

RAPID REPORT

Expression and function of dipeptidyl-aminopeptidase-like protein 6 as a putative β -subunit of human cardiac transient outward current encoded by Kv4.3

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Dipeptidyl-aminopeptidase-like protein 6 (DPPX) was recently shown in the brain to modulate the kinetics of transient A-type currents by accelerating inactivation and recovery from inactivation. Since the kinetics of human cardiac transient outward current (I_{to}) are not mimicked by coexpression of the α -subunit Kv4.3 with its known β -subunit KChIP2, we have tested the hypothesis that DPPX may serve as an additional β -subunit in the human heart. With quantitative real-time RT-PCR strong mRNA expression of DPPX was detected in human ventricles and was verified at the protein level in human but not in rat heart by a DPPX-specific antibody. Co-expression of DPPX with Kv4.3 in Chinese hamster ovary cells produced I_{to} -like currents, but compared with expression of KChIP2a and Kv4.3, the time constant of inactivation was faster, the potential of half-maximum steady-state inactivation was more negative and recovery from inactivation was delayed. Co-expression of DPPX in addition to Kv4.3 and KChIP2a produced similar current kinetics as in human ventricular myocytes. We therefore propose that DPPX is an essential component of the native cardiac I_{to} channel complex in human heart.

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The transient outward K^+ current (I_{to}) plays an essential role in initiating the early repolarization phase of the cardiac action potential (AP). In the human heart the respective channel-forming α -subunit is Kv4.3 (Dixon *et al.* 1996) and several accessory β -subunits like KChIPs, MiRPs and KChAPs can significantly modify the electrophysiological properties of I_{to} when coexpressed in heterologous expression systems (reviewed by Pourrier *et al.* 2003). Nevertheless, expression of Kv4.3 alone or in combination with the β -subunits KChIP2 or KCNE2 (Deschenes & Tomaselli, 2002; Jiang *et al.* 2004) does not reproduce the characteristic kinetics of the native human cardiac I_{to} , for instance fast time courses of inactivation and recovery from inactivation, suggesting that additional accessory β -subunits may be required to mimic the characteristics of native I_{to} . One potential candidate is the dipeptidyl-aminopeptidase-like protein 6 (DPPX; EC 3.4.14.5), which has recently been discovered in neuronal brain tissue of rat where it substantially accelerates inactivation and recovery from inactivation of A-type

currents encoded by Kv4.2 and Kv4.3, which are the brain correlates of I_{to} in rat (Nadal *et al.* 2003).

DPPX belongs to the prolyl-oligopeptidase family of serine proteases but lacks enzymatic activity (Wada *et al.* 1992; Kin *et al.* 2001). In rat, expression of DPPX mRNA was detected in several organs, including brain, kidney, ovary, prostate and testis, but not heart (Wada *et al.* 1992). While the long mRNA isoform (DPPX-L) is expressed almost exclusively in brain, the short isoform (DPPX-S) is detected also in the above-mentioned peripheral tissues (Wada *et al.* 1992). In human, DPPX has been identified in the brain (Yokotani *et al.* 1993), but it is not known whether it is also expressed in the heart.

In order to test the hypothesis that DPPX could serve as an I_{to} -modulating β -subunit in human heart we have examined the expression of the molecule at the mRNA and protein level in human right and left ventricular tissue and studied the functional effects of DPPX on I_{to} in a stable Chinese hamster ovary cell (CHO) expression system for Kv4.3 and KChIP2a.

Methods

Molecular biology

cDNAs encoding the long and the short isoforms of DPPX (DPPX-L; DPPX-S) cloned into pRC/RSV vector (Invitrogen) were a kind gift from Professor Keiji Wada (Tokyo, Japan). The pEGFP-N1 vector was from Clontech (Palo Alto, CA, USA). The DPPX-S isoform was used for transfection because in rat studies it was reported to be more widely expressed in different organs while the long isoform was found almost exclusively in brain (Wada *et al.* 1992; Kin *et al.* 2001).

