Pflügers Arch - Eur J Physiol (2000) 441:257–262
DOI 10.1007/s004240000406

ORIGINAL ARTICLE

Paola Imbrici · Stephen J. Tucker
Maria Cristina D'Adamo · Mauro Pessia

Role of receptor protein tyrosine phosphatase α (RPTPα) and tyrosine phosphorylation in the serotonergic inhibition of voltage-dependent potassium channels

Received: 26 April 2000 / Received after revision: 26 June 2000 / Accepted: 4 July 2000 / Published online: 21 October 2000

Abstract The activity of voltage-gated potassium (Kv) channels can be dynamically modulated by several events, including neurotransmitter-stimulated biochemical cascades mediated by G-protein-coupled receptors. By using a heterologous expression system, we show that activating the 5-HT_{2C} receptor inhibits both Kv1.1 and Kv1.2 channels through a tyrosine phosphorylation mechanism. The major molecular determinants of channel inhibition were identified as two tyrosine residues located in the N-terminal region of the Kv channel subunit. Furthermore, we demonstrate that receptor protein tyrosine phosphatase α (RPTPα), a receptor protein tyrosine phosphatase, co-ordinates the inhibition process mediated via 5-HT_{2C} receptors. We therefore propose that the serotonergic regulation of human Kv1.1 and Kv1.2 channel activity by the 5-HT_{2C} receptor involves the dual coordination of both RPTPα and specific tyrosine kinases coupled to this receptor.

Keywords 5-HT_{2C} · Kinase · Phosphatase · Phosphorylation · Potassium channel modulation · receptor protein tyrosine phosphatase α (RPTPα) · Serotonin receptor · Tyrosine

Introduction

Potassium-selective ion channels regulate numerous and heterogeneous cell functions. In particular, voltage-gated potassium (Kv) channels contribute to the action potential duration, modulate the release of neurotransmitters, and control the excitability and electrical properties of neurons in both the central and peripheral nervous systems [7]. On the other hand, the activity of Kv channels can be modulated by seven transmembrane domain receptors (GPCR). 5-HT_{2C} is a member of the GPCR family that couples to the $G\alpha q_{/11}$-protein [17]. This receptor stimulates phospholipase C, generating diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) which elicits the release of calcium ions from intracellular stores. The highest expression levels of the 5-HT_{2C} receptor subtype are in CA1, CA2 and CA3 hippocampal pyramidal neurons [13]. An overlapping expression pattern of Kv1.1 and Kv1.2 channels and 5-HT_{2C} receptors has been observed in several brain areas including the dentate gyrus, the substantia nigra, the vestibular nuclei and CA3 neurons [13, 21, 22].

Hippocampal pyramidal neurons are densely innervated by serotonergic terminals from the raphe nucleus. It has previously been reported that 5-hydroxytryptamine (5-HT) regulates the firing pattern of CA1 neurons by modulating several potassium conductances [4]. Moreover, the firing rate of some substantia nigra interneurons is accelerated upon 5-HT_{2C} receptor activation. Consequently, an increased frequency of $GABA_A$-receptor-mediated spontaneous inhibitory postsynaptic currents (IPSCs) could be recorded from dopaminergic cells in this area [16]. Activation of the 5-HT_{2C} receptor is also able to suppress RBK1 channel activity, a rat brain potassium channel homologue. However, the molecular mechanism(s) of channel inhibition remains poorly understood [8, 10]. Nevertheless, these observations demonstrate that a specific functional coupling between Kv channels and the 5-HT_{2C} receptor may occur in vivo, as well as in heterologous expression systems.

