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Human Kv1.6 current displays a C-type-like inactivation when re-expressed in cos-7 cells

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Abstract

The human Kv1.6 K^+ channel was functionally re-expressed in COS-7 cells at different levels. Voltage-activated K^+ currents are recorded upon cell membrane depolarization independently of the level of Kv1.6 expression. The current acquires a fast inactivation when Kv1.6 expression is increased. Inactivation was not affected by divalent cations or by extracellular tetraethylammonium. We have characterized the inactivation properties in biophysical terms. The fraction of inactivated current and the kinetics of inactivation are increased as the cell becomes more depolarized. Inactivated current can be reactivated according to a bi-exponential function of time. Additional experiments indicate that Kv1.6 inactivation properties are close to those of a conventional C-type inactivation. This work suggests that the concentration of Kv1.6 channel in the cell membrane strongly modulates the kinetic properties of Kv1.6-induced K^+ current. The physiological implications of these modifications are discussed.

Keywords: Action potential; Glial cell; Ion channel; Neuron; Patch-clamp; Repolarization

K^+ channels are involved in the regulation of neuronal membrane excitability and exocytosis of hormones and neurotransmitters. Mutations in gene-encoding K^+ channels have been shown to promote strong modifications in the biophysical properties of K^+ channels, resulting in drastic alterations of tissue functionality reviewed in [1]. The K^+ channel properties have largely been investigated using heterologous re-expression systems although such an approach may lead to divergent conclusions. Indeed, cell lines can express endogenous K^+ channels, thus generating undesired electrical signal [2]. Furthermore, the biophysical properties of K^+ channels can depend on the cell line used for their re-expression [3,4].

Kv1.6 gene encodes K^+ channel from the *shaker* family. It is mainly expressed in the central and peripheral nervous system [5–12], although some woks have reported Kv1.6 expression in heart tissue [13,14], in muscle cells [15], and in granulosa cells [16]. The biophysical properties of Kv1.6 channel have been established upon Kv1.6 re-expression in different heterologous re-expression systems [17–19]. On the basis of these data, Kv1.6 channel is considered to induce a voltage-activated, slow-inactivating K^+ current. Furthermore, Kv1.6 is not susceptible to Kvβ-induced inactivation [19], although Kvβ binds to Kv1.6 protein [20]. The data presented here describe the functional re-expression of human Kv1.6 K^+ channel (Kv1.6) in COS-7 cells. We have observed that the expression of Kv1.6 channel results in fast- and slow-inactivating K^+ currents. Fast inactivation correlates well with high level of functional Kv1.6 channel associated with the cell membranes whereas slow inactivation correlates with low level of functional Kv1.6 channel. These results strongly suggest that the inactivation of Kv1.6 current in COS-7 cells is related to the concentration of Kv1.6 channel in the cell membrane.

Experimental procedures

Cell culture and transfection. COS-7 cells (ATCC CRL 1651) were grown onto 35 mm petri dish at 37 °C in Dulbecco's medium

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supplemented with fetal bovine serum (10%, v/v), L-glutamine (1%, w/v), streptomycin (100 μg ml^{-1}), and penicillin (100 UI ml^{-1}). The transfection was realized when cell confluence reached 80–85%. Polyethylenimine (PEI)-was used as transfection vector [4]. In brief, human Kv1.6-encoding plasmid (pCDNA3-CMV-Kv1.6, a kind gift of Dr. O. Pongs, Hamburg University, DR) was mixed with GFP-encoding plasmid (pTR-CMV-GFP) (total DNA = 2.8 μg) in 100 μl sterile NaCl 150 mM. The proportion of hKv1.6-encoding plasmid varied from 10% to 40%. The plasmid solutions were incubated in the presence of 10 mM PEI for 10 min at room temperature prior to their addition to the cells. Plasmid exposition was run for 24 h. The cells were dissociated and plated at low density, and protein expression was run for 24–48 h. For control experiments, COS-7 cells were sham-transfected with GFP-encoding plasmid alone (total DNA = 2.8 μg). Alternatively, COS-7 cell nuclei were microinjected using an Eppendorf ECET microinjector 5246 system with plasmid solutions (final concentration 50 μg ml^{-1}) containing Kv1.6-encoding plasmid (10% or 90%) and pTR-CMV-GFP (90% or 10%) supplemented with FITC-Dextran 0.5% (w/v) [12]. Transfected or microinjected cells were visualized by using epifluorescence microscopy.