Cardiac tissue samples were collected from explanted hearts of NYHA IV patients who had given written consent or from non-failing donor hearts which could not be used for transplantation due to technical reasons. Tissues from other organs were collected from autopsies. (The study conformed with the Declaration of Helsinki.) Total RNA was isolated using the LiCl method (Sambrook & Russel, 2001). Real-time RT-PCR was performed using the QuantiTect SYBR[®] Green RT-PCR kit (Qiagen, Hilden, Germany) and Rotor Gene thermal cycler (Corbett Research, Mortlake, Australia). In order to detect total DPPX we used the primers DPPX_F (5'-AGGAGAAGGACCAGATGGAG-3') and DPPX_R (5'-GAAAACGCAGAGGCATAGAG-3') and for DPPX-S the primers DPPX-S_F (5'-AAATCCGTGCAGCAGCAGCA-GGAAC-3') and DPPX-S_R (5'-AGGTGACGATCAAGG-AGCAGATG-3'). Real-time RT-PCR conditions were: 50°C for 30 min, 95°C for 15 min 30 s at 94°C, 30 s at 61°C, 30 s at 72°C and 15 s at 80°C. cRNA standards were produced by T7 *in vitro* transcription with the mMessage machine kit (Ambion, Austin, TX, USA) from RT-PCR products produced as above but using a modified forward primer containing an additional T7 promoter sequence (GGCCGCGG). Standards of known concentration of RNA included in each dilution series (10^5 – 10^{12} molecules μl^{-1}) allowed calculation of cRNAs. Results were analysed by Rotorgene software version 4.6 (Corbett Research). Qualitative RT-PCR was performed following standard procedures.

DPPX antibody

cDNA encoding 69 amino acids located at the C-terminus of DPPX (EEKDQMEAVRTMLKEQYIDR-TRVAVFGKDYGGYLSTYILPAKGENQGQTFCTGSALSP-ITDFKLYASAF) was amplified by RT-PCR from human atrium total RNA. The PCR product was cloned in frame with the thioredoxin gene and a His-Tag in the pET32a(+) vector (Novagen, Madison, WI, USA). Recombinant protein was expressed in *E. coli* BL21(DE3), according to the pET System handbook (Novagen). The DPPX fusion protein was purified on a Ni-NTA agarose affinity resin (Qiagen) according to the manufacturer's instructions.

The purified protein was used to raise an antibody in rabbits and to produce an affinity column by coupling the recombinant DPPX protein to a HiTrap[™] NHS-activated HP column (Amersham, Freiburg, Germany) for further purification.

Western blotting

Tissue samples and DPPX-transfected cells were homogenized in phosphate-buffered saline (PBS) containing 8 M urea. Protein extracts were run on a 7.5% SDS-PAGE gel (18 μg of cells and 75 μg of tissues) and blotted onto a nitrocellulose membrane. Membranes were blocked in Tris-buffered-saline (TBS), 1% milk and probed overnight with the anti-DPPX antibody (diluted 1:200). Membranes were washed, probed with a secondary antibody (anti-rabbit conjugated with horseradish peroxidase (HRP), diluted 1:5000, Amersham), and detected by chemiluminescence. For the antigen competition assay, 15 μg of antibody was incubated with 150 μg of antigen for 3 h before probing the blotted membrane.