Voltage-gated potassium currents can be modulated by a number of receptors, including the M1 muscarinic acetylcholine receptor, the epidermal growth factor receptor (EGF) or insulin treatment [2, 3, 5, 6, 9, 12]. The phosphorylation of multiple tyrosine residues of the Kv subunit is involved in these processes [2, 5, 9, 12]. Moreover, downstream effectors, such as protein kinase C (PKC), the GTP-binding protein RhoA and the protein

P. Imbrici · M.C. D'Adamo · M. Pessia (✉)
Istituto di Ricerche Farmacologiche "Mario Negri",
Consorzio Mario Negri Sud,
Department of Vascular Medicine and Pharmacology,
66030 Santa Maria Imbaro (Chieti) Italy
e-mail: pessia@cmns.mnegri.it
Tel.: +39-0872-570283, Fax: +39-0872-578240

S.J. Tucker
University Laboratory of Physiology, Parks Road,
Oxford OX1 3PT, UK

tyrosine kinases PYK2 and Src, play an important role in suppressing Kv1.x currents [1, 3, 9, 12, 14, 15]. By contrast RPTPα, a receptor protein tyrosine phosphatase highly expressed in the central nervous system, stimulates the activity of Kv1.2 channels [18, 20].

The study reported here investigates the molecular mechanisms underlying the inhibition of human Kv1.1 and Kv1.2 channel activity mediated by activation of the human 5-HT_{2C} receptor. We show that specific tyrosine residues on the hKv1.1 subunit are the target for 5-HT_{2C}-receptor mediated inhibition, and we have identified a role for receptor protein tyrosine phosphatase α (RPTPα) in mediating this serotonergic modulation.

Materials and methods

Electrophysiology

Xenopus laevis care and handling were in accordance with the highest standards of institutional guidelines. Animals underwent no more than two surgeries, separated by at least 3 weeks. *Xenopus laevis* were deeply anaesthetised with an aerated solution containing 3-aminobenzoic acid ethyl ester methansulfonate salt (5 mM) and sodium bicarbonate (60 mM), pH=7.3. The ovary was dissected and the oocytes digested in OR-2 solution containing collagenase A (0.5 units/ml, Sigma). In vitro transcribed mRNAs were microinjected into the oocytes 24 h later by using a nanoliter injector (WPI) and incubated at 16°C.

The amount of mRNAs was quantified using a spectrophotometer and by ethidium bromide staining. In all cases, the amount of co-injected mRNAs and the volume injected per oocyte were held constant. Every oocyte was injected with 50 nl of a solution containing 1 ng 5-HT_{2C} mRNA and 0.1 ng of either hKv1.1 or hKv1.2 mRNA. The amount of Kv mRNA injected typically resulted in 10–15 μA of current after 24–36 h.

Two-electrode voltage-clamp recordings

Recordings were performed from *Xenopus* oocytes at 22°C, 1–8 days after injection. A GeneClamp 500 amplifier (Axon Instruments) interfaced to a Power Macintosh 7200/90 computer with an ITC-16 interface (Instrutech, N.Y., USA) was used. Mi-

