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ORIGINAL ARTICLE

The presence of mu-, delta-, and kappa-opioid receptors in human heart tissue

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Abstract Functional evidence suggests that the stimulation of peripheral and central opioid receptors (ORs) is able to modulate heart function. Moreover, selective stimulation of either cardiac or central ORs evokes preconditioning and, therefore, protects the heart against ischemic injury. However, anatomic evidence for OR subtypes in the human heart is scarce. Human heart tissue obtained during autopsy after sudden death was examined immunohistochemically for mu- (MOR), kappa- (KOR), and delta- (DOR) OR subtypes. MOR and DOR immunoreactivity was found mainly in myocardial cells, as well as on sparse individual nerve fibers. KOR immunoreactivity was identified predominantly in myocardial cells and on intrinsic cardiac adrenergic (ICA) cell-like structures. Double immunofluorescence confocal microscopy revealed that DOR colocalized with the neuronal marker PGP9.5, as well as with the sensory neuron marker calcitonin gene-related peptide (CGRP). CGRP-immunoreactive (IR) fibers were detected either in nerve bundles or as sparse individual fibers containing varicose-like structures. Our findings offer the first hint of an anatomic basis for the existence of OR subtypes in the human heart by demonstrating their presence in CGRP-IR sensory nerve fibers, small cells with an eccentric nucleus resembling ICA cells, and myocardial cells. Taken together, this suggests the role of opioids in both the neural transmission and regulation of myocardial cell function.

Keywords Human heart · Opioid receptor · Sensory neuron · Immunohistochemistry

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Introduction

There is a growing body of data supporting a peripheral role for opioids in the regulation of organ function, such as the gut [1], lungs [2, 3], and heart [4]. Experimental studies have shown that stimulation of both peripheral (cardiac) and central opioid receptors (ORs) is able to modulate heart function and evoke cardioprotection against ischemia [5–7]. The efficacy of opioids in inducing preconditioning and postconditioning not only in intact hearts in vivo [8] but also in isolated cardiomyocytes in vitro [9, 10], their inotropic effects [11–13], and their impact on growth factor expression [14, 15] in cell culture indicate the presence of ORs on cardiomyocytes and not only on neural elements.

The function of the heart is controlled by the autonomic nervous system. It is widely accepted that the heart has an intrinsic cardiac nervous system, or "heart brain," consisting of a complex network of intrinsic cardiac ganglia, sensory afferents, local circuits, and pre- and postganglionic parasympathetic and postganglionic sympathetic

efferents. We have consistently shown in rats that mu-OR (MOR), delta-OR (DOR), and kappa-OR (KOR) are expressed as mRNA and translated into specific receptor proteins on different components of the intrinsic cardiac nervous system [16, 17].

To date, anatomic evidence for the different OR subtypes in the human heart has been scarce. Most investigators have confirmed the presence of DOR mRNA and protein, but there is still conflicting evidence for MOR and KOR [18–20]. Moreover, immunohistochemical studies of their anatomic distribution are lacking. Therefore, we examined the presence of MOR, DOR, and KOR throughout human heart tissue sampled from victims of sudden death.

Materials and methods

Patients and the preparation of human heart tissue

The study protocol adhered to the International Guidelines of the Declaration of Helsinki 2004 (World Medical Association: http://www.wma.net) and was approved by the Ethics Committee of the Nicolaus Copernicus University in Torun, Poland. Human heart tissue samples were obtained from two subjects (females, 37 and 42 years of age) up to 48 h after sudden death during postmortem examination in the Forensic Medicine Department, Bydgoszcz. The younger female died as a result of suicide. The older female died unexpectedly and during autopsy, atherosclerotic changes in the coronaries and focuses of myolysis and nuclear fragmentation were identified, which gave the suspicion of a very recent myocardial infarction. Both were nonsmokers. Samples (at least 1 cm^3 each) were taken from five different regions, two from each location: the right atrium between the caval veins, the free wall of the right ventricle, the intraventricular septum, the anterior wall of the left ventricle including the left anterior descending coronary artery, and the inferior wall of the left ventricle. Tissue samples were fixed in 4 % (w/v) paraformaldehyde in 0.16 M phosphate buffer solution (pH 7.4) for 5 h and then cryoprotected overnight at 4 °C in phosphate-buffered saline (PBS) containing 10 % sucrose [21]. The tissues were embedded in Tissue-Tek compound (OCT; Miles, Elkhart, IN, USA). Then 50-µm thick sections were processed in a cryostat and collected in PBS (floating sections).

