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RESEARCH PAPER

Regulation of two-pore-domain (K_{2P}) potassium leak channels by the tyrosine kinase inhibitor genistein

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Background and purpose: Two-pore-domain potassium (K_{2P}) channels mediate potassium background (or 'leak') currents, controlling excitability by stabilizing membrane potential below firing threshold and expediting repolarization. Inhibition of K_{2P} currents permits membrane potential depolarization and excitation. As expected for key regulators of excitability, leak channels are under tight control from a plethora of stimuli. Recently, signalling via protein tyrosine kinases (TKs) has been implicated in ion channel modulation. The objective of this study was to investigate TK regulation of K_{2P} channels.
Experimental approach: The two-electrode voltage clamp technique was used to record K_{2P} currents in *Xenopus* oocytes. In addition, K_{2P} channels were studied in Chinese hamster ovary (CHO) cells using the whole-cell patch clamp technique.
Key results: Here, we report inhibition of human $K_{2P}3.1$ (TASK-1) currents by the TK antagonist, genistein, in *Xenopus* oocytes ($IC_{50} = 10.7\ \mu M$) and in CHO cells ($IC_{50} = 12.3\ \mu M$). The underlying molecular mechanism was studied in detail. $hK_{2P}3.1$ was not affected by genistin, an inactive analogue of genistein. Perorthovanadate, an inhibitor of tyrosine phosphatase activity, reduced the inhibitory effect of genistein. Current reduction was voltage independent and did not require channel protonation at position H98 or phosphorylation at the single TK phosphorylation site, Y323. Among functional hK_{2P} family members, genistein also reduced $K_{2P}6.1$ (TWIK-2), $K_{2P}9.1$ (TASK-3) and $K_{2P}13.1$ (THIK-1) currents, respectively.
Conclusions and implications: Modulation of K_{2P} channels by the TK inhibitor, genistein, represents a novel molecular mechanism to alter background K^+ currents.
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Keywords: background potassium current; cardiac arrhythmia; cardioprotection; cellular excitability; ischaemic preconditioning; K_{2P} channel; leak channel; membrane potential; tyrosine kinase; protein phosphorylation

Abbreviations: K_{2P}, two-pore-domain K^+ channel; TASK, TWIK-related acid sensitive K^+ channel; THIK, tandem pore domain halothane inhibited K^+ channel; TK, tyrosine kinase; TWIK, tandem of P domains in a weak inward rectifying K^+ channel

Introduction

Two-pore-domain potassium (K_{2P}) channels are recognized to be highly regulated leak pathways that control excitability, stabilizing membrane potential below firing threshold and expediting repolarization (Goldstein *et al.*, 2001, 2005). K_{2P} channels are identified by a unique structure of two pore-forming loop domains in each subunit. The channels assemble from two subunits to form a single ion conduction pathway. Expressed robustly throughout the cardiovascular, gastrointestinal, genitourinary and CNS, K_{2P} channels are implicated in multiple physiological processes, including neuromodulation, neuro- and cardioprotection, regulation of cardiac rhythm, anaesthesia, apoptosis, and sensation of oxygen tension, mechanical stress, taste and temperature (Patel *et al.*, 1998; Lauritzen *et al.*, 2003; Heurteaux *et al.*, 2004; Kemp *et al.*, 2004; Richter *et al.*, 2004; Chemin *et al.*, 2005; Kang *et al.*, 2005; Lalevee *et al.*, 2006; Putzke *et al.*, 2007). Because membrane potential is fundamental to neuronal and cardiac activity, leak current regulation is a primary and dynamic mechanism for control of cellular excitability (Goldstein *et al.*, 2001; Patel *et al.*, 2001; Bayliss *et al.*, 2003).

Unravelling signal-transduction mechanisms that control excitability is critical to our understanding of cardiac and neuronal electrophysiology. Signalling via tyrosine kinases

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(TKs) mediates hormone- and receptor-dependent signal transduction, regulation of cell growth, differentiation, metabolism and function. Specific cardiac functions associated with TK activity include ischaemic preconditioning (Fryer *et al*., 1998; Benter *et al*., 2005) and signal transduction in angiotensin II-associated cardiac hypertrophy (Haendeler and Berk, 2000). In the brain, TKs are involved in long-term potentiation in the hippocampus (O'Dell *et al*., 1991). At the molecular level, TKs regulate the activity of several ion channels, including a diverse group of voltage-gated K^+ channels (Hool *et al*., 1998; Missan *et al*., 2006). Although previous studies have established that K_{2P} channels are differentially regulated by protein kinases A and C (reviewed in Goldstein *et al*., 2001; Bayliss *et al*., 2003; Mathie, 2007), there is no information on TK-related modification of K_{2P} leak currents.

Here, K_{2P} family members 3.1, 6.1, 9.1 and 13.1 (TASK-1, TWIK-related acid-sensitive K^+ channel 1; TWIK-2, tandem of P domains in a weak inward rectifying K^+ channel 2; TASK-3; and THIK-1, tandem pore domain halothane-inhibited K^+ channel 1, respectively) are revealed to be inhibited by the TK inhibitor, genistein. The *International Union of Pharmacology* classification has accorded each K_{2P} channel gene with an ion channel subunit product (Goldstein *et al*., 2005); these identifiers are used and presented with common acronyms in this study. Originally isolated from the fermentation broth of *Pseudomonas* sp., the isoflavone compound genistein inhibits protein TKs by competing for the ATP-binding site with an IC_{50} of 20.4–111 μM while exhibiting little or no effects on serine/threonine kinases (Akiyama *et al*., 1987; Akiyama and Ogawara, 1991). Recent experimental and clinical data suggest that the phytooestrogen genistein is associated with reduced incidence of cardiovascular disease and breast, uterine and prostate cancer (Dixon and Ferreira, 2002; Park *et al*., 2005). Furthermore, genistein inhibits metastasis of prostate cancer in mice and enhances the efficacy of cancer therapeutics through modification of cell proliferation and survival pathways (Gescher *et al*., 2001; Sarker and Li, 2006; Lakshman *et al*., 2008).

Molecular determinants of genistein-dependent regulation of the most sensitive K_{2P} channel, K_{2P}3.1 (TASK-1), were studied in detail. Inhibitory effects on K_{2P}3.1 were abolished or reduced when genistin and daidzein, inactive or less potent analogues of genistein, were applied. The phosphotyrosine phosphatase inhibitor, perorthovanadate (PVN), attenuated the effect of TK inhibition on K_{2P}3.1. Genistein-associated blockade occurred independently of channel phosphorylation at the single TK phosphorylation site, Y323, suggesting that TK activity does not directly affect K_{2P}3.1 channel function. Modulation of K_{2P} channels by genistein is revealed to be a novel mechanism to alter background K^+ channel function.