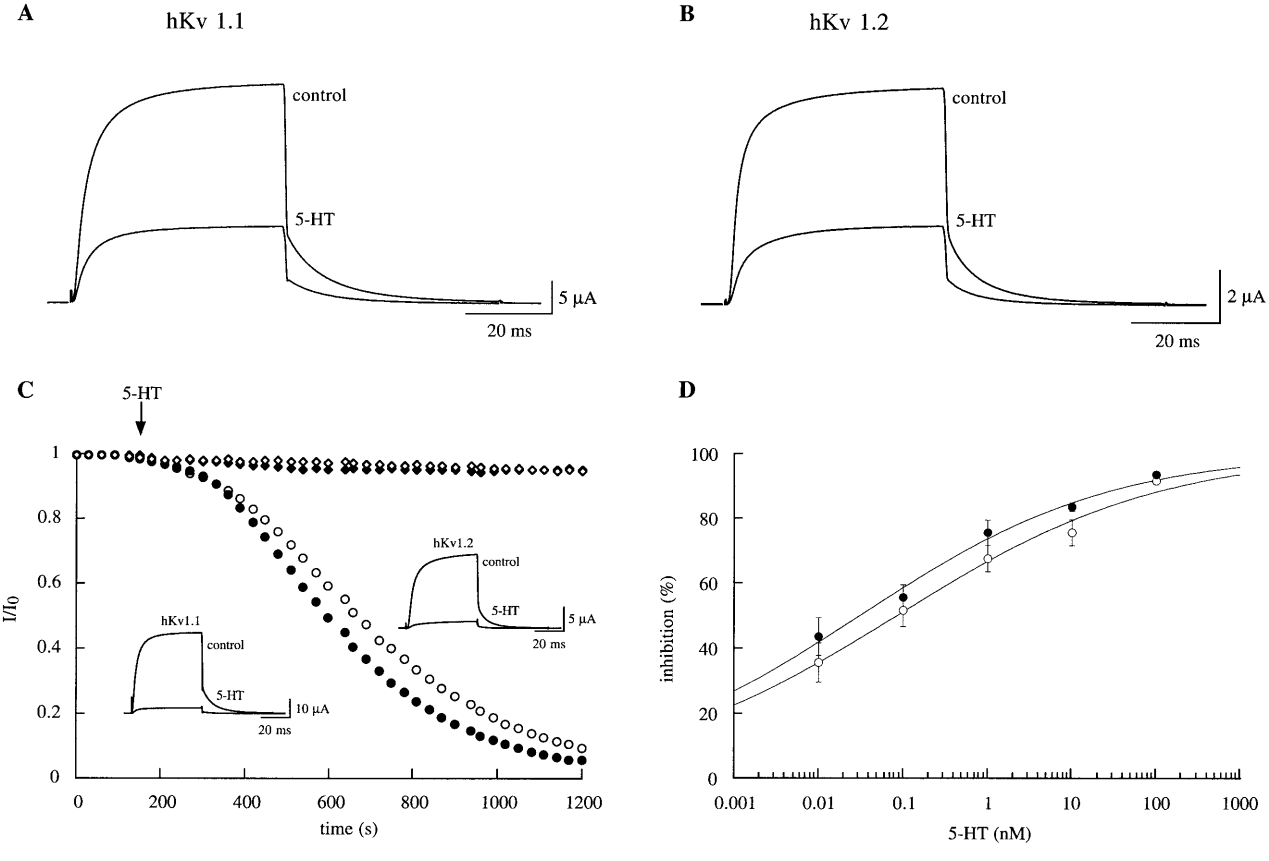

Fig. 1A–D 5-HT_{2C} receptor activation inhibits hKv1.1 and hKv1.2 currents. Current traces recorded from *Xenopus* oocytes expressing the human 5-HT_{2C} receptor and either hKv1.1 (**A**) or hKv1.2 (**B**) channels. Currents were evoked by depolarisations to +20 mV from the holding potential of –80 mV, before (*control*) and 20 min after the superfusion of 5-hydroxytryptamine (5-HT, 1 nM). Tail currents were recorded at –50 mV. **C** Time course of hKv1.1 (*closed circles*) and hKv1.2 (*open circles*) current inhibition caused by the activation of 5-HT_{2C} receptors. Current amplitudes were evoked every 30 s by depolarising pulses to +20 mV and normalised to the control current (I_0). The *arrow* indicates the time of 5-HT (100 nM) application. The effect of applying 5-HT (100 nM) on oocytes expressing hKv1.1 (*closed diamonds*) and hKv1.2 (*open diamonds*) channels, in the absence of the 5-HT_{2C} receptor, is also depicted. The *insets* show two additional recordings illustrating the inhibitory effect of 5-HT (100 nM). **D** Concentration–response relationships for 5-HT-evoked inhibition of hKv1.1 (*closed symbols*) and hKv1.2 (*open symbols*) currents. The data points represent the % of current inhibition, recorded at +20 mV, and plotted as a function of 5-HT concentration (mean ±SEM, n=6). *Lines* represent the fit with the equation: I=100·[5-HT]^n/([5-HT]^n+$K_D{}^n$) from which the K_D values were calculated

croelectrodes were filled with 3 M KCl and had resistances of 0.1–0.5 MΩ. The recording solution contained (mM): NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, pH=7.4. Currents were evoked by voltage commands from a holding potential of –80 mV as described in the figure legends. Data acquisition and analysis were performed using Pulse+PulseFit (HEKA Elektronik, Germany), KaleidaGraph (Synergy Software, USA) and IGOR (Wavemetrics) software. Leak and capacitative currents were subtracted using a P/4 protocol.