Western-blot analysis. COS-7 cells were transfected during 24 h by high (40% total DNA) and low (10% total DNA) levels of Kv1.6-encoding plasmid. Transfected cells were harvested and rinsed twice in the presence of cold PBS (1×) supplemented with proteases inhibitors (100 μM Pefabloc, 1 μM leupeptin, 1 μM pepstatin, 0.1 μM aprotinin, 1 mM DTT, and 1 mM EDTA). The cells were incubated for 30 min at 4 °C with 0.1× PBS in the presence of protease inhibitors and finally passed through a 22 gauge needle (20 strokes). The resulting homogenate was centrifuged at 15,000*g* for 30 min to remove nuclei, mitochondria and the supernatant was further centrifuged at 100,000*g* for 60 min in order to pellet the total microsomal fraction. Microsomes were resuspended in 150 mM KCl, 10 mM Hepes, pH 7.0, at a concentration of ~10 mg/ml.

Proteins (20 or 40 μg) were separated by SDS–PAGE (10% acrylamide) according to the Laemmli method [21]. Kv1.6 protein was probed by using conventional immuno-blotting. The revelation was performed by using anti-Kv1.6 antibody (Alomone Labs, Jerusalem, Israel) and an ECL kit from Amersham (Les Ulis, France). Quantification of protein signal intensity was performed by using IP-Lab Gel software.

Electrophysiology. Ionic currents were recorded at 35 ± 2 °C using an extracellular medium containing (in mM): NaGluconate 145, KGluconate 4, $Ca_{1/2}$Gluconate 7 (free Ca^{2+}: 1.05), $Mg_{1/2}$Gluconate 4 (free Mg^{2+}: 1.15), glucose 10, mannitol 30, and Hepes 10 (pH 7.4). [12]. Patch–clamp pipettes (2.5–3.5 MΩ) were filled with (in mM): KGluconate 142, NaGluconate 4, $Ca_{1/2}$Gluconate 0.8 (free Ca^{2+}: 95 nM), $Mg_{1/2}$Gluconate 2 (free Mg^{2+}: 535 nM), EGTA 1, $ATPK_2$ 3, and Hepes 5 (pH 7.2). Currents, membrane potentials, and cell capacitance were monitored using Acquis1 (4.0) software (Biologic, Pont-de-Clay, France). Current densities were deduced from current normalization by cell capacitance. Experimental data were analyzed with Sigma Plot 2000 software (Jandel Sci., San Raphael, CA). Statistical significance was determined on Sigma Stat software (Jandel Sci., San Raphael, CA) using ANOVA followed by a Bonferroni test (***$p < 0.001$).

Results

COS-7 cells were used to study the properties of Kv1.6K^+ channel at physiological temperature. As shown in Fig. 1, Kv1.6 protein could be expressed by the cells and was recovered associated with the microsomal fraction. A ~59 kDa protein was recognized by the anti-Kv1.6 antibody in cells transfected by Kv1.6-encoding plasmid but not in sham-transfected cells (Fig. 1A).