Single immunostaining for light microscopy

Floating tissue sections were processed for MOR, DOR, or KOR immunohistochemistry with a Vectastain avidin–biotin–peroxidase complex (ABC) kit (Vector Laboratories, Burlingame, CA, USA) as described previously [21, 22]. All incubations were carried out at room temperature, and PBS was used for washing (three times for 10 min) after each step. Sections were incubated for 45 min in PBS with 0.6 % H_2O_2 and 50 % methanol to block endogenous peroxidase, and for 60 min in PBS containing 0.3 % Triton X-100, 1 % bovine serum albumin, and 10 % goat serum (Vector Laboratories) (blocking solution) to prevent nonspecific binding. The sections were then incubated overnight with polyclonal rabbit antibodies against MOR (a gift from S. Schulz and V. Höllt, Magdeburg, Germany), DOR (a gift from R. Elde, Minneapolis, MN, USA), or KOR (a gift from S.J. Watson, MI, USA), and thereafter for 90 min with a goat antirabbit biotinylated secondary antibody (Vector Laboratories) and for another 90 min with ABC. Finally, specific immunostaining was detected with 3′,3′-disaminobenzidine tetrahydrochloride (Sigma, Taufkirchen, Germany). Rabbit polyclonal anti-MOR, anti-DOR, and anti-KOR have been thoroughly characterized previously [16, 17, 23]. Their immunoreactivity is lost upon gene deletion of the respective receptors in transgenic animals [24] or on Western blot following preincubation with the respective antigen peptide [16, 17, 23]. In addition, there is no cross-reactivity between these antisera, since they do not stain the other receptors following their respective transfection into cell lines [25–27].

Double immunofluorescence staining

Double immunofluorescence staining was performed as described previously [22]. In brief, floating tissue sections were incubated for 60 min in blocking solution. The sections were then incubated overnight with the following antibodies: (1) rabbit polyclonal anti-DOR (dilution: 1:1000) in combination with chicken polyclonal protein gene product 9.5 (PGP9.5) antibody (dilution: 1:500; EnCor Biotechnology, Gainesville, FL, USA) or guinea pig polyclonal calcitonin gene-related peptide (CGRP) antibody (dilution: 1:500; Peninsula Laboratories, San Carlos, CA, USA). After incubation with primary antibodies, the tissue sections were washed with PBS and then incubated with the appropriate secondary antibodies. After incubation with primary antibodies, the tissue sections were washed with PBS and then incubated with Texas Red conjugated goat antirabbit antibody (Vector Laboratories) in combination with Alexa Fluor 488 goat anti-guinea pig or antichicken antibody (Invitrogen, Karlsruhe, Germany). Thereafter, sections were washed with PBS, and the nuclei stained bright blue with 4′-6-diamidino-2-phenylindole (0.1 µg/ml in PBS) (Sigma). Finally, the tissues were

washed in PBS, mounted in Vectashield (Vector Laboratories), and imaged on a confocal laser scanning microscope, Zeiss LSM 510 (Carl Zeiss, Göttingen, Germany). To demonstrate specificity of staining, the following controls were included as described thoroughly in detail elsewhere [16, 17, 28–30]: (1) preabsorption of diluted antibody against MOR, DOR, or KOR with 5 μg/ml of synthetic peptide antigen for MOR (Gramsch Laboratories, Schwabhausen, Germany), DOR (Neuromics, Minneapolis, MN, USA), or KOR (S.J. Watson), respectively, for 24 h at 4 °C; and (2) omission of either the primary or secondary antibodies.