Molecular biology

The human 5-HT_{2C} cDNA was PCR amplified from a human brain cDNA library (Stratagene) using gene-specific primers [17]. All cDNAs were subcloned into the oocyte expression vector pBF (courtesy of Dr. B. Fakler), which provides 5′ and 3′ untranslated regions from the *Xenopus* β-globin gene, flanking a polylinker containing multiple restriction sites. In vitro mRNAs were generated using SP6 RNA polymerase. Unless otherwise stated, the 5-HT_{2C} receptor was always co-expressed in oocytes together with the specified channel.

Results

The superfusion of *Xenopus* oocytes, expressing 5-HT_{2C} receptors and either hKv1.1 or hKv1.2 channels, with solutions containing 5-HT inhibited both potassium currents (Fig. 1A, B). This process typically required 20 min to reach its steady-state level, when the currents were almost completely suppressed (Fig. 1C). Therefore, a cut-off time of 20 min was used in our study. The inhibition occurred in a concentration-dependent fashion yielding a K_D=33±8 pM for hKv1.1 and a K_D=91±23 pM for hKv1.2 channels, in our experimental conditions (Fig. 1D).

To determine the possible role of tyrosine phosphorylation mechanisms in this inhibitory coupling, *Xenopus* oocytes expressing either hKv1.1 or hKv1.2 alone were treated with solutions containing orthovanadate, a tyrosine phosphatase inhibitor. Either the superfusion of orthovanadate (1 mM, not shown) or its cytoplasmic microinjection (100 µM, presumed intracellular final concentration) inhibited both currents, albeit with slightly different time courses (Fig. 2A). Correspondingly, the cytoplasmic microinjection of genistein, a tyrosine kinase inhibitor, markedly slowed the time course of current inhibition mediated by the co-expressed 5-HT_{2C} receptors (Fig. 2B). This inhibition was antagonised by genistein in a concentration-dependent fashion (5–50 µM, presumed intracellular final concentration; Fig. 2C).

These results demonstrate the involvement of tyrosine phosphorylation/dephosphorylation mechanisms in this process. The data also suggest that the stimulation of the 5-HT_{2C} receptor inhibits channel activity by further activating an endogenous tyrosine kinase. Therefore, we attempted to identify the target residues for these kinases by sequential site-directed mutagenesis of all the intracellular tyrosine residues in hKv1.1 (Fig. 3A) and by co-expressing these mutants with the 5-HT_{2C} receptor in *Xenopus* oocytes. In summary, we found that the current inhibition caused by 100 pM 5-HT was markedly reduced