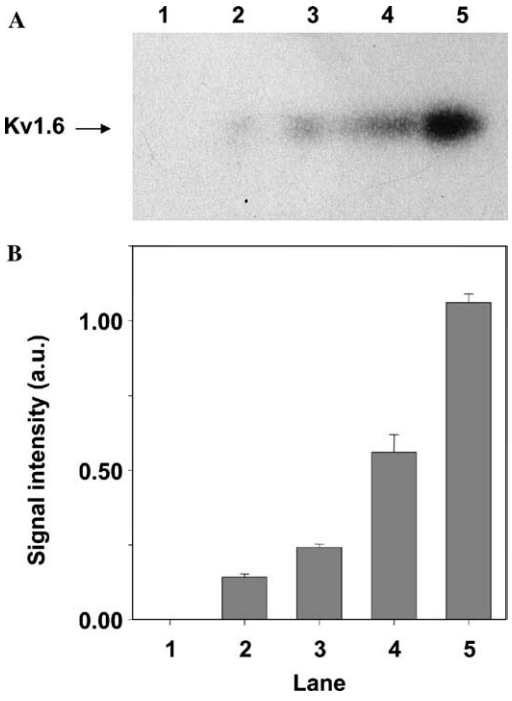

Fig. 1. Expression of Kv1.6 protein in COS-7 cells. (A) Microsomes were obtained from COS-7 cells as described in Experimental procedures. Kv1.6 protein was probed in sham-transfected cells (lane 1) and in Kv1.6-transfected cells (lanes 2, 3, 4, and 5). Forty micrograms (lanes 1, 3, and 5) and 20 μg (lanes 2 and 4) of microsomal protein were used in our conditions. (B) The quantification of the signal (expressed in arbitrary unit: a.u.) corresponding to Kv1.6-associated band was realized as described in Experimental procedures. Data are expressed as means ± SEM of three independent Western blots.

Furthermore, increasing the quantity of plasmid delivered to the cell from 10% (total DNA) to 40% (total DNA) resulted in a larger amount of Kv1.6 protein associated with the cell membranes (Fig. 1B).

Patch–clamp of transfected cells was performed to assay the functionality of Kv1.6 channel. Upon cell stimulation, a voltage-activated outward current was specifically observed in Kv1.6-expressing cells (Figs. 2A and B), but not in sham-transfected cells (Fig. 2C). Surprisingly, the time-course of Kv1.6-induced current was heterogeneous, since slow- (Fig. 2A) and fast-inactivating (Fig. 2B) currents were recorded in cells transfected in similar conditions. These results show that fast- and slow-inactivating currents corresponded to K^+ current originating from Kv1.6 channel expression in COS-7 cells. The addition of extracellular TEA (10 mM) reduced the amplitude of fast- (data not shown) and slow- (data not shown) inactivating currents. However, TEA did not modify the time-course of fast-inactivating current (data not shown).

The activation of slow-inactivating Kv1.6-induced current was voltage-dependent and occurred at a membrane potential threshold close to −50 mV (Fig. 3). Furthermore, it was characterized by a half-maximal activation potential $V_{0.5\,act} = 12.3 \pm 7.0$ mV ($n = 8$)