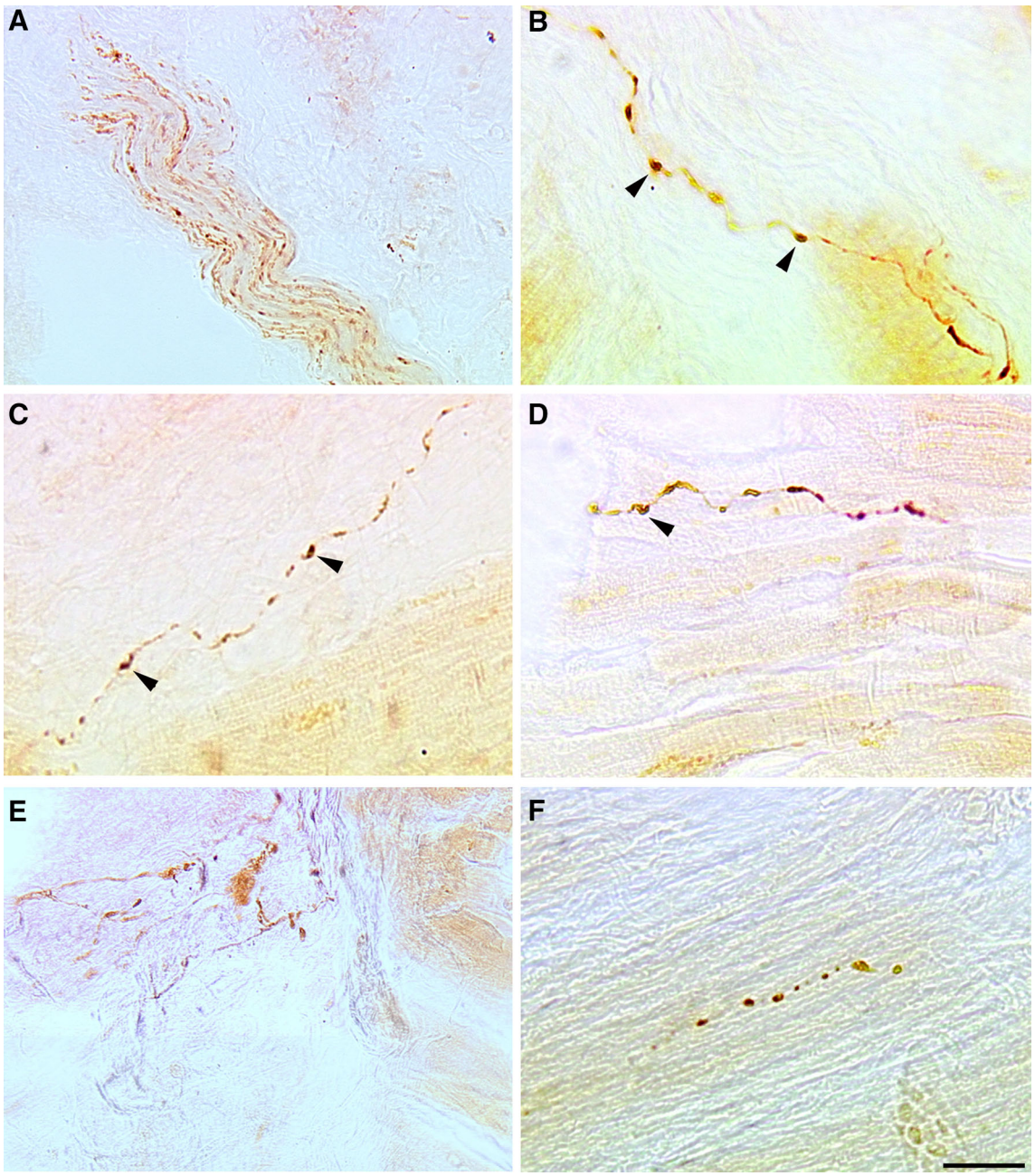

Fig. 1 Immunohistochemical localization of calcitonin gene-related peptide (CGRP) in the right and left ventricles of human heart tissue. **a** CGRP immunoreactivity expressed in nerve processes coursing in nerve bundles. **b–d** CGRP immunoreactivity localized to some nerve processes arborizing throughout the right ventricle tissue, containing localized bead-like enlargements of the axoplasm (*arrowheads*) along their lengths. **c**, **f** CGRP immunohistochemical localization in the left ventricle. Note that CGRP is expressed in sparse, fine, and varicose nerve fibers. *Bar* 20 μm

Results

Identification of CGRP-immunoreactive sensory nerve fibers in human myocardial tissue

In the right ventricle of the human heart, the majority of CGRP immunoreactivity consisted of nerve processes coursing in nerve bundles together with nonstained nerve processes (Fig. 1a–d). In addition, CGRP-stained individual nerve fibers arborizing throughout the right ventricle myocardium contained localized, bead-like enlargements of the axoplasm along their course. Histologically, these enlargements resembled varicosities. In the free wall of the left ventricle, sensory nerve fibers were usually sparse, fine, and varicose, and exhibited CGRP immunoreactivity (Fig. 1e, f). An abundant supply of CGRP-immunoreactive (IR) nerve bundles and varicose nerve fibers was distributed throughout the intraventricular