Methods

Molecular biology

Drug target nomenclature conforms with British Journal of Pharmacology's Guide to Receptors and Channels (Alexander *et al*., 2007). Human K_{2P}4.1 (B), K_{2P}5.1 (B), K_{2P}6.1 (B), K_{2P}10.1 (B), K_{2P}13.1 (B), K_{2P}16.1 (P) and K_{2P}17.1 (B) were amplified from brain (B) or pancreas (P) cDNA libraries, inserted into pCR2.1-TOPO and subcloned into pRAT, a dual-purpose expression vector, and containing a CMV promoter for mammalian expression and a T7 promoter for cRNA synthesis.

Mutations described in this study were made with a QuikChange Site-Directed Mutagenesis kit and synthetic mutant oligonucleotide primers. All cDNA constructs were confirmed by DNA sequencing. Procedures for *in vitro* transcription and oocyte injection were performed as published previously (Kiehn *et al*., 1999). Briefly, cRNAs were transcribed after vector linearization using T7 RNA polymerase and the mMessage mMachine kit. Transcripts were quantified using a spectrophotometer and by comparison with control samples separated by agarose gel electrophoresis. Stages V and VI defolliculated *Xenopus* oocytes were injected with 2–46 ng cRNA encoding study channels.

Tissue culture

Chinese hamster ovary (CHO) cells were cultured in minimum essential medium α (MEM α) supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin G sodium, and 100 μg mL^{-1} streptomycin sulphate in an atmosphere of 95% humidified air and 5% CO_2 at 37 °C. Cells were passaged regularly and subcultured prior to treatment. Transient transfections of CHO cells were performed using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions.

Electrophysiology

Two-electrode voltage clamp measurements were performed as described earlier (Thomas *et al*., 1999). This study has been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. Ovarian lobes were surgically removed in aseptic technique from female *Xenopus laevis* frogs anaesthetized with 1 g L^{-1} tricaine solution (pH = 7.5). Frogs were not fed on the day of surgery to avoid emesis during anaesthesia. After surgery, the frogs were allowed to recover consciousness, followed by at least 2 months of recovery period. Oocyte collection was alternated between left and right ovaries, and no more than three surgeries were performed on one individual frog. After the final taking of oocytes, the anaesthetized frog was killed by decerebration and pithing. Following collagenase treatment, stages V and VI defolliculated oocytes were manually isolated under a stereomicroscope. Whole-cell currents were measured 1–3 days after injection with an Oocyte Clamp amplifier (Warner Instruments, Hamden, CT, USA) using pCLAMP (Axon Instruments, Foster City, CA, USA) and Origin 6 (OriginLab, Northampton, MA, USA) software for data acquisition and analysis. Data were sampled at 2 kHz and filtered at 1 kHz. Current recordings from CHO cells were performed using the whole-cell patch clamp configuration as previously reported (Thomas *et al*., 2001). All experiments were carried out at room temperature

(20–22 °C), and no leak subtraction was done during the experiments.

Solutions and drug administration

Two-electrode voltage clamp electrodes were filled with 3 M KCl and had tip resistances of 1–5 MΩ. Recordings were performed under constant perfusion at room temperature. The standard physiological extracellular solution contained 96 mM NaCl, 4 mM KCl, 1.1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid) (pH was adjusted to 7.4 with NaOH). Human K_{2P}16.1 and K_{2P}17.1 were activated by adjusting extracellular pH to 8.5. Currents were evoked in oocytes by step depolarization from −140 to +60 mV (500 ms) in 20-mV increments at 2-s intervals (5-s intervals for hK_{2P}18.1) from the holding potential (−80 mV).

For whole-cell patch clamp recordings from CHO cells, electrodes were filled with the following solution (in mM): 100 K-aspartate, 20 KCl, 2.0 $MgCl_2$, 1.0 $CaCl_2$, 10 EGTA, 2 ATP, 10 HEPES (pH adjusted to 7.2 with KOH). The external solution for these experiments contained (in mM): 140 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, 10 glucose (pH adjusted to 7.4 with NaOH). Families of K_{2P}3.1 currents were recorded during step depolarization from −120 to +80 mV (500 ms) in 20-mV increments at 5-s intervals. The holding potential was −80 mV. To quantify the inhibitory effects of genistein, currents were activated by a 500 ms test pulse to +60 mV (holding potential −80 mV). Pulses were applied in 10-s intervals during superfusion with drug solution. Current amplitudes were recorded at the end of the depolarizing test pulse once steady state had been reached (usually within 3–4 min).

Genistein, genistin and daidzein were prepared as 100 mM stock solutions in dimethylsulphoxide and stored at −20 °C. The sodium orthovanadate stock solution was prepared by adding H_2O_2 (30% wt wt^{-1} solution, 10 mM final concentration) to an aqueous solution containing 10 mM Na_3VO_4 and 50 mM HEPES (pH = 7.4). This solution was mixed and allowed to stand at room temperature for 20 min. Excess H_2O_2 was eliminated by adding 200 μg mL^{-1} catalase (5 min incubation). The stock solution containing a mixture of vanadate and peroxovanadium complexes was made immediately before use. The orthovanadate concentration used in our experiments is based on the Na_3PO_4 concentration used in preparing the stock solution.

On the day of experiments, aliquots of the stock solutions were diluted to the desired concentrations with the bath solution. Human K_{2P}3.1 currents recorded from oocytes were not significantly altered upon application of 0.6% dimethylsulphoxide (v v^{-1}; maximum bath concentration) for 6 min ($n = 7$; data not shown).

Materials

Human cDNA clones encoding K_{2P}1.1-K274Q. (Rajan *et al*., 2005), K_{2P}2.1 (EF165334), K_{2P}3.1 (NM_002246), K_{2P}3.1-H98N (Lopes *et al*., 2001) and K_{2P}9.1 (NM_016601) were provided by Dr Steve Goldstein (Chicago, IL, USA). cDNA encoding human K_{2P}18.1 (NM_181840) was generously donated by Dr C Spencer Yost (San Francisco, CA, USA). Brain (B) and pancreas (P) cDNA libraries were obtained from Clontech (Palo Alto, CA, USA), pCR2.1-TOPO from Invitrogen (Carlsbad, CA, USA) and pRAT was kindly provided by Dr Steve Goldstein (Chicago). The QuikChange Site-Directed Mutagenesis kit was from Stratagene (La Jolla, CA, USA) and the mMessage mMachine kit from Ambion (Austin, TX, USA). MEM α, fetal bovine serum and Lipofectamine 2000 transfection reagent were from Invitrogen (Karlsruhe, Germany). Genistein, genistin daidzein and Na_3VO_4 were all from Sigma.

Data analysis and statistics

Concentration–response relationships for drug-induced block were fit with a Hill equation of the following form: $I_{drug}/I_{control} = 1/[1 + (D/IC_{50})^n]$, where I indicates current, D is the drug concentration, n is the Hill coefficient and IC_{50} is the concentration necessary for 50% block. Data are expressed as mean ± s.e.mean. We used Student's t-test (two-tailed tests) to compare the statistical significance of the results: $P < 0.05$ was considered statistically significant. Multiple comparisons were performed using one-way ANOVA. If the hypothesis of equal means could be rejected at the 0.05 level, pairwise comparisons of groups were made and the probability values were adjusted for multiple comparisons using the Bonferroni correction.

