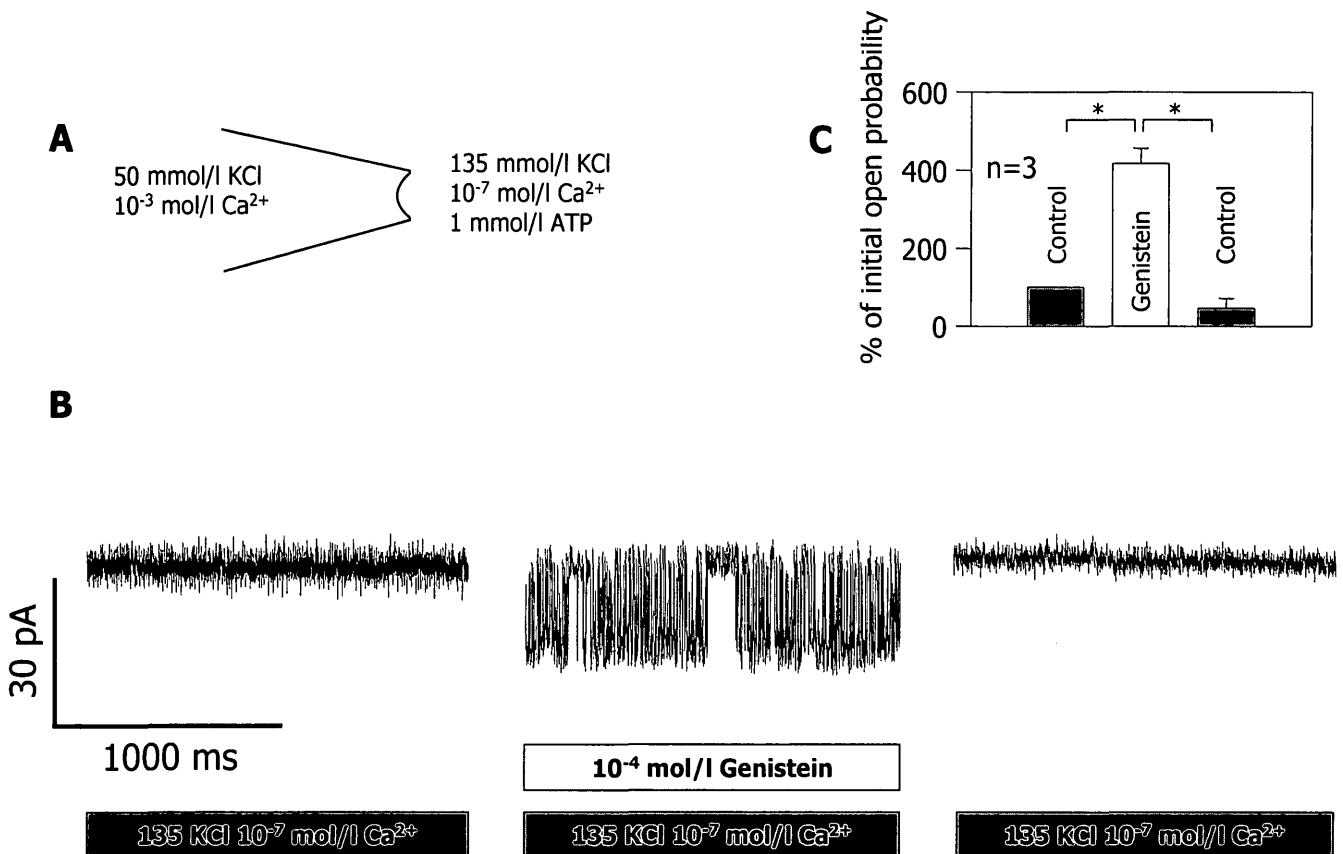


FIGURE 12. The effect of genistein on the open probability of maxi-K channels in the inside-out configuration. (A) Schematic representation of the inside-out patch-clamp configuration. (B) Traces from one patch showing stimulation of open probability by genistein. (C) Summary of the results of three experiments. Similar to preparations with an intact cytosolic compartment, genistein stimulated maxi-K channels. * $P < 0.05$.