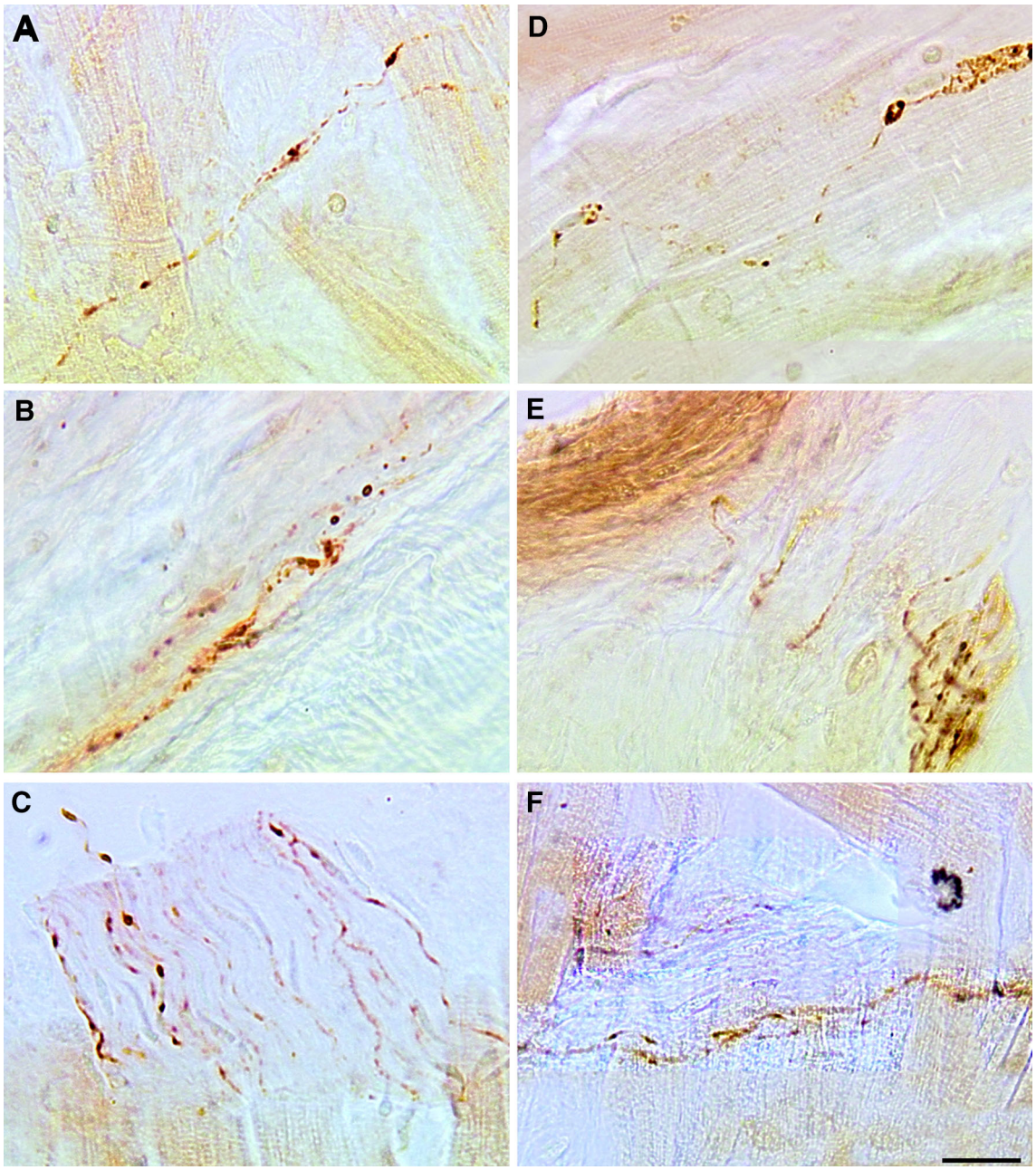

Fig. 2 Immunohistochemical localization of CGRP immunoreactivity in the intraventricular septum (**a**–**e**) and right atrium (**f**) of human myocardial tissue. **a**–**c** An abundant supply of CGRP-immunoreactive nerve trunks (**b**, **c**), nerve fascicles (**e**), and varicose nerve fibers (**a**, **d**). **f** CGRP in the right atrium. Note that CGRP is expressed in sparse, fine, and varicose nerve fibers. *Bar* 20 μm

septum (Fig. 2a–e). Single CGRP-IR nerve fibers in the right atrium were usually sparse, fine, and varicose (Fig. 2f).

Identification of MOR, DOR, and KOR immunoreactivity in human myocardial tissue

Light microscopy pictures of human myocardium immunostained with a specific antibody against MOR identified a high degree of MOR immunoreactivity inside myocardial cells in the inferior wall of the left ventricle (Fig. 3a, b). In addition, MOR immunoreactivity was demonstrated in sparse fine nerve fibers taking their course through human myocardium in the intraventricular septum and inferior wall of the left ventricle (Fig. 3c).