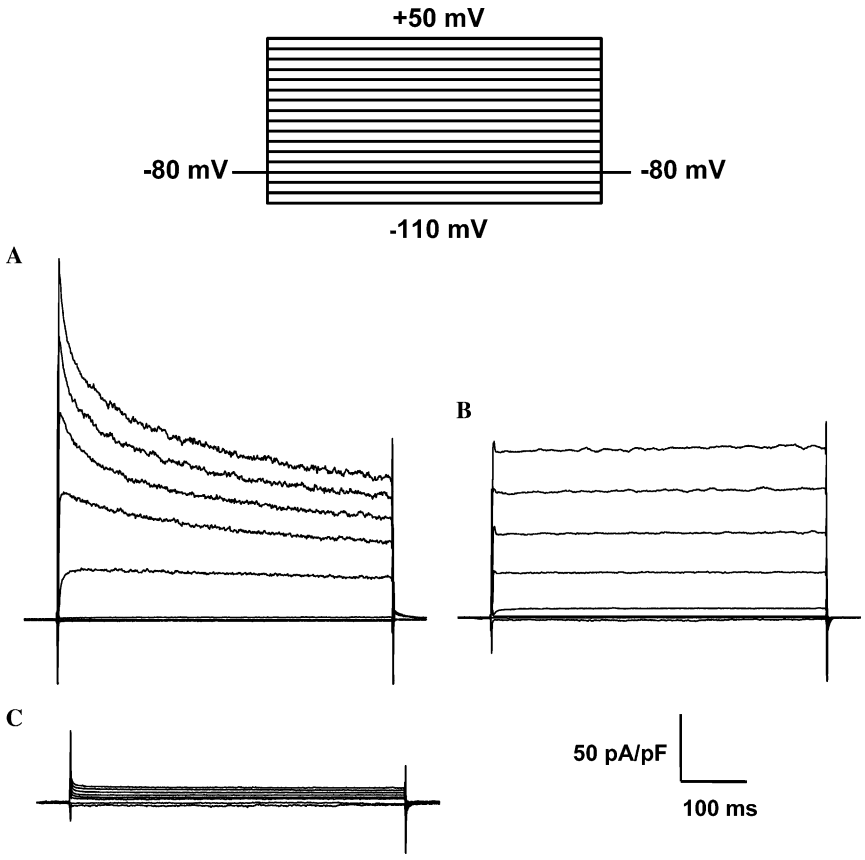

Fig. 2. Kv1.6 channel expression induced outward current in COS-7 cells. Cells were transfected as described in Experimental procedures. The macroscopic current was recorded after the cell stimulation according to the protocol depicted in the figure (upper panel). For the sake of clarity of illustrations, one trace over two is given. (A) and (B) Current traces recorded in two independent cells transfected with Kv1.6-encoding plasmid (40% total DNA). (C) Current traces obtained in sham-transfected cell.

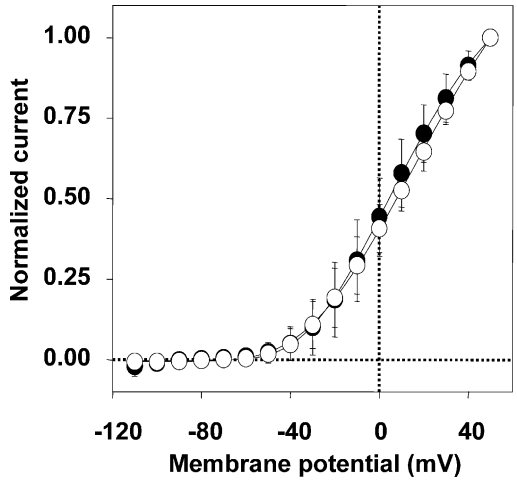

Fig. 3. Kv1.6-induced currents are voltage-activated. Cells were transfected and stimulated as described in Fig. 2. Current densities were measured at a time corresponding to the peak of current. They were normalized to that measured at +50 mV. Closed and open circles correspond to the mean ± SEM of current density found in fast- ($n = 19$) and slow-inactivating currents ($n = 8$). Curves were fitted (solid lines) using the Boltzmann's equation.

(Fig. 3). This indicates that the expression of Kv1.6 channel resulted in a voltage-activated K^+ current, the properties of which are similar to those reported for functional re-expression of Kv1.6 channel in other re-expression systems [17–19].

However, a fast-inactivating Kv1.6-induced current was also observed in cells transfected by Kv1.6-encoding plasmid. A recent report has described the strengthening by Mg^{2+} of the inactivation of ROMK2-induced current upon the re-expression of ROMK2 channel in the same conditions [4]. Here, the removal of Ca^{2+} and Mg^{2+} from the extracellular or from the intracellular media neither modified the occurrence of fast-inactivating Kv1.6-induced current nor changed its biophysical properties (data not shown). Thus, the fast inactivation of Kv1.6-induced current cannot be related to an effect of intra- or extracellular divalent cations.