Results

Genistein reduces K_{2P}3.1 (TASK-1) background currents

The effects of TK inhibition on human K_{2P}3.1 (TASK-1) leak channels were studied in *X. laevis* oocytes. Genistein, a broad range TK inhibitor, reduced hK_{2P}3.1 potassium currents in a concentration-dependent manner, as displayed in Figure 1. Currents were elicited by a 500 ms depolarizing step to +20 mV from a holding potential of −80 mV. The degree of block was determined after 6 min (Figure 1a). To study the concentration dependence of hK_{2P}3.1 inhibition by genistein, currents in the presence of the drug were normalized to their respective control values and plotted as relative current amplitudes in Figure 1b ($n = 4$–13 cells were investigated at each concentration). The half-maximal inhibitory concentration (IC_{50}) for block of hK_{2P}3.1 leak channels yielded 10.7 ± 0.8 μM with a Hill coefficient n_H of 1.5 ± 0.3. The time course is shown in Figure 1c ($n = 4$). After a control period of 6 min with no significant changes in current amplitude, hK_{2P}3.1 current reduction by 100 μM genistein reached steady-state conditions after 6 min. Upon washout (20 min), inhibitory effects of genistein on hK_{2P}3.1 were partially reversible.

Human K_{2P}3.1-related K_{2P} family members K_{2P}6.1, K_{2P}9.1 and K_{2P}13.1 are sensitive to genistein

Over the past 11 years, 15 different human K_{2P} channels have been cloned. Some genes failed to produce currents (K_{2P}7.1, K_{2P}12.1 and K_{2P}15.1), leaving 12 functional leak channels accessible to electrophysiological investigation. To

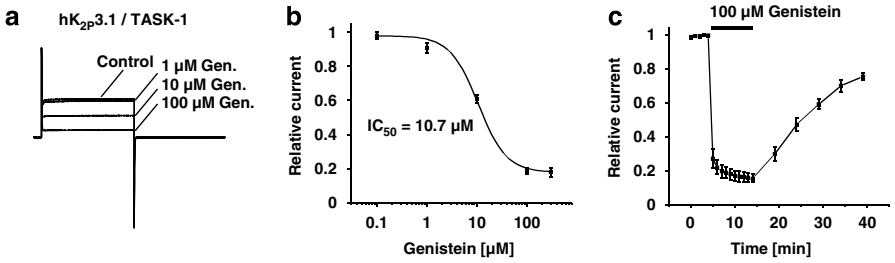

Figure 1 Inhibition of human $K_{2P}3.1$ (TASK-1) channels expressed in *Xenopus* oocytes by genistein. Representative current traces recorded from the same cell under control conditions and after superfusion with genistein (1, 10 and 100 µM, respectively) are displayed in (**a**). (**b**) Concentration–response relationships for the effect of genistein on $hK_{2P}3.1$ outward currents measured at +20 mV ($n = 4$–13 cells; mean ± s.e.mean). The IC_{50} yielded 10.7 µM. (**c**) Time course of $hK_{2P}3.1$ current inhibition by 100 µM genistein ($n = 4$).

assess specificity of genistein-induced K_{2P} current inhibition, all functional human K_{2P} channels cloned to date were studied (Figure 2). From a holding potential of −80 mV, depolarizing pulses were applied for 500 ms to voltages between −140 and +60 mV in 20-mV increments (0.5 Hz). This protocol was used in all experiments performed in this study, unless indicated otherwise. $K_{2P}1.1$ (TWIK-1) channels, previously shown to be non-functional, were recently revealed to produce leak currents when a lysine residue is removed by mutation to glutamine ($K_{2P}1.1$-K274Q; Rajan *et al*., 2005). This effect has been suggested to be caused by lack of covalently bound small ubiquitin-like modifier protein, SUMO. However, this hypothesis is currently being discussed controversially (Feliciangeli *et al*., 2007). Here, we expressed $hK_{2P}1.1$-K274Q cRNA to achieve sufficient $hK_{2P}1.1$ current levels in *Xenopus* oocytes. Both $hK_{2P}1.1$-K274Q and $hK_{2P}6.1$ displayed very low current levels under control conditions (0.36 ± 0.02 µA ($n = 15$) and 0.31 ± 0.02 µA ($n = 5$), respectively). However, these current amplitudes were significantly larger than mean current levels in uninjected control oocytes (0.19 ± 0.01 µA; $n = 12$), indicating successful channel expression. The effects of 100 µM genistein on K_{2P} family members are summarized in Figures 2 and 3a. The most pronounced effect was observed with $hK_{2P}3.1$. In addition, genistein significantly reduced $hK_{2P}6.1$, $hK_{2P}9.1$ and $hK_{2P}13.1$ currents, respectively (Figures 2 and 3a). A phylogenetic tree illustrates that leak channels affected by TK regulation are related (Figure 3b).

$hK_{2P}3.1$ current reduction by TK inhibition is voltage independent

Molecular mechanisms of TK-dependent regulation of the most sensitive K_{2P} channel, $hK_{2P}3.1$, were studied in detail. The effect of genistein on $hK_{2P}3.1$ current–voltage (I–V) relationship was investigated under isochronal recording conditions using the protocol described in Figure 2. Families of current traces from one cell are shown for control conditions and after exposure to 100 µM genistein (6 min) in Figure 4a, revealing electrophysiological characteristics typical for a potassium-selective background leak conductance, that is, a voltage-independent portal showing Goldman–Hodgkin–Katz, or open, rectification (Figure 4b and c; Goldstein *et al*., 2001). Potassium channels that display open rectification pass current more readily in one direction (rectify) owing to unequal ion concentration across the membrane. Relative inhibition of $hK_{2P}3.1$ currents (plotted as a function of the test pulse potential in Figure 4d) was not significantly different among the voltage steps applied ($n = 13$ cells were studied at each potential), indicating that genistein application reduced leak currents in a voltage-independent manner.

To differentiate between TK-dependent actions of genistein and direct inhibition of $hK_{2P}3.1$ channels, structurally similar analogues were applied under similar experimental conditions (Figure 4e). Currents were recorded at the end of depolarizing voltage steps to +20 mV under control conditions and after drug application for 6 min. The inactive analogue, genistin (10 and 100 µM), did not cause significant current reduction ($n = 8$ and 6 cells, respectively), whereas genistein application reduced mean currents by 38.9 ± 1.8% ($n = 22$) and 81.1 ± 1.7% ($n = 12$) under similar conditions (10 and 100 µM, respectively). Daidzein is a different structural analogue of genistein that is commonly used as negative control similar to genistin. However, in contrast to genistin, daidzein inhibits TKs at higher drug concentrations. Accordingly, 10 µM daidzein did not induce significant $hK_{2P}3.1$ current reduction ($n = 7$), as expected, whereas 100 µM daidzein reduced outward current amplitudes by 18.2 ± 1.3% ($n = 9$).