Delta opioid receptors (DOR) immunoreactivity was located in the free wall of the right ventricle and in the intraventricular septum, predominantly on longitudinal structures corresponding to nerves. Some of the DOR-IR thin nerve fibers were in close proximity to small artery walls (Fig. 3e), whereas others lay between cardiomyocytes (Fig. 3f). DOR immunoreactivity was also identified inside myocardial cells of the right ventricle (Fig. 3g). KOR immunoreactivity was demonstrated in the right ventricle and intraventricular septum of human myocardium in sparse fine nerve fibers between cardiomyocytes (Fig. 4a),

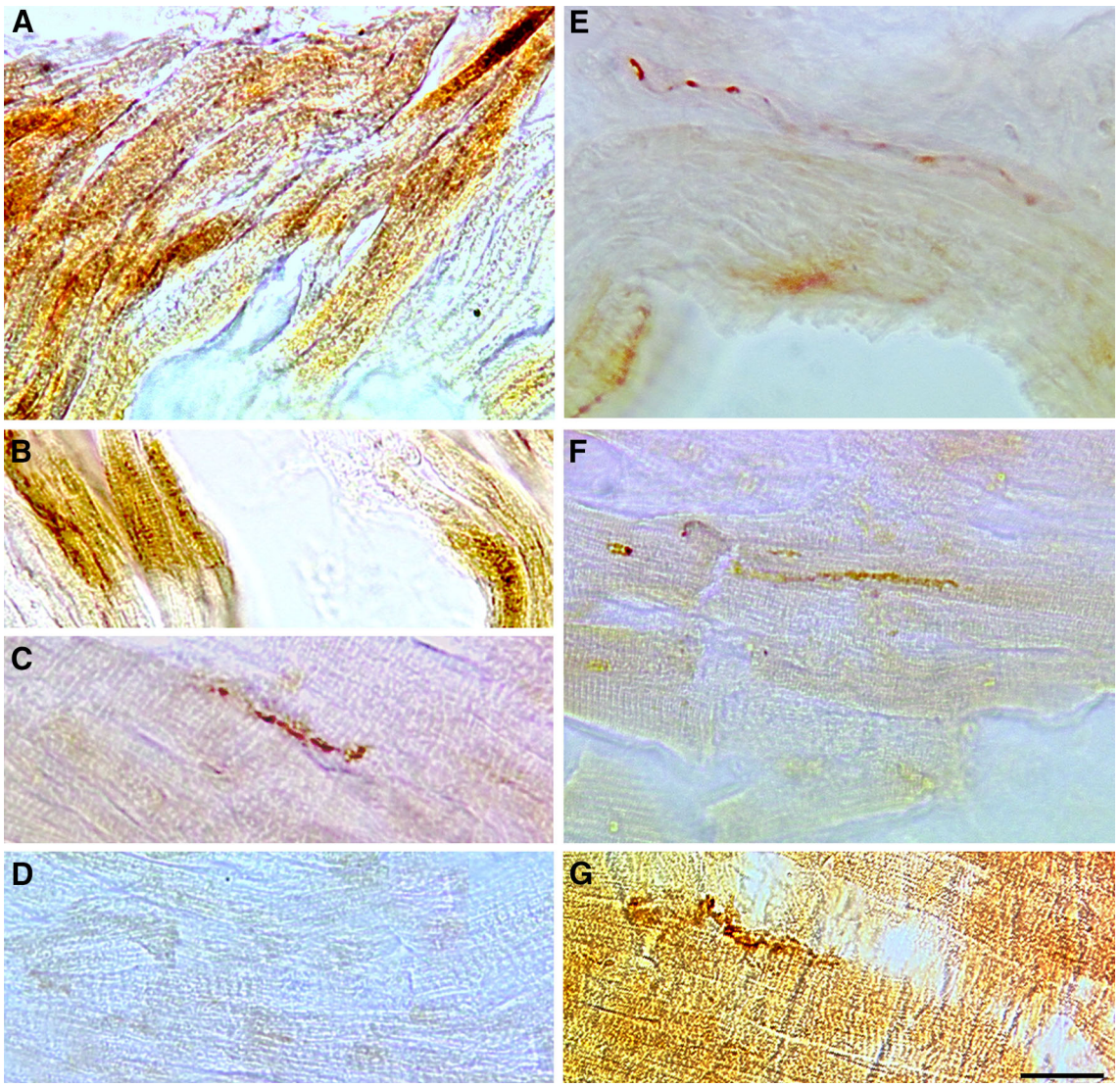

Fig. 3 Immunohistochemical localization of mu opioid receptor (MOR) (**a**–**d**) or delta opioid receptor (DOR) (**e**–**g**) in human myocardial tissue. **a**, **b** MOR expressed in myocardial cells of the human heart. **c** MOR expressed in sparse solitary nerve processes in cardiac tissue. **d** Preabsorption of antibody against MOR with 5 μg/ml of synthetic peptide antigen for MOR revealed no significant immunoreactivity. **e**–**g** DOR-immunoreactive nerve fibers in human myocardial tissue was usually sparse, fine, and varicose. **g** DOR was expressed in cardiomyocytes of the human heart. *Bar* 20 μm