To our knowledge, only two studies exist concerning the tyrosine kinase sensitivity of maxi-K channels. In Chinese hamster ovary cells, Prevarskaya et al.¹⁵ report an inhibition of the channel by tyrosine kinase inhibitors, whereas Xiong et al.³¹ report the opposite effect using a preparation of rat aortic cells.

In a previous study, we were able to show the presence of maxi-K channels in bovine trabecular meshwork.²¹ The important role of maxi-K channels (high-conductance calcium-activated potassium channels, BK_{Ca}) in smooth muscle cells is undisputed.^{56,57} An important function is to hyperpolarize cells after cytosolic calcium increases, thus serving as a mechanism for negative feedback. One of the paradoxes is how high calcium levels have to be before significant stimulation of maxi-K channels can be observed. Although carbachol (10⁻⁴ M) and endothelin (10⁻⁸ M) are reported to increase cytosolic calcium to levels of 300 nM to 500 nM in trabecular meshwork,^{18,40,41} cytosolic calcium has to exceed 10⁻⁵ M before an appreciable enhancement of open probability in the inside-out configuration can be observed.²¹ Under physiological conditions, in the presence of cytosolic components such as specific enzymes or ATP, this level may well be lower. In addition, submembranous compartments have been shown to have calcium levels sufficient to stimulate associated maxi-K channels.⁵⁶ Nevertheless, the disparity between calcium levels physiologically observed in the main compartment of cells and the levels needed to stimulate maxi-K channels is disturbing.

In this article, we present a new model for the regulation of maxi-K channels in trabecular meshwork (Fig. 13) that

involves tyrosine phosphorylation, unrelated to the stimulation by elevating cytosolic calcium. The action of genistein on channels in the inside-out configuration speaks for a relatively direct signaling cascade involving membrane-bound constituents. Possibly, the open probability of maxi-K channels is modulated by direct phosphorylation of the channel protein on a tyrosine residue by a tyrosine kinase. Membrane-bound tyrosine kinases have been found in the membranes of various cellular preparations, including membrane receptor tyrosine kinases (such as the EGF receptor) and membrane-associated nonreceptor tyrosine kinases related to pp60^{c-src}.¹³ Holmes et al.⁵⁸ report an association of a tyrosine kinase with a potassium channel. Interestingly, voltage-dependent potassium channels from the Shaker family are reported to have a tyrosine kinase site on the inactivating N-terminal.⁵⁹ Possibly, maxi-K channels, which have the same basic structure with six membrane-spanning domains and the same voltage sensor,⁶⁰ share this feature. Although extensive literature exists on the regulation of maxi-K channels by calcium or voltage,^{21,56,60} we believe that the regulation by tyrosine phosphorylation may be an important additional feature that explains the physiological role of these channels.

The way in which potassium channels affect smooth muscle tone has been amply described.^{61,62} In smooth muscle cells,^{62,63} but also in many nonmuscle cells,⁶⁴ calcium is known to activate contractile proteins through a cascade involving the binding of calcium to calmodulin which in turn activates myosin light-chain kinase. Myosin light-chain kinase,