TK-mediated $hK_{2P}3.1$ current block does not depend on channel protonation

$K_{2P}3.1$ channels are sensitive to extracellular pH variations in the physiological range, displaying a pKa value of 7.2 (Lopes *et al*., 2000, 2001). A single histidine residue adjacent to the potassium selectivity filter within the first pore-forming loop, H98, represents the extracellular proton-binding site in $hK_{2P}3.1$ (Figure 4f). Effects of 10 µM genistein (6 min) on wild-type and mutant $hK_{2P}3.1$-H98N channels insensitive to pH modulation were compared. Reduction of wild-type currents (39.2 ± 2.4%; $n = 13$) was not significantly different from $hK_{2P}3.1$-H98N channels (33.0 ± 1.8%; $n = 8$), indicating that channel protonation is not required for TK regulation of $hK_{2P}3.1$.

Direct TK-dependent phosphorylation of $hK_{2P}3.1$ α-subunits is not involved in genistein-dependent leak channel regulation

The significance of tyrosine phosphorylation in genistein-associated $hK_{2P}3.1$ current inhibition was further analysed using PVN, a membrane-permeable inhibitor of protein

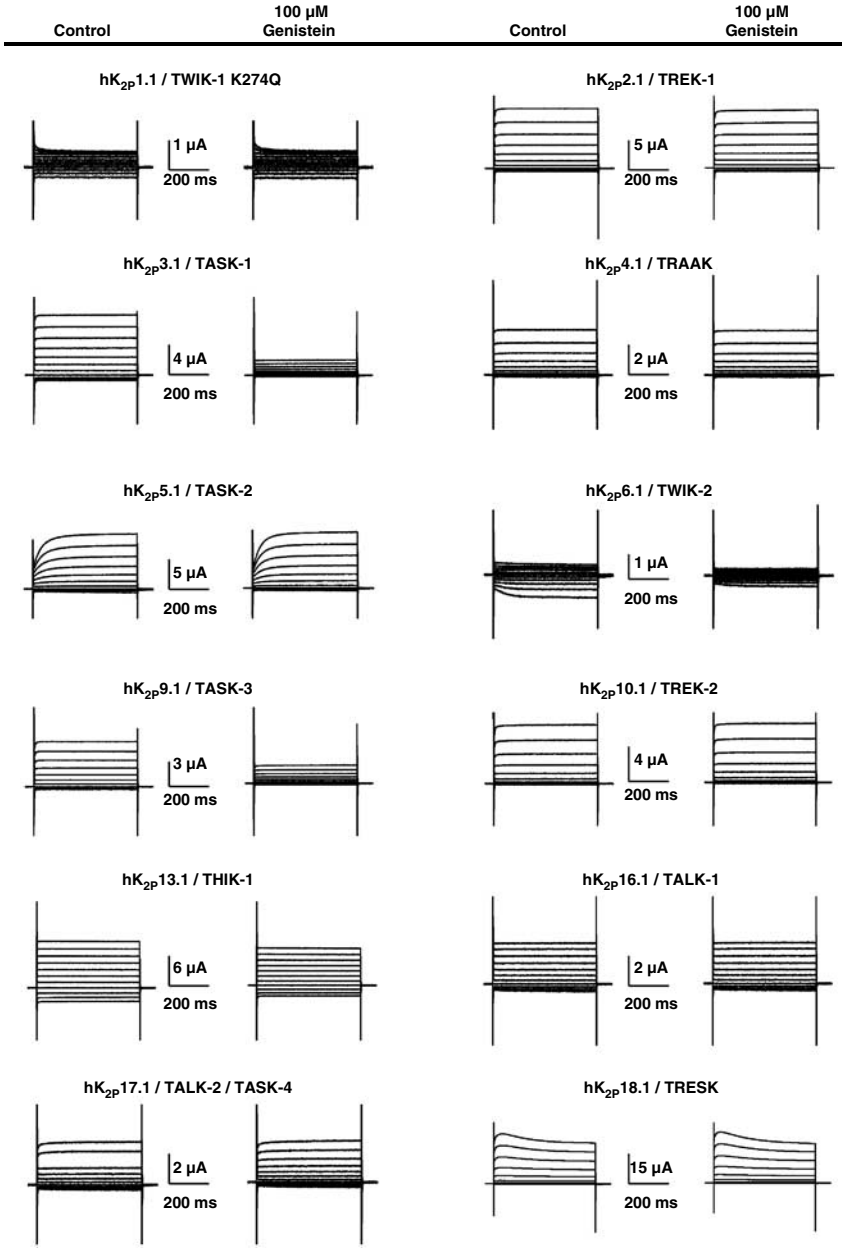

Figure 2 Synopsis of genistein effects on functional human K_{2P} family members. Representative control measurements and recordings after application of 100 µM genistein (6 min) are shown. As wild-type $hK_{2P}1.1$ (TWIK-1) produces relatively small currents, a mutant previously shown to display increased macroscopic currents in oocytes ($hK_{2P}1.1$-K274Q; Rajan *et al*., 2005) has been used. See text for voltage protocol.

tyrosine phosphatases. After application of 100 µM PVN for 6 min, that is, when dephosphorylation of TK substrates was prevented, the effect of TK inhibition by 10 µM genistein (co-administered for additional 6 min) was significantly reduced compared with PVN-free conditions (Figure 5a; current reduction of 26.9 ± 1.2 versus 38.9 ± 1.8%, respectively; $n = 6$ and 22 cells). The difference in current reduction by 100 µM genistein in the presence (76.4 ± 1.1%; $n = 9$) or absence of 100 µM PVN (81.1 ± 1.7%; $n = 12$) did not achieve statistical significance.

Perorthovanadate application alone (100 µM; 6 min) did not cause significant $hK_{2P}3.1$ amplitude changes (Figure 5a; $n = 9$). The lack of response to pharmacological inhibition of tyrosine phosphatases suggests a saturated level of basal TK substrate phosphorylation under the given experimental conditions.