The biophysical characteristics of the fast-inactivating current were further characterized. The activation properties of the fast-inactivating Kv1.6-induced current are shown in Fig. 3. The current was clearly voltage-activated with an activation threshold close to −50 mV. A half-maximal activation potential $V_{0.5\,act} = 3.4 \pm 6.0$ mV

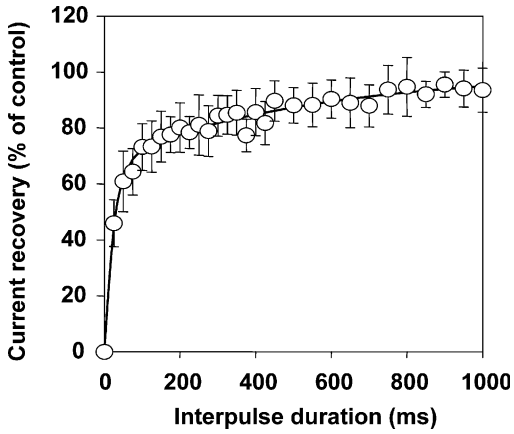

Fig. 4. Reactivation of inactivated hKv1.6-induced current. Current reactivation was assayed by using a protocol consisting of two depolarizations from −80 mV to +20 mV separated by a variable inter-pulse duration (increment: 25 and 50 ms for intervals lasting from 0 to 400 ms and from 450 to 1000 ms, respectively). Current recovery (expressed in %) corresponded to $100 \times (I_{peak2}/I_{peak1})$. Data are expressed as means ± SEM ($n = 9$). Solid line corresponds to a bi-exponential fit of the data.

($n = 19$) was determined from activation curve (Fig. 3) and this value was not significantly different from that calculated for slow-inactivating Kv1.6-induced current. Moreover, we observed a recovery of Kv1.6-induced current from its inactivated state (Fig. 4). This recovery was characterized as a function of time and the data were best fitted by a bi-exponential function. In these conditions, we determined $\tau_{recov1} = 19.6 \pm 5.0$ ms ($n = 9$) and $\tau_{recov2} = 214.5 \pm 43.1$ ms ($n = 9$). A steady-state inactivation protocol consisting of two depolarizing pulses was applied to COS-7 cells expressing Kv1.6 protein. As shown in Fig. 5A, KV1.6-induced current inactivation was not complete and a residual current (~20% of the peak value) was maintained. From curve fitting, we calculated a $V_{0.5\,inac} = -43.1 \pm 3.1$ mV ($n = 8$). The time constants of the current decay were calculated considering the fact that the current inactivation was best fitted by a bi-exponential function of time. The values for time constants were plotted as a function of membrane potential. As shown in Fig. 5B, an increase in membrane depolarization mainly resulted in a reduction of τ_{inac1}, although a reduction τ_{inac2} was also seen for potentials above +20 mV. Furthermore, we calculated the fraction of inactivated Kv1.6-induced current (see Fig. 5C, inset) at any membrane potentials and for transfections realized with high (i.e., 40% total DNA) or low (i.e., 10% total DNA) levels of Kv1.6-encoding plasmid. For both conditions, the fraction of inactivated current increased as membrane potential became more positive (Fig. 5C). The data were mono-exponentially fitted (Fig. 5C). We deduced voltage constants of 19.1 ± 2.0 and $33.7 \pm 1.5\,mV^{-1}$ for cells transfected by high and low levels of Kv1.6-encoding plasmid, respectively. Interestingly, the amplitude of the inactivated fraction was influenced by the level of Kv1.6-encoding plasmid used for cell transfection. Indeed, the fraction of inactivated current measured for high (i.e., 40% total DNA) level of plasmid was systematically larger than that measured for low (i.e., 10% total DNA) level of plasmid. Taken as a whole, these results establish that expression of Kv1.6 channel induced a K^+ current that displays a strong, but incomplete, inactivation as a function of time, membrane potential, and Kv1.6 protein expression.