Cell culture, transfection and patch clamp experiments

Chinese hamster ovary (CHO) cell lines stably transfected with hKv4.3 or hKv4.3/hKChIP2a were gifts from Dr K. Steinmeyer (Sanofi-Aventis Pharma, Frankfurt, Germany). Cells were maintained at 37°C, 5% CO₂, in basal Iscove's medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS), 1% glutamine, 100 $\mu\text{g ml}^{-1}$ Zeocin and 400 $\mu\text{g ml}^{-1}$ G418. Cells were seeded on 24-well plates at a density of 75 000 cells per well. Transfections were done with the Roti[®]-Fect transfection reagent (Carl Roth, Karlsruhe, Germany) according to the manufacturer's instructions, using 2.5 μl of transfection reagent, 0.5 μg of DPPX-S vector and 0.2 μg of pEGFP-N1 per well. After 48 h of incubation to allow protein synthesis, cells were harvested by trypsinization. Transient outward currents in CHO cells were measured with standard patch clamp techniques. Raw data were digitized at 10 kHz sampling rate, pulse protocols were generated by ISO2 software (MFK, Niedernhausen, Germany). Mean series resistance was $8.4 \pm 0.1 \text{ M}\Omega$ ($n = 66$) and compensated by at least 70%. Mean cell capacitance was $33.7 \pm 2.7 \text{ pF}$ ($n = 61$) in non-transfected and transfected cells. Currents were normalized to cell capacitance and expressed in pA pF⁻¹. Superfusion solution contained (mM): NaCl 150, KCl 5.4, MgCl₂ 2, CaCl₂ 1.8, Hepes 10, glucose 11, pH 7.4. Pipette solution contained (mM): KCl 40, potassium aspartate 80, NaCl 8, CaCl₂ 2, MgATP 5, EGTA 5, GTP 0.1, Hepes 10, pH 7.4 adjusted with KOH. The liquid junction potential for this solution was 11 mV as calculated with the software JPCalc (P. H. Barry, Sydney, Australia). Data are not corrected for the junction potential.

Statistical analysis

All results are given as means \pm s.e.m. Statistical analysis was performed with Student's *t* test or one-way ANOVA with Bonferroni's *post hoc* test (GraphPad Prism software, V4.1; San Diego, CA, USA). Differences were considered to be significant if $P < 0.05$.

Results

mRNA expression of DPPX in human heart

Qualitative RT-PCR exhibited clear positive mRNA expression of DPPX in human brain (3/3), pancreas (3/3), testes (2/2) and heart (5/5). No expression was detected in bladder (0/2), liver (0/3) and spleen (0/3), and uncertain results were found for lung (2/3), ovary (1/2) and kidney (1/3), the numbers in brackets indicating number of positive samples from number of autopsies (Fig. 1A). The short isoform of DPPX has been shown to be expressed not only in brain but also in peripheral tissues (Wada *et al.* 1992). Since this isoform was used for the expression experiments we also tested cardiac expression of DPPX-S, which could clearly be detected by RT-PCR (Fig. 1B).

Since cardiac I_{to} exhibits a transmural gradient (sub-epicard > subendocard; Wettwer *et al.* 1994) distribution

of DPPX expression between subepi- and subendocardial tissue layers of left and right ventricles was determined. Large amounts of DPPX were detected in all samples investigated and differences between the regions did not reach the level of statistical significance. Pooled data yielded a mean mRNA content of 453 ± 59 fg (ng of total RNA)⁻¹ (Fig. 1D). DPPX was also detected in tissue samples from non-failing hearts, however, the pooled data yielded a significantly lower expression level of 340 ± 20 fg ng⁻¹ (Fig. 1C).

Western blot analysis of DPPX

Our antibody was validated with total protein extracts from human and rat brain and DPPX-transfected CHO cells as positive controls. In both human and rat brain, the antibody recognized a band with an apparent molecular mass of about 100 kDa as expected (Kin *et al.* 2001; Nadal *et al.* 2003; Zaghera *et al.* 2005) (Fig. 2A). The antibody recognized two bands at about 107 and 115 kDa in CHO cells, corresponding to the short and long isoforms, respectively. In human heart preparations, the antibody recognized one band at 100 kDa which was absent in rat heart and differed from bands in brain preparations by about 2 kDa (all apparent molecular