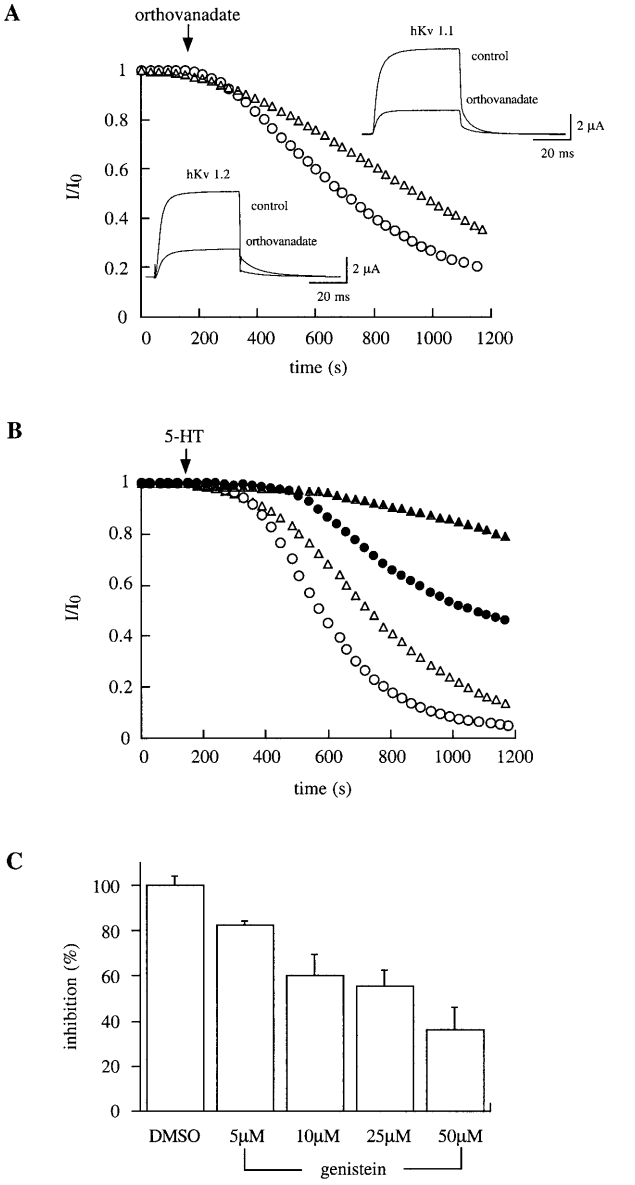

Fig. 2A–C Role of protein tyrosine phosphatase and kinase in hKv1.1 and hKv1.2 current inhibition. **A** Time course of hKv1.1 (*open circles*) and hKv1.2 (*open triangles*) current inhibition, before and after the cytoplasmic microinjection of orthovanadate (indicated by the *arrow*). The *insets* show representative hKv1.1 and hKv1.2 current traces recorded as in Fig. 1, before (*control*) and after orthovanadate microinjection. **B** Effects of 5-HT receptor activation (application of 5-HT 1 nM indicated by the *arrow*) on hKv1.1 (*closed circles*) and hKv1.2 (*closed triangles*) current amplitudes recorded from cells previously microinjected with genistein (10 µM, presumed intracellular final concentration). Representative recordings from mock-injected control cells are shown for comparison: hKv1.1 (*open circles*) and hKv1.2 (*open triangles*). **C** Normalised percentage of hKv1.1 current inhibition induced by the application of 5-HT (1 nM) on oocytes microinjected with either the drug vehicle alone, dimethyl sulfoxide (*DMSO*), or genistein at the indicated presumed intracellular concentration. The data are the mean ±SEM of 4–6 cells