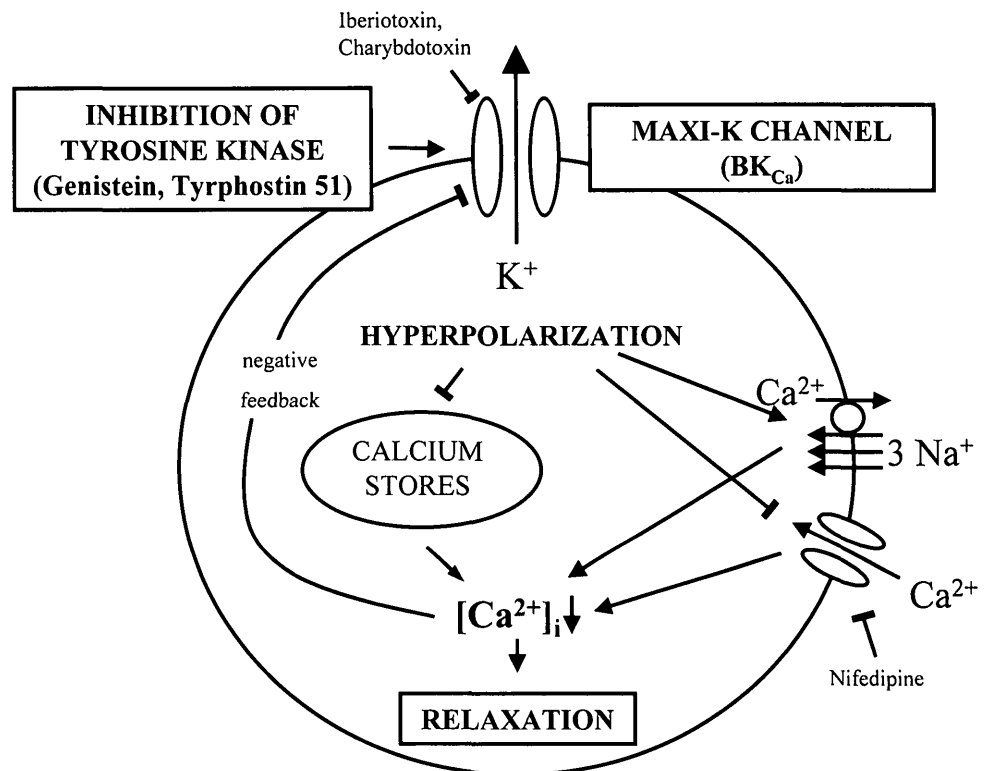


FIGURE 13. Schematic model of the function of maxi-K channels in mediating relaxation of trabecular meshwork cells by tyrosine kinase inhibitors. Inhibition of tyrosine kinase leads to an increase in the open probability of maxi-K channels. This leads to hyperpolarization, which in turn closes L-type channels and decreases the release of calcium from cytosolic stores. Both effects lower cytosolic calcium leading to relaxation. Lower levels of cytosolic calcium also tend to shut down the maxi-K channel, constituting a negative-feedback loop.

in turn, phosphorylates myosin, enabling it to attach to actin, generating force. Conversely, a decline in cytosolic calcium leads to relaxation. Considering that trabecular meshwork cells contain smooth muscle α -actin and smooth muscle myosin,^{43,44,65,66} it is not unreasonable to assume that trabecular meshwork contractility is also regulated in this way. In previous studies, application of muscarinic agonists to trabecular meshwork cells led to depolarization³⁷ and to an increase in cytosolic calcium.⁴¹ Possibly, this induces a latching of actin and myosin leading to contraction, as observed in contractility experiments.²⁷ In contractility experiments, application of tyrosine kinase inhibitors to trabecular meshwork strips precontracted by carbachol led to relaxation.⁸ The data presented here suggest that this relaxation is caused by an opening of maxi-K channels with subsequent efflux of potassium, leading to hyperpolarization (Fig. 13). This, in turn, leads to a closure of L-type calcium channels¹⁸ and to a decrease in calcium efflux from cytosolic calcium stores through ryanodine-sensitive channels^{56,67,68} and a stimulation of calcium efflux through the sodium-calcium exchanger.⁴⁹ All these mechanisms should lead to a decline in cytosolic calcium and to relaxation through uncoupling of actin and myosin.

In addition to the pathway outlined above, many smooth muscle cells also exhibit a calcium-independent mechanism of contraction.^{69,70} Data from previous studies^{46,71} suggest that trabecular meshwork, too, possesses one or more pathways of this type.

For better comparability with the contraction experiments,⁸ all data presented here were recorded using bovine trabecular meshwork cells. Preliminary results from a study we are currently engaged in show that human trabecular meshwork cells react in the same way. Future work should show the link of maxi-K channel activation to a decrease in cytosolic calcium by directly measuring intracellular calcium.

In summary, we present evidence for the relaxation of trabecular meshwork by tyrosine kinase-dependent activation of maxi-K channels. Tyrosine kinase inhibitors, with their potential for relaxing both vascular smooth muscle^{10,13} and trabecular meshwork⁸ seem to be promising new candidates in the search for a more effective therapy for glaucoma.

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