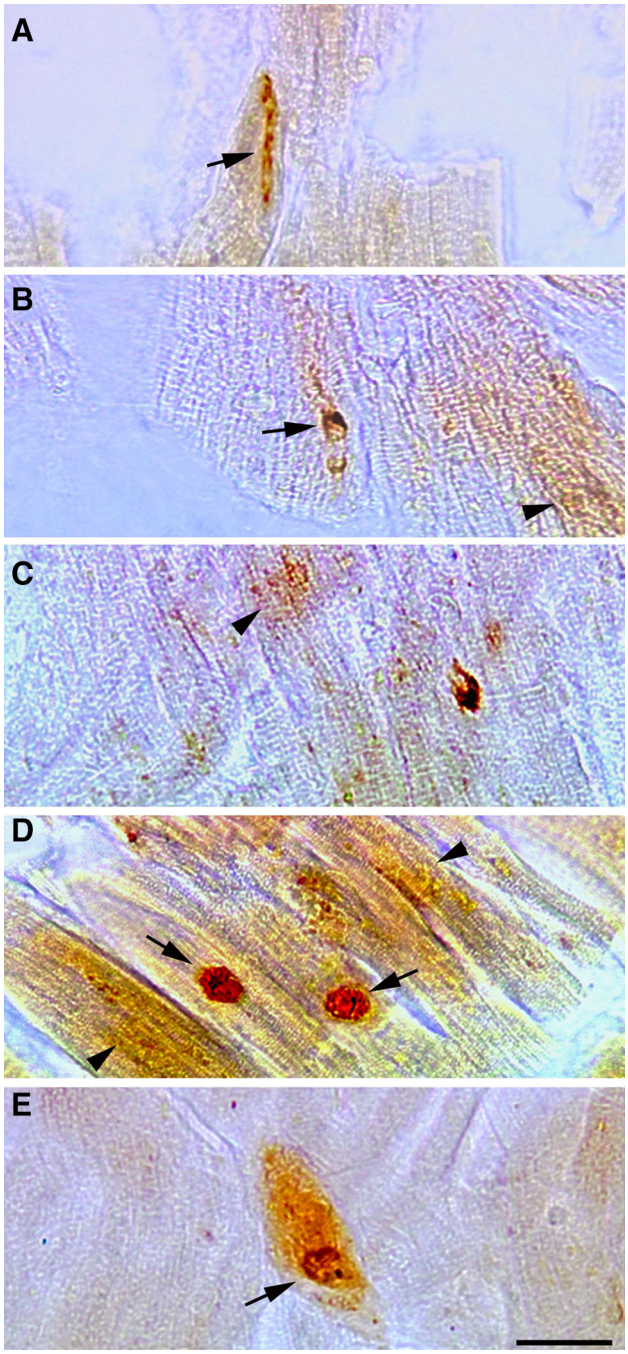

Fig. 4 Immunohistochemical localization of kappa opioid receptor (KOR) (**a**–**e**) in human myocardial tissue. **a**, **b** KOR was expressed in sparse solitary nerve processes in cardiac tissue (*arrow*). **d**, **e** KOR was expressed on long fusiform cells with an eccentrically located large nucleus (*arrow*) of the human heart. **b**–**d** KOR is expressed in cardiomyocytes (*arrowhead*) of the human heart. *Bar* 20 μm

inside myocardial cells (Fig. 4b–d), and occasionally on long fusiform cells with an eccentrically located large nucleus resembling intrinsic cardiac adrenergic (ICA) cell-like structures (Fig. 4d, e). Preabsorption of primary antibodies against MOR, DOR, or KOR with 5 μg/ml of the respective synthetic peptide antigen for MOR, DOR, or KOR as well as the omission of the primary antibody revealed no significant immunoreactivity (Fig. 3d).

Coexpression of DOR in CGRP-IR and PGP9.5-IR neurons of human myocardial tissue

Double immunofluorescence confocal microscopy demonstrated that human myocardium was densely innervated by the general neuronal marker PGP9.5-IR (fluorescein isothiocyanate (FITC) green) axons (Fig. 5b, e), some of which also displayed DOR (Texas Red) immunoreactivity (Fig. 5a, c, d, f). Some nerve fibers were immunolabeled for PGP9.5 alone (Fig. 5c, f). In addition, double immunofluorescence confocal microscopy of human heart tissue demonstrated afferent nerve fibers coexpressing the sensory neuronal marker CGRP (FITC green) (Fig. 5h, k), which were colocalized with DOR immunoreactivity (Fig. 5g, i, j, l).