The antagonism of genistein-induced $hK_{2P}3.1$ current reduction by PVN (Figure 5a) can be associated in the first instance with direct TK-dependent phosphorylation of the channel α-subunit at its single predicted TK consensus site, Y323 (Figure 5b). To differentiate between direct effects of a TK on the channel protein from intermediate actions within signal-transduction cascades, we performed site-directed mutagenesis to generate mutant $hK_{2P}3.1$ channels that lack the consensus TK phosphorylation site (Y323F). In addition, a different mutant was generated to mimic phosphorylation at residue 323 (Y323E). Under control conditions, current amplitudes recorded from $hK_{2P}3.1$-Y323F ($n = 10$) and

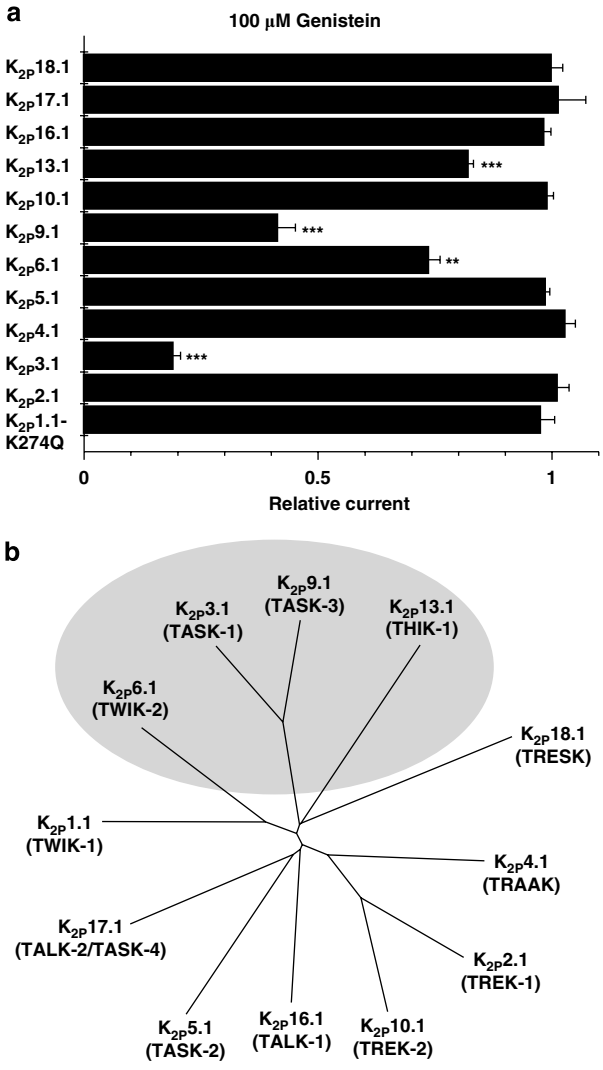

Figure 3 Protein tyrosine kinase inhibitor genistein reduces currents of related hK_{2P} family members. (**a**) Significant current reduction was observed with $hK_{2P}3.1$, $hK_{2P}6.1$, $hK_{2P}9.1$ and $hK_{2P}13.1$, respectively (**$P<0.01$, ***$P<0.001$ versus respective controls; $n=5$–15 cells were measured for each K_{2P} channel). Data are given as mean ± s.e.mean. (**b**) Phylogenetic tree of functional human K_{2P} channels. Nucleotide sequence alignment and phylogenetic analysis were generated using ClustalW software version 1.83 (BLOSUM method) and TreeView. Human K_{2P} channels affected by genistein are indicated.

$hK_{2P}3.1$-Y323E channels ($n=10$) were not significantly different from wild-type currents (Figure 5c; $n=10$). Furthermore, the inhibitory action of 10 μM genistein was not significantly altered by Y323 mutations compared with wild-type $hK_{2P}3.1$ (Figure 5d). Taken together, these data reveal that TK regulation of $hK_{2P}3.1$ channels is not mediated by direct channel phosphorylation.

Indirect actions of TK inhibition on $hK_{2P}3.1$ channels are not mediated via PKA- or PKC-dependent pathways

Indirect TK-dependent actions may affect ion channel function by regulating serine/threonine kinase activity (Schröder *et al*., 2004; Zhou *et al*., 2007). To determine whether two serine/threonine kinases, protein kinases A and C, contribute to TK-dependent $hK_{2P}3.1$ regulation, cells were incubated with the PKA and PKC inhibitor, staurosporine (1 μM), for 1–3 h prior to electrophysiological recordings. Figure 6a illustrates that inhibition of protein kinases A and C by staurosporine did not significantly affect $hK_{2P}3.1$ current reduction by 10 μM genistein (6 min; $n=11$) when compared with oocytes from the same batch without staurosporine pretreatment ($n=9$), arguing against a significant role of PKA and/or PKC in $hK_{2P}3.1$ regulation by TKs. It is noteworthy, however, that basal $hK_{2P}3.1$ current amplitudes were increased by incubation with 1 μM staurosporine compared with untreated cells (mean current amplitudes at +20 mV: 7.7 ± 0.5 versus 5.2 ± 0.5 μA; $n=11$ and 9 cells, respectively). This is in line with previous studies reporting $K_{2P}3.1$ current reduction by PKC-dependent mechanisms, resulting in cardiac repolarization abnormalities (Besana *et al*., 2004).

To further confirm that PKA- and/or PKC-dependent phosphorylation of $hK_{2P}3.1$ channels is not required for TK regulation, we generated $hK_{2P}3.1$ clones where all putative PKA- and/or PKC-dependent phosphorylation sites were mutated to alanine residues to prevent phosphorylation (Figure 6b). The resulting constructs were $hK_{2P}3.1$ ΔPKA (S392A–S393A), $hK_{2P}3.1$ ΔPKC (S358A–T383A), and $hK_{2P}3.1$ ΔPKA ΔPKC (S358A–T383A–S392A–S393A), respectively. In addition, a construct lacking all putative PKA-, PKC- and TK-dependent phosphorylation sites was generated, $hK_{2P}3.1$ ΔPKA ΔPKC ΔTK ($hK_{2P}3$ ΔPKA ΔPKC-Y323F). When compared to wild-type $hK_{2P}3.1$, mutant clones were equally sensitive to 100 μM genistein (Figure 6c; 6 min incubation; $n=4$–8 cells were studied), ruling out a significant role of direct phosphorylation by PKA, PKC and/or TK in genistein-mediated $hK_{2P}3.1$ current reduction.

Under baseline conditions, mean $hK_{2P}3.1$ wild-type current amplitudes (recorded at +20 mV) yielded 3.8 ± 0.8 μA ($n=4$), whereas $hK_{2P}3.1$ ΔPKC channels displayed significantly increased currents (5.5 ± 1.4 μA; $n=4$). This is in accordance with our results obtained from cells following staurosporine treatment and reflects PKC-dependent inhibition of $hK_{2P}3.1$ channels (Besana *et al*., 2004). Of note, currents recorded from cells expressing $hK_{2P}3.1$ ΔPKA channels were significantly smaller (0.5 ± 0.04 μA; $n=4$) compared with $hK_{2P}3.1$ WT, indicating PKA-dependent activation of $hK_{2P}3.1$. Mutant $hK_{2P}3.1$ ΔPKA ΔPKC channels displayed an intermediate phenotype; mean current amplitudes yielded 1.6 ± 0.3 μA ($n=4$).