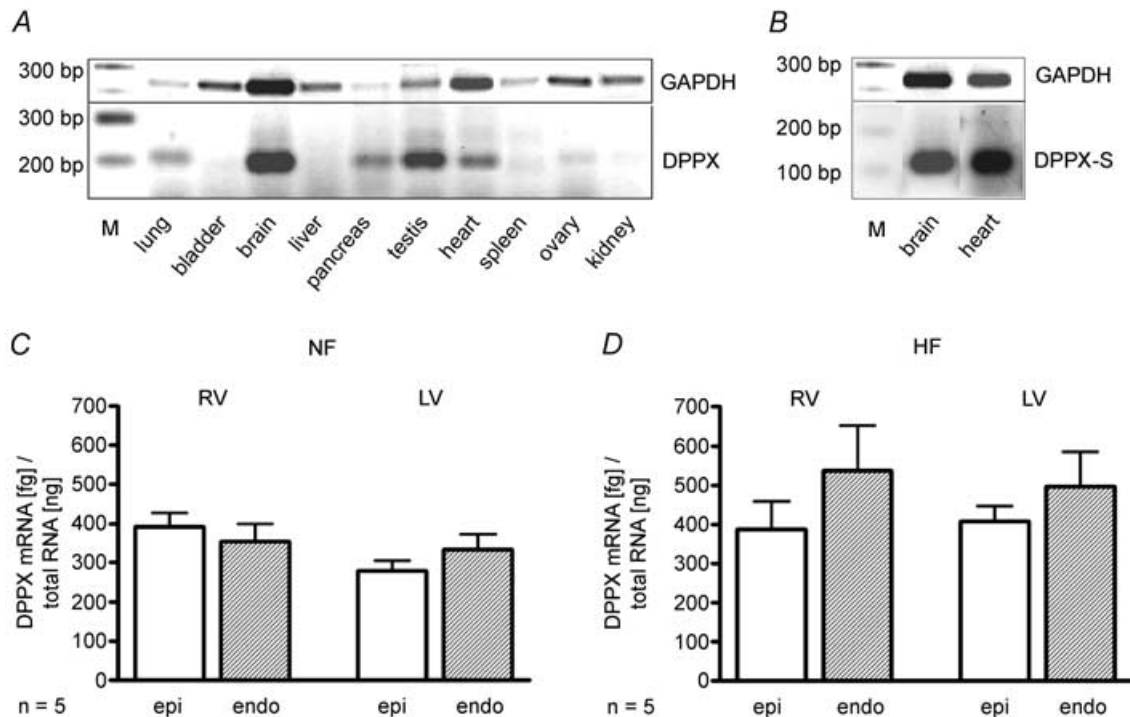


Figure 1. mRNA expression of DPPX in human tissues

A, distribution of DPPX mRNA among different human tissues as analysed by RT-PCR. Lanes: M: DNA ladder (Roche XIV); 1–10: organs as indicated. Expression of GAPDH served as control for mRNA integrity. B, expression of DPPX-S isoform in human brain and heart. mRNA expression of DPPX in 5 non-failing (C) and 5 failing human hearts (D), Quantification by real-time RT-PCR in samples from epi- and endocardial layers of right (RV) and left ventricles (LV).

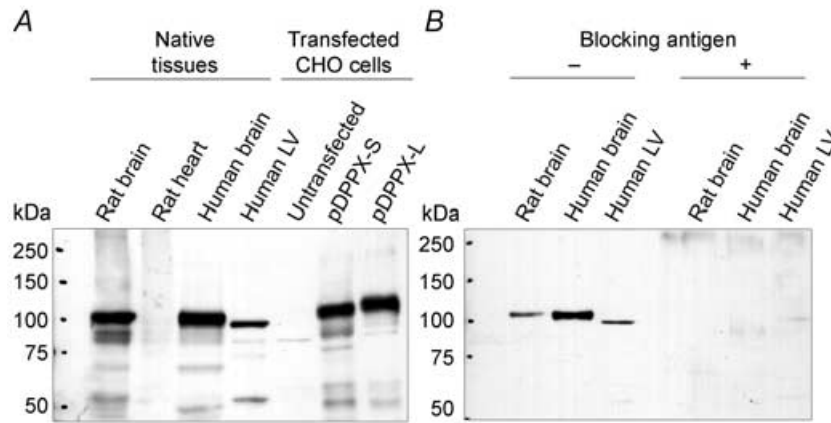


Figure 2. Western blot analysis of DPPX
 A, total protein extracts from the indicated tissues (75 µg) or CHO cells (18 µg) transfected with DPPX as positive controls were separated by 7.5% SDS-PAGE, blotted onto nitrocellulose membranes and probed with our DPPX antibody. B, test for antibody specificity by preincubation with the antigen; 150 µg of antigen were used to block 15 µg of antibody. Protein standards: Precision Plus Protein™ (Biorad). LV: left ventricle.

weights were calculated with the Phoretics 1D gel analysis software). Specificity of the DPPX antibody was tested by Western blot after preincubation with the blocking antigen (Fig. 2B), which completely abolished all bands from brain and heart preparations.