Discussion

The findings of this study demonstrate for the first time the immunohistochemical localization of all three ORs (MOR, DOR, and KOR) in human myocardial tissue. MOR and DOR immunoreactivity was mainly found in myocardial cells as well as on sparse individual nerve fibers. KOR immunoreactivity was found predominantly in myocardial cells and on ICA cell-like structures. Double immunofluorescence microscopy revealed that DOR colocalized with the neuronal marker PGP9.5 as well as with the sensory neuron marker CGRP. CGRP-IR fibers were detectable either in nerve bundles or as sparse individual nerve fibers containing varicose-like structures.

In earlier studies, the neuropeptide CGRP was demonstrated in nerve fibers of the human heart. The CGRP content in the atria was found to be three- to fourfold higher than that in the ventricles [31]. Wharton et al. [32] confirmed these early findings and reported CGRP immunoreactivity mainly on scattered individual neurons, ganglion neurons, and nerve bundles. Interestingly in the developing human heart, sensory CGRP-IR innervation occurs later (18th–24th week of gestation) than the autonomic parasympathetic and sympathetic ones (7th week of gestation), suggesting a chronological development of the autonomic and sensory nerves [33, 34]. Peptidergic CGRP-IR nerve fibers are part of a complex sensory network that transmits relevant sensory information from the heart to nearby intrinsic cells as well as to the spinal cord [35].

Consistent with our previous studies in rats [16], we can now demonstrate that DORs in the human heart are expressed in CGRP-IR sensory neurons as well as in