Genistein regulates $hK_{2P}3.1$ channels expressed in mammalian cells

It has been indicated that phosphorylation levels within *Xenopus* oocytes are highly variable (Cohen and Zilberberg, 2006). Thus, we expressed $hK_{2P}3.1$ potassium channels heterologously in CHO cells to demonstrate modulation of $hK_{2P}3.1$ currents in mammalian cells (Figure 7), that is, in a more stable environment. From a holding potential of −80 mV, depolarizing pulses were applied for 500 ms to voltages between −120 and +80 mV in 20-mV increments (0.2 Hz). The degree of block was determined at +60 mV

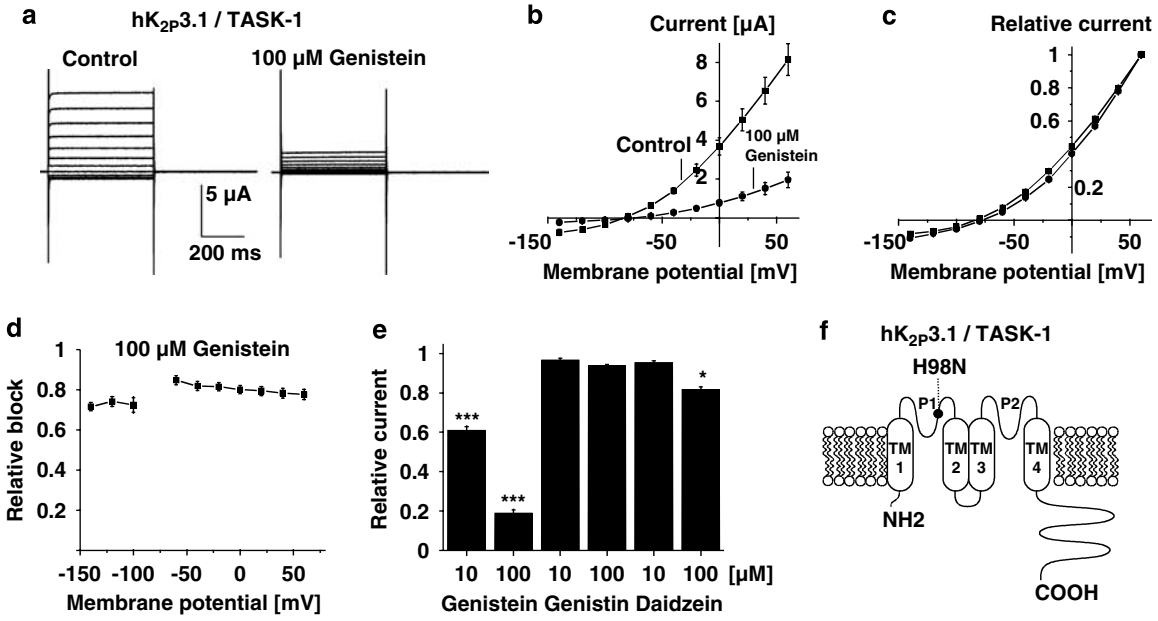

Figure 4 Biophysical and pharmacological determinants of genistein-induced $hK_{2P}3.1$ current reduction. (**a**) Effects of genistein on the voltage dependence of activation. Control measurement and the effect of 100 µM genistein (6 min) are shown in one representative oocyte. (**b**, **c**) Activation curves, that is, step current amplitudes as a function of the pulse potential, recorded under isochronal conditions (**b**, original current amplitudes; **c**, values normalized to peak currents) ($n=13$). There was no apparent shift in the current–voltage relationship. (**d**) Attenuation of $hK_{2P}3.1$ currents by genistein is not voltage dependent. The fraction of blocked step currents is plotted as a function of the respective test pulse potentials. Channel block did not display significant differences between −140 and +60 mV ($n=13$ cells). (**e**) The inactive analogue genistin (10 and 100 µM; $n=8$ and 6 cells, respectively) did not reproduce inhibitory effects of 10 and 100 µM genistein ($n=22$ and 12 oocytes, respectively). Similarly, 10 µM of the less potent TK inhibitor daidzein had no significant effect on $hK_{2P}3.1$ currents ($n=7$). At higher concentrations (100 µM) daidzein weakly reduced $hK_{2P}3.1$ currents ($n=9$). (**f**) Predicted topology of $hK_{2P}3.1$ subunits. TM, transmembrane domain; P, pore loop domain. N and C termini are intracellular. The histidine residue that allows protons to block in the physiological pH range (H98) is indicated. Data are expressed as mean ± s.e.mean. $^*P<0.05$, $^{***}P<0.001$ versus respective controls (see text for voltage protocol).

after steady state had been reached (usually within 3–4 min) (Figure 7b), and the half-maximal inhibitory concentration was calculated as described in Figure 1 (Figure 7d; $n=3$–4 cells were investigated at each concentration). The IC_{50} for block of $hK_{2P}3.1$ leak channels in CHO cells yielded 12.3 ± 5.4 µM with a Hill coefficient n_H of 0.83 ± 0.21.

Discussion and conclusions

K_{2P} channels stabilize membranes of excitable cells at hyperpolarized potentials below the threshold for action potential firing. Here, we describe genistein-dependent modulation of K_{2P} leak channels as a novel regulatory mechanism in addition to known G-protein-dependent pathways (for review, see Patel and Honore, 2001; Mathie, 2007).

K_{2P} leak channels are modulated by genistein

Genistein reduces potassium leak currents in *Xenopus* oocytes and CHO cells (Figures 1 and 7). This mechanism is conserved among related K_{2P} family members (Figures 2 and 3). In addition to $hK_{2P}3.1$, the potassium leak channels $hK_{2P}6.1$, $hK_{2P}9.1$ and $hK_{2P}13.1$ are blocked by genistein. In the present study, molecular mechanisms of genistein-dependent regulation of the most sensitive human K_{2P} channel, $hK_{2P}3.1$, were analysed in detail. The inhibitory action of genistein was voltage independent, that is, current inhibition occurred with similar potency at membrane potentials between −140 and +60 mV. $K_{2P}3.1$ channels are sensitive to extracellular pH (pKa = 7.2; Lopes *et al.*, 2001). However, protein protonation is not required: mutation of a single histidine residue adjacent to the potassium selectivity filter that represents the extracellular proton-binding site in $hK_{2P}3.1$, H98 (Figure 4f), did not alter genistein sensitivity of the channel.

Molecular mechanisms

Genistein may modulate ion channel and receptor function either through inhibition of TKs (Yu *et al.*, 2004; Cho *et al.*, 2005; Missan *et al.*, 2006) or by direct blockade (Paillart *et al.*, 1997; Belevych *et al.*, 2002; Altomare *et al.*, 2006). These pathways are not mutually exclusive, as illustrated by the work of Ogata *et al.* (1997), suggesting a combination of both mechanisms.