Electrophysiological recordings of I_{to} in CHO cells

Typical test pulses from -80 mV to test pulses between -40 and $+60$ mV did not activate any I_{to} in wild-type CHO control cells or minute currents in cells transfected with Kv4.3 (2.3 ± 0.7 pA pF $^{-1}$ steady-state current at $+50$ mV, $n = 6$, Fig. 3A). In comparison,

coexpression of Kv4.3 and KChIP2a yielded large transient outward currents (495 ± 86 pA pF $^{-1}$ at $+50$ mV, $n = 26$, Fig. 3B). Transient outward current in cells coexpressing Kv4.3 with DPPX (Fig. 3C) exhibited smaller current density (399 ± 90 pA pF $^{-1}$ at $+50$ mV, $n = 6$) and significantly different kinetics: the time constant of inactivation at $+50$ mV was smaller (25 ± 6 ms, versus 55 ± 4 ms, $P < 0.05$), $V_{0.5}$ of steady-state inactivation was shifted to more negative potentials (-57.0 ± 3.1 mV versus -31.3 ± 2.4 mV, $P < 0.01$), and recovery from inactivation was slower (52 ± 16 ms versus 42 ± 8 ms). In cells expressing Kv4.3 and DPPX-S in addition to KChIP2a (Fig. 3D) current density was smaller compared to cells with KChIP2a expression alone (373 ± 50 pA pF $^{-1}$,