Increasing the level of plasmid for cell transfection resulted in a higher expression of Kv1.6 channel in the cell membrane (see Fig. 1). Thus, it is tempting to establish a relation between the fast inactivation of Kv1.6-induced current and the amount of Kv1.6 protein synthesized by the cells. This is strengthened by the data illustrated in Fig. 6. Indeed, we calculated the

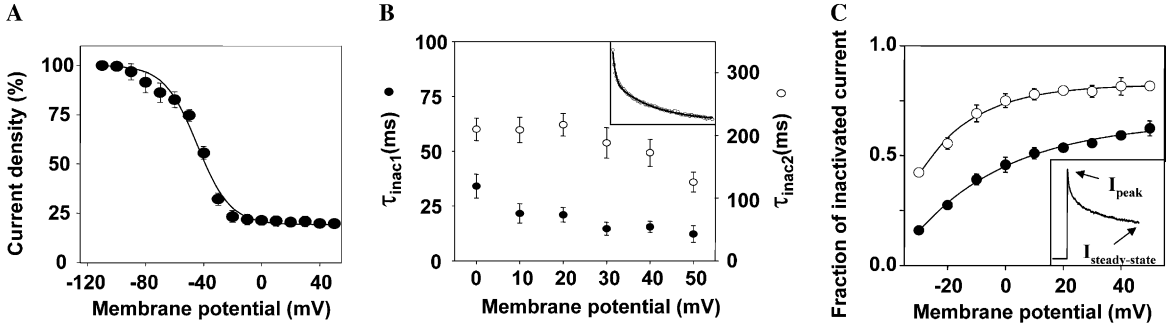

Fig. 5. Inactivation of hKv1.6-induced current is voltage-dependent. Transfected cells were submitted to a steady-state inactivation protocol consisting of a pre-pulse from −110 to +50 mV (holding potential −80 mV, voltage increment 10 mV, and 1500 ms duration) followed by a pulse to +50 mV (500 ms duration). (A) Peaks of current density were normalized to that measured after a pre-pulse to −110 mV. They were plotted as a function of membrane potential. Data are expressed as means ± SEM ($n = 8$). The curve was fitted by using the Boltzmann function. (B) Time constants of the inactivation process were plotted as a function of membrane potential after their calculation from a bi-exponential fit of the current decay (see inset: open circles and solid line correspond to the current values and data fit, respectively). Solid lines correspond to manual fit of the data. Data are expressed as means ± SEM ($n = 8$). (C) The fraction of inactivating current was defined as $(I_{peak} - I_{steady\ state})/I_{peak}$ (see inset) and plotted as a function of membrane potential. Open and closed circles correspond to cells transfected with 40% (total DNA) ($n = 8$) and with 10% (total DNA) ($n = 8$) of hKv1.6-encoding plasmid, respectively. Data are expressed as means ± SEM. Solid lines correspond to mono-exponential fits of the data.