Here, several lines of evidence support the hypothesis that $hK_{2P}3.1$ current reduction is mediated by TK inhibition. First, genistin, a structurally similar analogue of genistein, did not exert inhibitory effects. Second, a different structural analogue that only weakly inhibits TKs (daidzein) did not markedly affect $hK_{2P}3$. Daidzein caused weak current inhibition only at high drug concentrations, as predicted. This is in

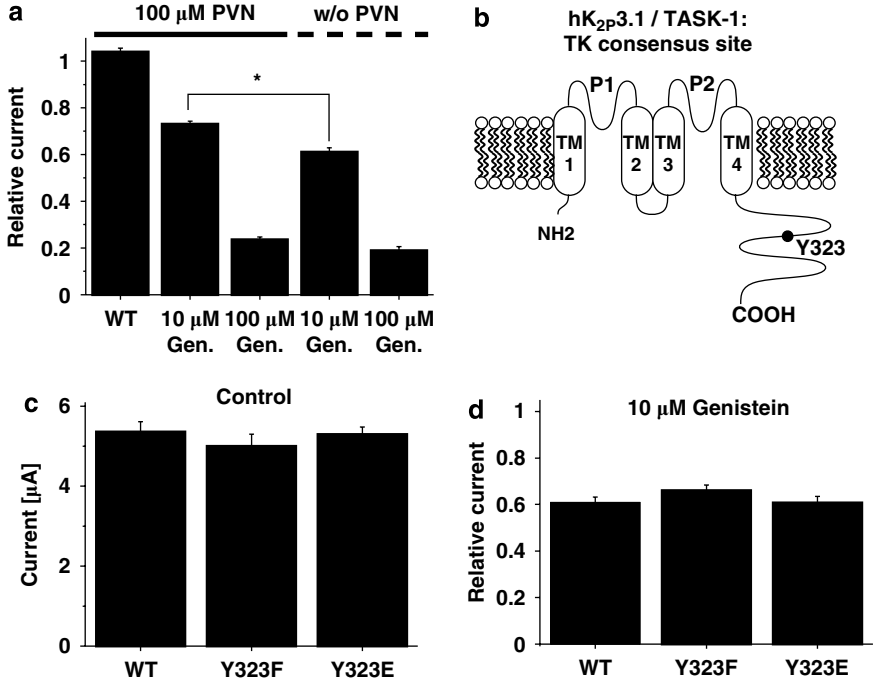

Figure 5 Human $K_{2P}3.1$ regulation by tyrosine kinases (TKs) does not require direct TK-dependent phosphorylation of the channel α-subunit. (**a**) Mean (± s.e.mean) relative current amplitudes in the presence or absence of the tyrosine phosphatase inhibitor, peorthovanadate (PVN; 100 μM), after application of 10 or 100 μM genistein (6 min) or compared to PVN-free control conditions (w/o PNV) for $hK_{2P}3.1$ wild type ($n = 6$–22 cells studied). Although PVN did not alter basal $hK_{2P}3.1$ current amplitudes, the inhibitory effect of 10 μM genistein was significantly attenuated by simultaneous PVN treatment (*$P < 0.05$). (**b**) Predicted membrane topology of $hK_{2P}3.1$ subunits illustrating the location of the putative TK phosphorylation site, Y323. (**c**) Exchange of the tyrosine residue at position 323 for phenylalanine (to prevent phosphorylation; F; $n = 10$) or glutamate (to mimic phosphorylation; E; $n = 10$) did not affect basal current amplitudes under control conditions (WT, wild type; $n = 10$). Furthermore, $hK_{2P}3.1$ current reduction induced by 10 μM genistein was not altered (**d**; $n = 8$–13). See text for voltage protocol.

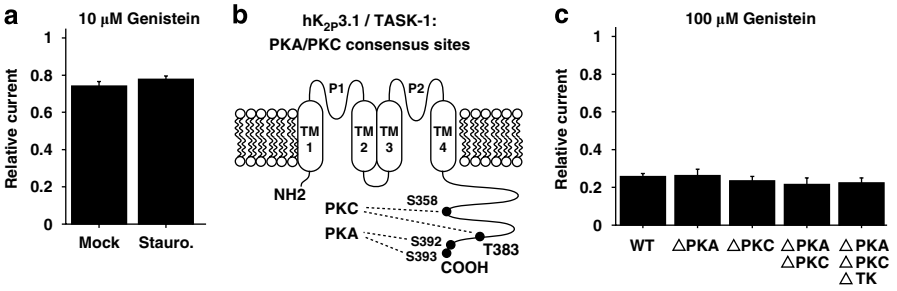

Figure 6 Protein kinases A and C are not involved in tyrosine kinase (TK)-dependent $hK_{2P}3.1$ regulation. (**a**) Oocytes were treated with 1 μM staurosporine, a serine/threonine kinase inhibitor, for 1–3 h prior to current recordings (stauro.; $n = 11$). The effect of 10 μM genistein was then compared to control cells from the same batch without staurosporine (mock; $n = 9$). The genistein response was not significantly altered by pretreatment with staurosporine. Predicted PKA and PKC phosphorylation sites are indicated in (**b**). To prevent phosphorylation, respective residues were mutated to alanine, producing $hK_{2P}3.1$ ΔPKA (S392A–S393A), $hK_{2P}3.1$ ΔPKC (S358A–T383A), $hK_{2P}3.1$ ΔPKA ΔPKC (S358A–T383A–S392A–S393A) and $hK_{2P}3.1$ ΔPKA ΔPKC ΔTK ($hK_{2P}3.1$ ΔPKA ΔPKC including the Y323F mutation to prevent TK phosphorylation), respectively. Mutant channels were sensitive to 100 μM genistein similar to wild-type $hK_{2P}3.1$ (**c**; $n = 4$–8 cells studied). Data are given as mean ± s.e.mean. See text for voltage protocol.

line with studies demonstrating TK-dependent effects of genistein (Yu *et al.*, 2004; Cho *et al.*, 2005; Missan *et al.*, 2006). Furthermore, this hypothesis is reinforced by the observation that PVN, an inhibitor of tyrosine phosphatase activity, attenuated the inhibitory effect of 10 μM genistein on $hK_{2P}3.1$ currents.

In contrast, PVN did not significantly affect $hK_{2P}3.1$ inhibition by 100 μM genistein. This lack of effect argues in favour of TK-independent mechanisms (Belevych *et al.*, 2002). Moreover, the onset of current inhibition was fast (Figure 1). Rapid onset of block is consistent with direct channel blockade from the extracellular side. However, genistein and PVN have been shown to act rapidly, within 5 min, on ion channels and receptors via TK-dependent mechanisms as well (Wischmeyer *et al.*, 1998; Cho *et al.*, 2005), indicating that no definitive conclusions may be drawn from this observation. Finally, the signal-transduction mechanism does not involve direct TK phosphorylation of