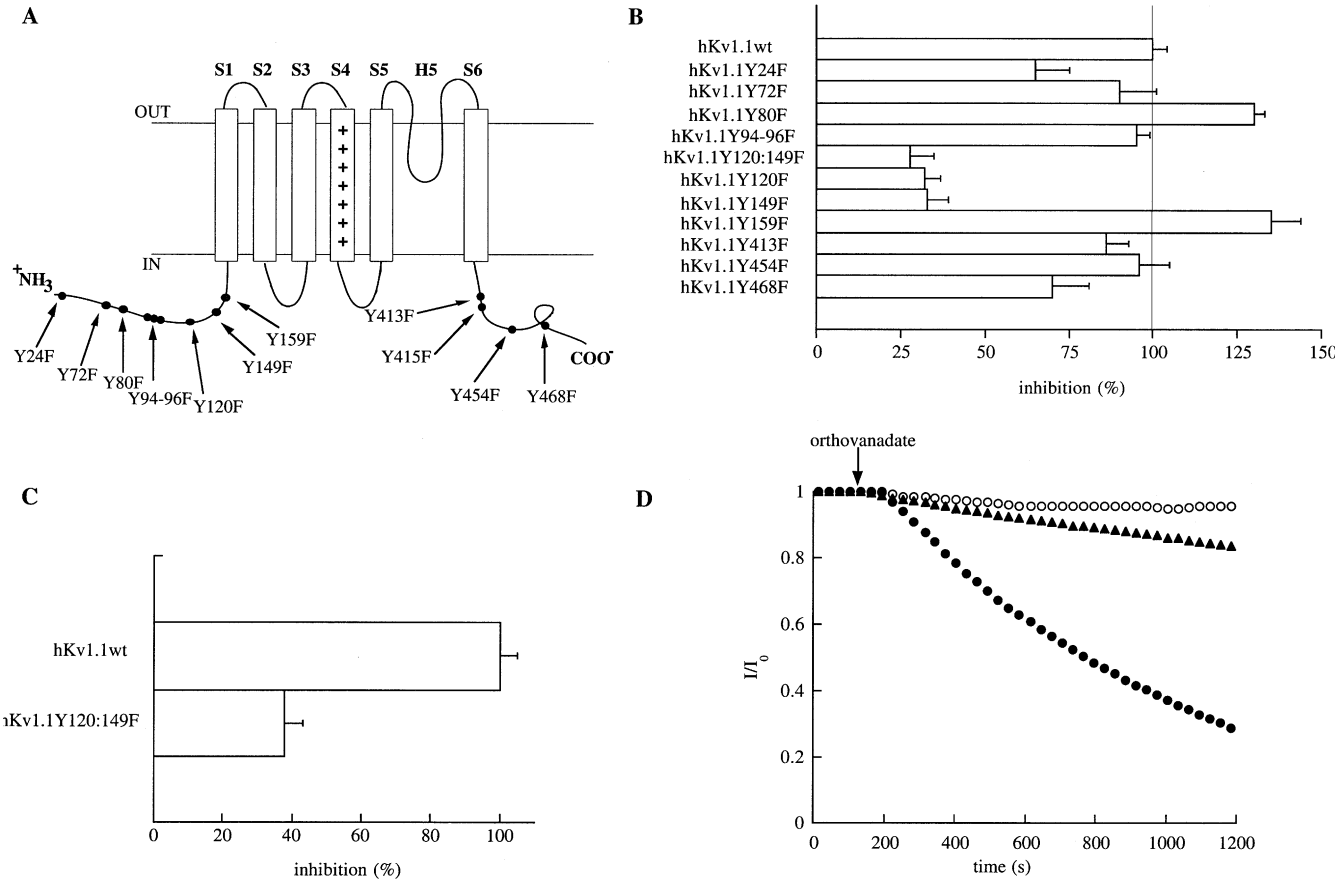

Fig. 3A–D Identification of residues involved in 5-HT_{2C}-receptor-mediated current suppression. **A** Schematic representation of a hKv1.1 subunit indicating the relative position of all intracellular tyrosine residues changed to phenylalanine. **B** Normalised percentage of current inhibition produced by 5-HT_{2C} receptor activation for the mutant channels. **C** Normalised current inhibition induced by orthovanadate (100 µM) microinjection. **D** Time course of hKv1.1 wild-type (*closed circles*) and hKv1.1Y120:149F (*closed triangles*) current inhibition recorded as in Fig. 1. The *arrow* indicates the time of orthovanadate (100 µM) microinjection. Control currents, showing the stability of the recordings, are also shown (*open circles*)

in both the Y120F and Y149F hKv1.1 channels (Fig. 3B). The ability of 5-HT to inhibit the double mutant, Y120F/Y149F Kv1.1 channel was reduced slightly further (Fig. 3B). Consistent with the involvement of these residues in the tyrosine kinase-dependent suppression of Kv1.1 channel activity, we found that orthovanadate's inhibition of the Y120F/Y149F hKv1.1 potassium currents was also markedly reduced (Fig. 3C, D).

Recently, it has been shown that expression of the tyrosine phosphatase (RPTPα) in *Xenopus* oocytes increases Kv1.2 channel activity [20]. Based on this evidence we investigated whether RPTPα plays a role in the 5-HT_{2C} receptor-mediated inhibition of hKv1.1 and hKv1.2 channel activity. We found that the additional co-expression of RPTPα in *Xenopus* oocytes markedly slowed the time course of hKv1.1 and hKv1.2 current inhibition (Fig. 4A) and significantly reduced the total amount of current inhibited by 5-HT_{2C} receptor activation (Fig. 4B). By contrast, co-expression of the tyrosine phosphatase SHP2 had no effect on channel activity (not shown), suggesting a specific role for RPTPα in this phenomenon.