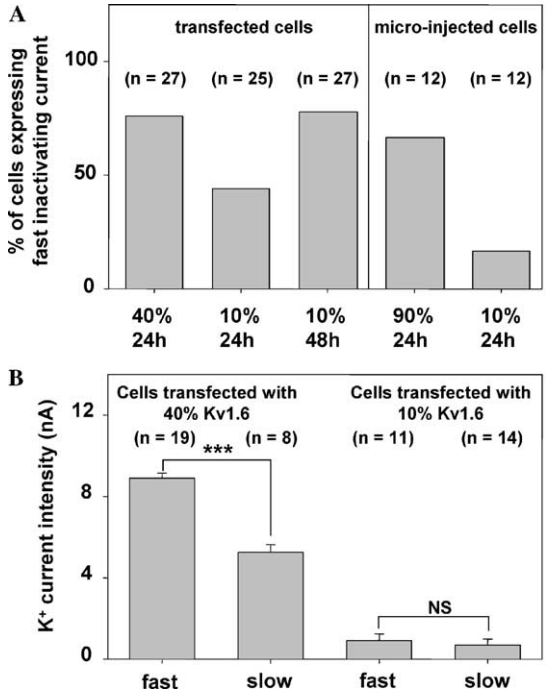

Fig. 6. Kv1.6-induced current inactivation is promoted by an increase in the expression of Kv1.6 protein. (A) The percentage of cells exhibiting an inactivating current was determined after the cell transfections by high level (40% total DNA) and low level (10% total DNA) of Kv1.6-encoding plasmid and after the intra-nuclear injection of high and low amounts of Kv1.6-encoding plasmid. The number of analyzed cells is given into brackets. Data correspond to means ± SEM. (B) The intensity of the current was measured after a depolarization of the cell membrane at +50 mV, for cells exhibiting fast- and slow-inactivating currents. Transfection conditions were: high level (40% total DNA) or low level (10% total DNA) of Kv1.6-encoding plasmid. The number of analyzed cells is given in brackets. Data correspond to means ± SEM.

percentage of cells expressing fast inactivation in different conditions. On the one hand, the level of Kv1.6 plasmid was changed from 10% to 40% (total DNA) and the time for protein expression was run for 24 h. Fig. 6A shows that the percentage of cells with inactivating current raised from 44% to 76% in these conditions. Similar results were obtained when intra-nuclear injection of the plasmid instead of transfection was used (Fig. 6B). On the other hand, the cells were transfected by 10% (total DNA) of Kv1.6-encoding plasmid and the current was recorded after 24 or 48 h expression time (Fig. 6A). In these conditions, we observed that the percentage of cells with inactivating Kv1.6-induced current increased from 44% to 77.8% (Fig. 6A). Finally, the current intensity was measured after a depolarization to +50 mV in the population of cells expressing fast- and slow-inactivating currents after their transfection by high (i.e., 40% total DNA) or by low (i.e., 10% total DNA) level of Kv1.6-encoding plasmid. At low (10% total DNA) level of Kv1.6-encoding plasmid, the current intensities were 0.91 ± 0.33 nA ($n = 11$) and 0.70 ± 0.29 nA ($n = 14$) for fast- and slow-inactivating current (Fig. 6B). The difference was not statistically significant (NS). On the contrary, the intensities after the cell transfection by high (40% total DNA) level of Kv1.6-encoding plasmid were 8.9 ± 0.25 nA ($n = 19$) and 5.2 ± 0.38 nA ($n = 8$) for fast- and slow-inactivating Kv1.6-induced current (Fig. 6B). In these conditions, the difference reached statistical significance with $p < 0.001$.

Discussion

The biophysical properties of recombinant human Kv1.6K^+ channel were investigated at physiological temperature in mammalian COS-7 cells. We have used two conditions of transfections, i.e., high and low levels of Kv1.6-encoding plasmid. In these conditions, our data show that the amount of Kv1.6 channel found in the microsomal fraction is proportional to the level of Kv1.6-encoding plasmid used for cell transfection. Our study shows that transfected cells display an outward K^+ current upon their stimulation. Furthermore, it is reported that increasing the expression of Kv1.6 channel resulted in a larger K^+ current intensity. These results show that part, if not totality, of Kv1.6 channels expressed here are functional and are properly targeted to the plasma membrane.

Kv1.6-induced currents are activated by a cell membrane depolarization. This property is consistent with that reported for Kv1.6-induced current in other re-expression systems [17–19]. However, two populations of cells can be identified on the basis of the current time course. A first population exhibits virtually no, or a slow, decay of the current during time. A second population displays a fast time-dependent decay upon cell membrane depolarization. We have demonstrated that the current discrepancy corresponds to an inactivation process. Indeed, as it has been established for other inactivating K^+ currents [22], the decay of Kv1.6-induced current is clearly conditioned by membrane depolarization and inactivated Kv1.6-induced current can be reactivated as a function of time.