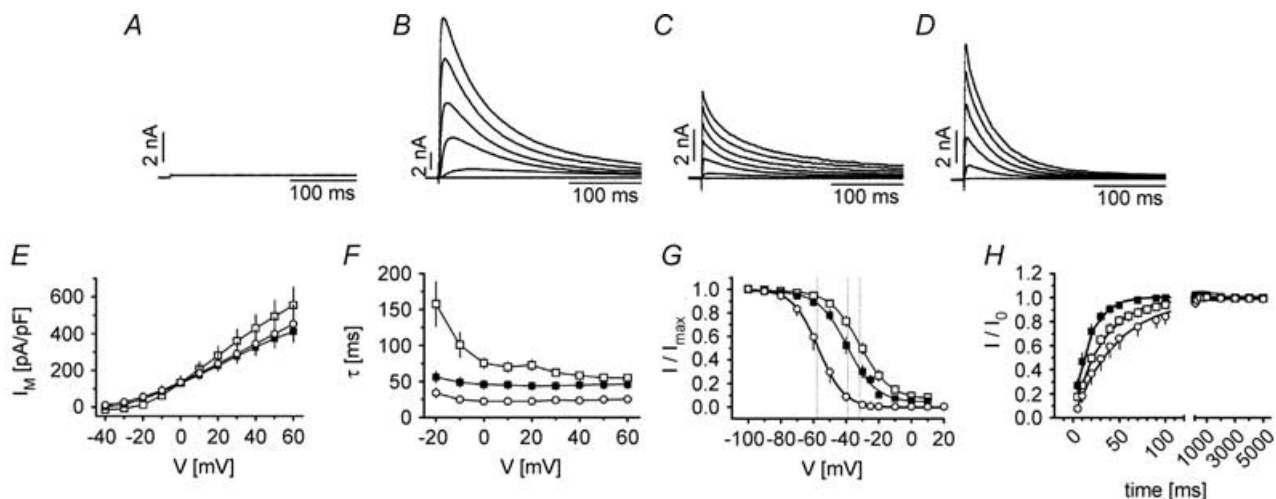


Figure 3. Effects of KChIP2a and DPPX on Kv4.3 currents expressed in CHO cells

A–D, current tracings (initial 300 ms) elicited by test steps (1000 ms) from -80 mV to -40 , -20 , 0 , $+20$, $+40$ and $+60$ mV in CHO cells stably transfected with Kv4.3 (A), Kv4.3 plus KChIP2a (B, □), Kv4.3 plus DPPX (C, ○), and Kv4.3 plus KChIP2a plus DPPX (D, ■). E, current–voltage relations of I_{to} in the transfected CHO cells. F, voltage dependence of fast time constant of I_{to} inactivation determined from fitting single exponential curves to individual current traces. G, normalized steady-state inactivation (I/I_0) curves for I_{to} . Fitting of a Boltzmann function to mean data yielded the following potentials of half maximum inactivation ($V_{0.5}$, dotted lines): -31.5 mV for Kv4.3 plus KChIP2a, -57.0 mV for Kv4.3 plus DPPX, and -39.2 mV for Kv4.3 plus KChIP2a plus DPPX, with slope factors (k) of -9.2 , -7.8 and -8.9 mV, respectively. H, time course of recovery from inactivation of I_{to} , test pulse $+50$ mV, recovery potential -80 mV. Curve fitting yielded τ -values of 33 ms, 46 ms and 17 ms, respectively.

Table 1. Ito current characteristics in CHO expression systems with coexpression of KChIP2a and DPPX, 22°C

	Kv4.3 + KChIP2a ^a	<i>n</i>	Kv4.3 + DPPX ^b	<i>n</i>	Kv4.3 + KChIP2a + DPPX ^c	<i>n</i>	Human ventricular myocytes ^d	<i>n</i>
<i>C_m</i> (pF)	33 ± 3	40	12 ± 2	8	39 ± 6	18	292 ± 22	28
<i>I_m</i> current density at +50 mV (pA pF ⁻¹)	495 ± 86	26	399 ± 90	6	373 ± 50	15	7.9 ± 0.7	28
τ_{fast} of inactivation at +50 mV (ms)	55 ± 4	11	25 ± 6*	8	45 ± 6	10	54 ± 3 (58 ± 3)	5 (28)
τ_{slow} of inactivation at +50 mV (ms)	392 ± 160	10	177 ± 16	8	427 ± 213	10	n.d.	—
<i>V</i> _{0.5} of steady-state inactivation (mV)	-31.3 ± 2.4	24	-57.0 ± 3.1*	8	-39.8 ± 2.8	9	-45.5 ± 1.2 (-30.4 ± 1.2)	5 (26)
Slope <i>k</i> of steady-state inactivation (mV)	-5.0 ± 0.5	24	-5.5 ± 0.4	8	-6.6 ± 0.6	9	-4.6 ± 0.8 (-4.5 ± 0.2)	5 (26)
τ_{fast} of recovery from inactivation at +50 mV, from -80 mV (ms)	42 ± 8	11	52 ± 16	8	18 ± 3*	11	24 ± 3 at +20 mV, from -100 mV	6

*Values significantly different from other groups $P < 0.05$. ^aStable expression of Kv4.3 and KChIP2a. ^bStable expression of Kv4.3 and transient expression of DPPX. ^cStable expression of Kv4.3 and KChIP2a and transient expression of DPPX. ^dData from Wettwer *et al.* (1994) for comparison, not included in statistical analysis; subepicardial myocytes, nifedipine (1 μM) or Cd²⁺ (0.1 mM, data in brackets) was used for block of *I_{Ca}*. n.d. values not determined.

$n = 15$). The time constant of inactivation became slightly smaller (45 ± 6 ms, $n = 10$ at +50 mV), *V*_{0.5} of inactivation was more negative (-39.8 ± 2.8 mV) and the time constant for recovery from inactivation was smaller than in cells with KChIP2a expression (18 ± 3 ms, $P < 0.05$). Mean values for the measured parameters are depicted in Fig. 3E–H and summarized in Table 1.

Discussion

Here we provide evidence that the dipetidyl-aminopeptidase-like protein 6 (DPPX) is expressed in human cardiac tissue on mRNA and protein level. Functional experiments in Kv4.3 expressing CHO cells revealed that additional expression of KChIP2a or DPPX alone produced transient outward current, but the characteristic *I_{to}* kinetics of native human ventricular cells were most closely mimicked in cells coexpressing both subunits in addition to Kv4.3.