Discussion

In this study we show that the human 5-HT_{2C} receptor modulates the activity of delayed-rectifier potassium channels. A detailed mutational analysis indicates a major role for phosphorylation of tyrosine residues 120 and 149 of hKv1.1 in the current suppression mediated by the 5-HT_{2C} receptor. Furthermore, we demonstrate that this coupling involves tyrosine phosphorylation processes that are regulated by RPTPα. These results demonstrate that the co-ordinated action of tyrosine kinases and phosphatases fine tunes the activity of Kv1.1 and Kv1.2 channels. Moreover, the observation that orthovanadate by itself is able to inhibit both channels implies that some basal level of tyrosine kinase activity must be present in *Xenopus* oocytes. Therefore, these channels probably exist in a phosphorylated/dephosphorylated equilibrium.

Both of the tyrosine residues on hKv1.1, identified as targets for the serotonergic suppression of channel activity, are located in the juxtamembrane N-terminal region of the channel. The three-dimensional structure of the N-terminal tetramerization domain (T1) of the *Shaker* potassium channel has recently been elucidated [11]. The Y120 and Y149 residues are located within a region

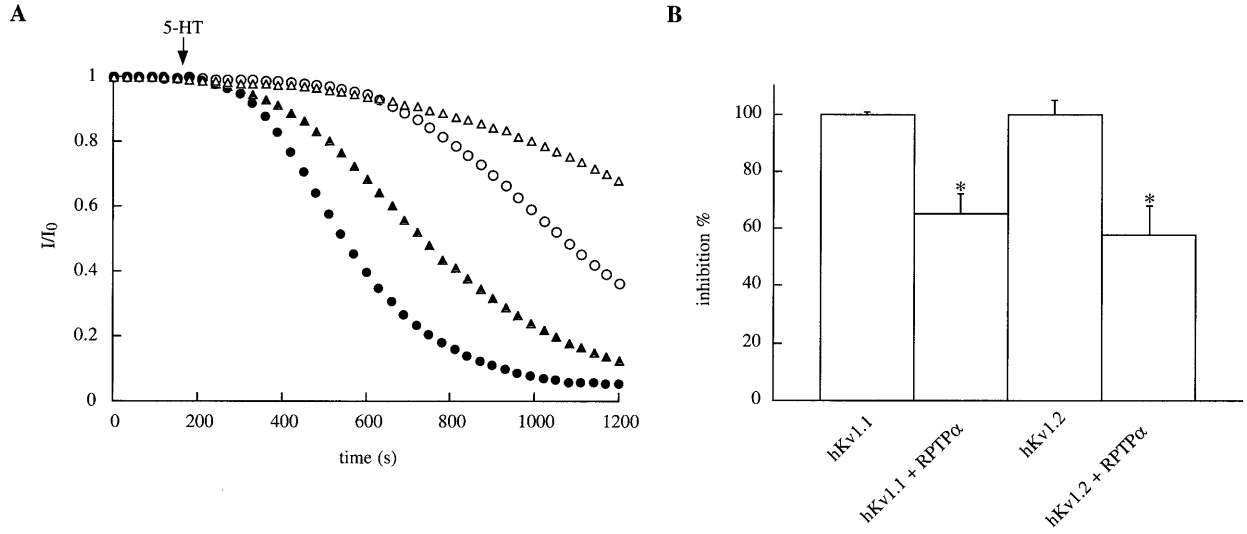

Fig. 4A, B RPTPα reduces hKv1.1 and hKv1.2 current inhibition by the 5-HT_{2C} receptor. **A** Representative time course of current inhibition induced by 5-HT (1 nM) for both hKv1.1+RPTPα (*open circles*) and hKv1.2+RPTPα (*open triangles*). Control currents are also shown for comparison: hKv1.1 (*closed circles*) and hKv1.2 (*closed triangles*). **B** Normalised percentage of current inhibition obtained in the absence and by the co-expression of RPTPα. $^{*}P<0.01$ compared with controls