The inactivation of K^+ current proceeds according to different mechanisms reviewed in [23]. The interaction of the K^+-conducting channel with ancillary β subunits is known to promote a current inactivation [24,25]. In contrast with HEK293 cells [2], no endogenous Kv α and β subunits have been described in COS-7 cells, yet. It is thus unlikely that Kv1.6-induced current inactivation results from Kv1.6 protein interaction with β subunit. Alternatively, K^+ current inactivation can be promoted by specific domains of the K^+ channel itself [23]. In N-type inactivation, an N-terminal motif of the channel is committed to interact with the intracellular face of the channel and to plug the pore. In most of the cases, this leads to a complete abolition of the current. In C-type inactivation, the current decay originates from

a pore constriction in a region corresponding to the pore's mouth [26–28]. Most of C-type inactivation so far observed in Kv-induced current maintained a residual current at the end of the pulse. Interestingly, extracellular TEA discriminates between N-type, which is insensitive to TEA, and C-type inactivation, which is slowed by TEA [27]. Structural and functional evidences argue against N-type inactivation in Kv1.6-induced current [19]) and because of this, one could explain our observations considering a C-type inactivation. This is supported by the fact that the inactivation of Kv1.6-induced current is incomplete in our conditions. However, the absence of effect of extracellular TEA on the kinetics of inactivation is in contradiction with a canonical C-type inactivation. Taken as a whole, our data indicate that the inactivation of Kv1.6-induced current is likely to result from a C-type inactivation, although it is promoted by an unconventional mechanism.

The mechanism underlying Kv1.6-induced current inactivation is presently unknown. Diacylglycerol-induced inactivation of Kv1.6-induced current has been reported upon re-expression of Kv1.6-channel in HEK 293 cells and in oocytes [29]. In this work, DAG induces a complex bi-exponential discrepancy of Kv1.6-induced current. The data presented here describe a similar inactivation, even though the current is recorded in whole cell condition and in the absence of exogenous regulators. We show that the inactivation of Kv1.6-induced current is large in amplitude and is accelerated when high level of Kv1.6 protein is associated with the cell membrane. On the opposite, when Kv1.6 is expressed at low level in the cell membrane, the current presents virtually no or slow inactivation. This clearly establishes a relation between Kv1.6-induced current inactivation and the expression of the protein in the cell membrane. A similar relation had already been postulated in the case of Kv1.3 channel, the over-expression of which leads to a faster inactivation of the current [30]. Thus, we propose that the concentration of Kv1.6 channel in the membrane controls the biophysical properties of Kv1.6-induced current.

The consequences of these modifications in Kv1.6 channel biophysical properties by the level of protein found in the plasma membrane can be important for the cell physiology. Indeed, it was recently reported that Kv1.6 mRNA level increased in oligodendrocyte progenitors treated with platelet-derived growth factor (PDGF) [31]. This up-regulation correlates with the PDGF-dependent stimulation of progenitor cell division [32] and the change in KV1.6-induced current properties may participate in the cell proliferation scheme. Furthermore, Kv1.6 channel activity contributes to the repolarization of neuronal action potential and its inhibition leads to an increase in the action potential duration [8]. Previous results have shown that Kv1.6 expression is up-regulated in neurons from the spinal chord upon chronic administration of morphine [9]. In these conditions, it is predicted that the action potential should be shortened. Nevertheless, the inactivation in Kv1.6-induced current described here suggests a decrease in repolarizing Kv1.6-induced K^+ current and thus will result in an increase of the action potential duration. Thus, the over-expression of Kv1.6 will deeply reduce the ability of these cells for multiple firing responses, and this may participate in the long-term attenuation of the neuronal excitability induced by opiates.

In summary, our data show that a modification of the biophysical properties of Kv1.6-induced K^+ current is triggered by the expression level of Kv1.6 channel in the cell membrane. The inactivation properties are close to-, although not exactly similar to, canonical C-type inactivation, indicating that a new molecular mechanism is responsible for it.

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