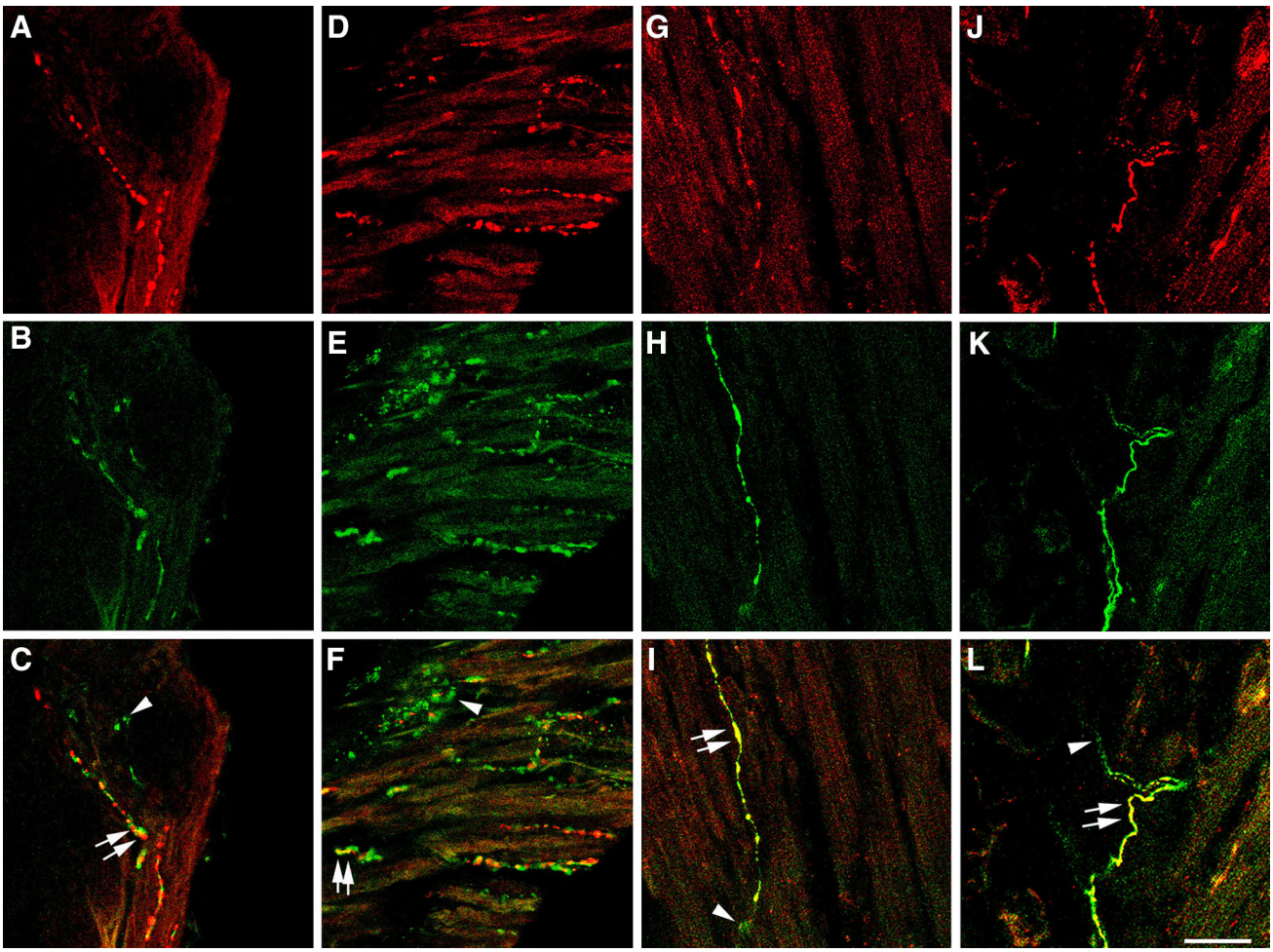

Fig. 5 Confocal microscopy of DOR (**a**, **d**, **g**, **j**) (*red fluorescence*) with polyclonal protein gene product 9.5 (PGP9.5) (**b**, **e**) or CGRP (**h**, **k**) (*green fluorescence*) double immunofluorescence in human myocardial tissue. **c**, **f**, **i** and **l** are combined images showing colocalization (*double arrows*) of DOR with PGP9.5 (**c**, **f**) or CGRP (**i**, **l**). Some PGP9.5- or CGRP-immunoreactive nerve fibers (*arrowheads*) do not express DOR. *Bar* 20 μm (color figure online)

myocardial cells, suggesting that opioids regulate both the transmission of sensory information and the function of myocardial cells. In addition, we can show the presence of the other ORs (MOR and KOR) on single nerve fibers and on myocardial cells, confirming that all three ORs are expressed in the human heart. Until now, MOR, DOR, and KOR have been mainly identified by the detection of their mRNA in cardiomyocytes [18, 19, 36–38]. Moreover, DOR protein was detected by Western blot and immunohistochemistry in ICA cells [37, 38]. The most interesting previous finding is the demonstration of MOR- and DOR-specific binding sites in the heart of human volunteers by the use of MOR- and DOR-specific radioligands and positron emission tomography [39]. The distribution of these binding sites was homogeneous throughout the myocardium, with relatively higher uptake in the lateral wall than in the septum. The specificity of binding was confirmed with naloxone pretreatment, which decreased radioligand uptake.

There is a growing body of evidence from experimental studies of the rodent heart supporting the notion that activation of the local ORs affects cardiac function. Indeed, ORs such as DOR in the heart elicit cardioprotective effects against myocardial infarction and trigger processes similar to ischemic preconditioning [40, 41]. DOR stimulation also reduces arrhythmias, and preserves the viability of isolated cells and organs [42, 43]. In addition, DOR activation enhances the growth of neonatal rat ventricular myocytes via the extracellular signal-regulated kinase pathway [44] and mediates antiapoptotic effects in cardiomyocytes [45]. KOR stimulation is proarrhythmic in swine [46], but in rats it can exert a pro- or antiarrhythmic influence [47–50]. In rabbit hearts, KOR agonists inhibit electric stimulation-induced sympathomimetic effects [51], whereas in rats MOR activation did not induce preconditioning [52, 53].

It is well established that endogenous ligands for ORs, i.e., the opioid peptides encephalin and dynorphin and their

precursor peptides proenkephalin and prodynorphin, are expressed in peripheral neuronal terminals of the human heart [54, 55] and that of other mammalian species, including the rat (for a review, see [56, 57]). However, the release of opioid peptides (i.e., OR ligands) from heart tissue has not been conclusively demonstrated although preliminary evidence supports this view [58, 59]. Thus, in the human heart there exists an intrinsic opioid system that may regulate the parasympathetic and sympathetic control of the heart as well as myocardial performance. However, our study has its limitations as tissue samples were taken within 48 h post mortem, which may contribute to a potential loss of antigen recognition. Nevertheless, our results remain in concordance with previous studies showing the existence of ORs in the human heart by other techniques [18, 19, 32, 36, 37].

Taken together, our findings demonstrate that ORs in the human heart are expressed in CGRP-IR sensory nerves, as well as in myocardial cells and small cells resembling ICA cells, suggesting a modulatory role of opioids both in the neural transmission and in the function of myocardial cells. This study may stimulate further experiments to investigate systematically the precise subcellular localization of each OR subtype in the human heart.

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