linking the T1 domain to the first transmembrane segment (T1–S1 linker). The phosphorylation of these tyrosine residues may cause allosteric changes within this region leading to channel closure. Additionally, Y120 is highly conserved throughout the Kv1.x family and resides directly within a region of the channel which has been shown to form the interaction site for the Kvβ1 subunit in Kv1.5 [19]. Given the well documented ability of Kvβ subunits to modify the gating of Kv channels and the importance of the above mentioned region in the N-type inactivation mechanisms, it is perhaps not surprising that phosphorylation of a residue within this region has the ability to alter channel activity.

Neither of the two residues identified completely abolished the current inhibition mediated by the 5-HT_{2C} receptor. A possible explanation for this is that either phosphorylation of additional tyrosine residues is required for complete current suppression or that other pathways, not necessarily involving tyrosine phosphorylation, may be simultaneously activated by 5-HT_{2C} receptors in *Xenopus* oocytes. The relatively long time course required for complete current suppression would also be consistent with the requirement for phosphorylation of multiple residues. Nevertheless, this study clearly identifies the two single tyrosine residues that play the main role in tyrosine-kinase-mediated Kv current suppression.

Kv channel inhibition mediated via 5-HT_{2C} receptor activation is almost irreversible, especially at higher 5-HT concentrations. The rate of protein phosphorylation/dephosphorylation, specific proteolysis or channel internalisation phenomena may play a role. However, the mechanism(s) involved is not known.

Expression of RPTPα antagonises the effects of the 5-HT_{2C} receptor on hKv1.1 and hKv1.2 channel activity. These results suggest a mechanism by which RPTPα directly dephosphorylates Kv1.1, thereby regulating the properties described here. This view is consistent with the recently reported involvement of this phosphatase in the M1 muscarinic acetylcholine-receptor-induced suppression of Kv1.2 channel activity [20], in which the physical association of RPTPα with the intracellular domains of Kv1.2 reduces the tyrosine phosphorylation of the channel [20]. Here we have functionally shown that the human homologues Kv1.1 and Kv1.2 may be modulated by RPTPα and have identified this phosphatase as a likely intermediate in serotonergic signalling.

The serotonergic modulation of voltage-gated potassium channels described in this study is likely to have profound functional consequences for neuronal activity. For example, inhibition of hKv1.1 and hKv1.2 would increase neuronal excitability, augment the release of neurotransmitter, prolong the action potential duration and modify the firing pattern of specific neurons [4, 16]. Therefore, serotonergic modulation of this class of channels would affect the transmission of action potentials between specific neurons.

In conclusion, these results demonstrate that the 5-HT_{2C} receptor is able to inhibit voltage-dependent potassium channels through tyrosine-phosphorylation-dependent pathways. Importantly, we have also identified specific tyrosine residues on hKv1.1 which play a major role in the serotonergic suppression of channel activity and have identified RPTPα as a key player in this signalling process.

Acknowledgements We thank Fabio Sponcichetti for early experiments and enthusiastic interaction. We are grateful to Dr. Mark Tanouye for the Kv clones, to Dr. Jan Sap for the RPTPα clone, to Dr. Alan Hall for the RhoA clone and to Dr. J. Schlessinger for the PYK2 clone. We thank the Animal Care Unit and the Art Department for valuable technical assistance. The financial support of Telethon – Italy (Grant no.1083) and The Wellcome Trust is gratefully acknowledged. Paola Imbrici is the recipient of a fellowship from M.U.R.S.T. "Corso Biennale per Esperto in Biotecnologie Applicate alla Ricerca Scientifica Biomedica", programma operativo 94–98, obbiettivo 1, asse 7.1b cod.1245/134.

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