Tissue distribution of DPPX

DPPX was first discovered in rat and bovine brain (Wada *et al.* 1992). In rat, the long isoform DPPX-L was found only in brain, whereas the short isoform DPPX-S was also detected in some peripheral tissues like kidney, ovary and testis, but transcripts of either isoform are absent in rat heart, liver or spleen (Wada *et al.* 1992). Human DPPX possesses more than 90% homology to bovine and rat DPPX (Yokotani *et al.* 1993). The predominance of DPPX expression in rat brain is confirmed by Western blot, but detection failed in other tissues particularly in rat heart (Kin *et al.* 2001; this study).

We found significant amounts of DPPX mRNA expressed in the human heart as well as in human brain, testes and pancreas and to a lesser extent in lung, kidney and ovary. No transcripts were detected in bladder, spleen and liver. In fact, one preliminary communication confirms our positive finding in human heart (Kloos *et al.* 2005). Interestingly, DPPX was significantly up-regulated in failing hearts, which could contribute to the observed alteration of *I_{to}* in heart failure (Kaab *et al.* 1998).

Western Blots reveal clear bands at about 100 kDa in human ventricle in good agreement with data previously reported in rat brain (Kin *et al.* 2001; Nadal *et al.* 2003; Zaghera *et al.* 2005). However, the apparent molecular mass of DPPX in human heart differs from that of DPPX in rat brain by 2 kDa, which is probably due to a different glycosylation pattern. A similar explanation may hold true for the even higher apparent molecular weight found for DPPX expressed in CHO cells.

Functional experiments

The detection of DPPX in human heart and the functional data from coexpression experiments suggest an important role for DPPX also in native *I_{to}* although direct evidence as could be provided by gene silencing techniques is still pending.

In *Xenopus* oocytes expression of DPPX in addition to Kv4.3 accelerates inactivation, shifts *V*_{0.5} to more negative membrane potentials and hastens recovery from inactivation compared with Kv4.3 expression alone or with coexpression of Kv4.3 and KChIP2 (Nadal *et al.* 2003). In our study with CHO cells, negligible *I_{to}* was obtained with expression of Kv4.3 alone. For robust *I_{to}* amplitudes, coexpression of KChIP2a or DPPX was required (compare Fig. 3A, B and C). This difference in functional outcome

of channel subunit expression between the two expression systems is most likely due to differences in endogenous expression of chaperones that promote translocation of Kv4.3 to the surface membrane. Both KChIP2a and DPPX have been associated with enhanced trafficking of the ion conducting subunit to the cell surface (Shibata *et al.* 2003; Nadal *et al.* 2003).

Co-expression of Kv4.3 with the β -subunits affected the kinetics of I_{to} ; however, the effects were different in CHO cells and *Xenopus* oocytes. Unexpectedly, DPPX retarded recovery from inactivation in comparison to KChIP2a whereas recovery from inactivation was accelerated in *Xenopus* oocytes. The time course of inactivation was accelerated and $V_{0.5}$ was substantially more negative. These effects of DPPX are consistent with those in *Xenopus* oocytes (Nadal *et al.* 2003). We hypothesized that the coexpression of two important β -subunits, KChIP2a and DPPX, should more closely reproduce I_{to} current characteristics of human ventricular myocytes, i.e. time constant of inactivation $\tau_i = 54$ ms (+ 50 mV), $V_{0.5}$ of steady-state inactivation -45 mV, and time constant of recovery from inactivation $\tau_{rec} = 24$ ms (+ 20 mV; recovery potential -100 mV; Wettwer *et al.* 1994). The respective values obtained in CHO cells expressing both KChIP2a and DPPX in addition to Kv4.3 were $\tau_i = 45$ ms (+ 50 mV), $V_{0.5}$ of inactivation -40 mV, and $\tau_{rec} = 18$ ms (+ 50 mV, recovery potential -80 mV). From this surprisingly good agreement with the data reported for native cardiomyocytes we suggest that both KChIP2a and DPPX determine I_{to} kinetics in human ventricular myocytes.

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