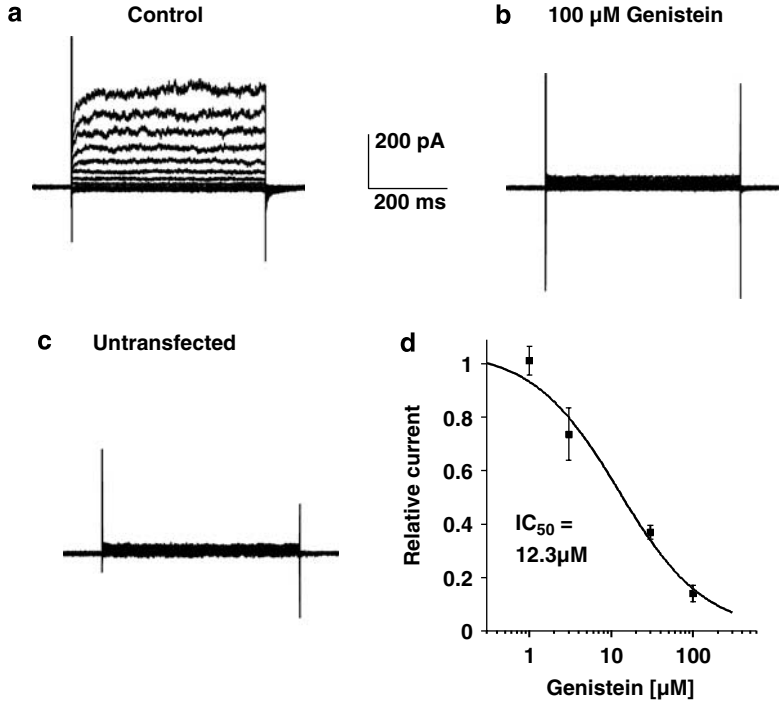

Figure 7 Genistein-induced inhibition of human $K_{2P}3.1$ (TASK-1) channels expressed in Chinese hamster ovary (CHO) cells. Families of current traces recorded under control conditions and after superfusion with 100 µM genistein are displayed in (**a**, **b**), respectively. (**c**) Representative recording from an untransfected CHO cell display little endogenous K^+ conductance. (**d**) Concentration–response relationships for the effect of genistein on $hK_{2P}3.1$ currents measured at +60 mV ($n = 3$–4 cells; mean ± s.e.mean). The IC_{50} yielded 12.3 µM (see text for voltage protocol).

the channel protein. Mutant $hK_{2P}3.1$ channels lacking the single putative TK-dependent phosphorylation site ($hK_{2P}3.1$-Y323F) or mimicking phosphorylation ($hK_{2P}3.1$-Y323E) were still modulated by genistein (Figure 5). Direct TK-dependent phosphorylation can be ruled out for other genistein-sensitive K_{2P} family members, as $hK_{2P}6.1$, $hK_{2P}9.1$ and $hK_{2P}13.1$ do not harbour any intracellular TK phosphorylation sites.

Indirect actions may affect K_{2P} channel function via cross-talk with protein kinase A and/or protein kinase C, as suggested previously for L-type calcium currents and voltage-gated potassium channels (Schröder *et al*., 2004; Zhou *et al*., 2007). However, inhibition of PKA and PKC by preincubation with staurosporine did not affect genistein-induced $hK_{2P}3.1$ current reduction. Furthermore, $hK_{2P}3.1$ proteins lacking putative PKA- and PKC-dependent phosphorylation sites were sensitive to genistein similar to wild-type channels. Thus, PKA and PKC are not essential for genistein-induced inhibition of $hK_{2P}3.1$, and direct phosphorylation of $hK_{2P}3.1$ protein by TK, PKA and PKC is not required.

The rapid onset of block argues against increased protein turnover and protein degradation as molecular mechanisms of action. However, cross-talk with intracellular second messengers or lipid-dependent pathways (for example, diacylglycerol, phospholipase C, PIP_2 and IP_3) may be involved in genistein-dependent K_{2P} regulation. In addition, accessory β-subunits or interacting proteins such as 14-3-3, endogenously expressed in *Xenopus* oocytes and CHO cells, may mediate inhibitory effects of genistein. Recently, association of activated Gαq subunits with $hK_{2P}3.1$ and $hK_{2P}9.1$ channels has been revealed to cause leak current inhibition (Chen *et al*., 2006; Veale *et al*., 2007). We may speculate that genistein-induced TK inhibition stimulates Gαq pathways, ultimately leading to association of activated Gαq proteins with $hK_{2P}3.1$ channels and current inhibition.

In summary, it is reasonable to assume that genistein modulates $hK_{2P}3.1$ channels, at least in part, via TK-dependent mechanisms. These pathways include intermediate signal-transduction factors. TK-dependent phosphorylation of $hK_{2P}3.1$ protein was not observed. In addition, direct inhibitory effects of genistein on $hK_{2P}3.1$ currents may contribute to the inhibitory effect of genistein on K_{2P} channels as well. Future studies including chimeric approaches and analyses of the putative drug-binding site in $hK_{2P}3.1$ are required to characterize the underlying molecular mechanism in detail.

Physiological and clinical implications of TK-dependent background K^+ current regulation in heart and CNS

The cardiac plateau current I_{K_P} is time independent, potassium selective and influences the amplitude and duration of the cardiac action potential and, consequently, the duration of myocardial contraction (Marban, 2002). On the basis of common distribution and biophysical attributes, it has been suggested that $K_{2P}3.1$ channels contribute to I_{K_P} (Lopes *et al*., 2000). In mouse, $K_{2P}3.1$ is expressed throughout the heart with prominence in the ventricles. $K_{2P}3.1$ mRNA and protein have been demonstrated in rabbit heart in both atrial and ventricular cardiomyocytes. Endogenous

K_{2P}3.1-like currents have been recorded in rat cardiomyocytes, and inhibition of these currents has been proposed to mediate proarrhythmic effects. Recently, cardiac K_{2P} channels have been associated with ischaemic preconditioning and cardioprotection (Lu *et al*., 2007). TK inhibition reduces beneficial effects of cardiac ischaemic preconditioning (Imagawa *et al*., 1997; Fatehi-Hassanabad and Parratt, 1997; Fryer *et al*., 1998). Thus, it is tempting to hypothesize that reduction of cardioprotective hK_{2P}3.1 currents by TK inhibition attenuates ischaemic preconditioning in the presence of TK antagonists. Moreover, TK-related block of cardiac K_{2P} leak currents may cause prolonged repolarization and, perhaps, dysregulation of cardiac pacemaker activity (Barbuti *et al*., 2002; Lalevee *et al*., 2006; Putzke *et al*., 2007).

TK-sensitive K_{2P} channels are strongly expressed in the CNS. In particular, K_{2P}3.1 and K_{2P}9.1 (with K_{2P}1.1 and K_{2P}10.1) are believed to contribute to the potassium standing outward current ($I_{K(SO)}$) important in cerebellar granule neurons (Millar *et al*., 2000; Han *et al*., 2002; Clarke *et al*., 2004). Furthermore, K_{2P}3.1 is responsive to volatile anaesthetics and has been implicated in oxygen sensation in both the brain and the carotid body. Inhibition of background potassium conductances in multiple areas of the CNS (including brainstem aminergic neurons, cerebellar granule neurons, cortex, thalamus, hippocampus and hypothalamus) is a significant mechanism by which hormones and neurotransmitters may enhance excitability and contribute to neuronal plasticity (Patel and Honore, 2001; Mathie, 2007). TK-related inhibition of K_{2P} currents in the CNS may lead to increased excitability at the cellular level, ultimately translating into differential regulation of central nervous function.

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Conflict of interest

The authors state no conflict of interest.

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