Isolation and Morphology of Single Purkinje Cells from the Porcine Heart

T. Stankovičová^{1,3}, V. Bito¹, F. Heinzel^{1,4}, K. Mubagwa² and K. R. Sipido¹

¹ Laboratory of Experimental Cardiology, Catholic University of Leuven, Belgium

² Center of Experimental Surgery, Catholic University of Leuven, Belgium

³ Department of Pharmacology and Toxicology, Faculty of Pharmacy, Comenius University, Bratislava, Slovakia

⁴ Institute of Pathophysiology, University of Essen, Germany

Abstract. Purkinje cells were isolated from both ventricles of young adult domestic pigs and examined by transmitted light or laser scanning confocal microscopy. Purkinje cells in free running Purkinje fibres were organised in multicellular strands where individual cells were tightly connected end-to-end and closely side-to-side. After isolation, single cells gradually lost the elongated appearance and became more rounded, but the cell membrane remained smooth and undamaged. The contractile material was not very dense and was seen most clearly in the submembraneous area. Staining of the cell membrane with the lipophilic fluorescent dve di-8-ANNEPS, and visualization with confocal microscopy, confirmed that the cell surface membrane was smooth without blebs. This staining also showed that Purkinje cells had no transversal tubules. We reconstructed the three-dimensional geometry of the Purkinje cells and determined the cell size. The average values were $62 \pm 9 \ \mu \text{m}$ for length, $32 \pm 3 \ \mu \text{m}$ for width, and $41 \pm 4 \ \mu \text{m}$ for depth (n = 7). Calculated cross-section area and volume were $1047 \pm 167 \ \mu m^2$ and $47 \pm 14 \ pl$. Compared to ventricular cells, the morphology of the Purkinje cells reflects their specific role in impulse conduction.

Key words: Purkinje cell — Pig — Morphology — Confocal microscopy

Introduction

The cardiac conduction system coordinates and synchronizes cardiac contraction (Sommer and Johnson 1979). The impulses pass from the sino-atrial node across the atria and atrio-ventricular node to Purkinje fibres which complete the activation

Correspondence to: Dr. Tatiana Stankovičová, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Comenius University, Kalinčiakova 8, 832 32 Bratislava 3, Slovakia. E-mail: Tatiana.Stankovicova@fpharm.uniba.sk

pathway through the interventricular septum, penetrate into the heart apex, and turn superiorly into the ventricular walls. They supply the papillary muscles before supplying the lateral walls of the ventricles. The ventricular depolarisation depends critically on the presence of these large, barrel-shaped fibres, first described by Jan Evangelista Purkinje in 1845. Because the mass of the left ventricle is much larger than that of the right ventricle, the Purkinje network is more elaborate in that side of the heart.

The primary function of cardiac Purkinje cells is a fast conduction of the impulse, rather than production of contractile force. The cellular architecture is known to differ from that of working myocytes (reviewed by Sommer and Johnson 1979), e.g., by the less dense network of contractile filaments. This feature is very pronounced in cells from larger mammals (sheep, cattle, dog) where the contractile material is restricted to a small rim under the sarcolemma, in contrast to smaller mammals (rabbit, rat, guinea pig) that can have abundant myofilaments in their Purkinje fibres. In the present report we have examined the morphology of single Purkinje cells isolated from pig hearts.

Materials and Methods

Isolation of Purkinje cells

The experimental procedures were approved by the Ethical Committee of the Catholic University of Leuven and conform to the principles outlined in the Declaration of Helsinki. Healthy domestic pigs (30–35 kg, sex indeterminate) were used. Pigs were fully anaesthetised with pentobarbital (20 mg/kg i.v.) after premedication with azaperone (4 mg/kg i.m.) and atropine (0.35 mg/kg i.v.). Under artificial ventilation, the chest was opened, the heart was rapidly removed, and collected into a beaker of cold Tyrode solution to be rinsed of blood.

To dissociate Purkinje cells from both ventricles two approaches were used. From the left ventricle we dissected free-running Purkinje fibres and subjected these to the enzymatic isolation procedure described by Glitsch et al. (1989), combined with the procedure for tissue chunk isolation described by Curtet et al. (2000), and modified for our experimental conditions. Purkinje fibres were placed into 10 ml beakers with Ca^{2+} -free Tyrode solution at 37 °C and agitated with a stream of oxygen. After 35–40 min Purkinje fibres were transferred into the freshly prepared enzymatic solution: 1.0–1.5 mg/ml collagenase A (Roche Diagnostics, Brussel, Belgium) and 0.1 mg/ml protease XIV (Sigma-Aldrich, Bornem, Belgium), dissolved in Ca^{2+} -free Tyrode solution. After 20–40 min the fibrous tissue sheath was largely digested, revealing bundles of Purkinje cells. The digestion medium with Purkinje fibres was gently triturated and regularly checked on an inverted microscope at low magnification (at first at 20 min intervals, more frequently as digestion progressed) for dissociation of single cells. When these were found to be abundant, the undigested strands were removed from the Purkinje-cells enriched suspension and transferred to another beaker with a fresh enzymatic solution. To stop digestion,

low-Ca²⁺ (0.18 mmol/l) Tyrode solution was added to the cell suspension. After cells had settled on the bottom of the beaker, 60-70% of the solution was exchanged with a fresh batch of the low calcium solution. These steps were repeated several times to remove the enzymes. At the end, normal Tyrode solution was added and cells were stored at the room temperature. Morphology was measured within 90 minutes after isolation.

To isolate Purkinje fibres from the right heart side, we first perfused the right ventricle through its supplying coronary artery, as a variant of the isolation technique described for rabbit Purkinje fibres (Sipido et al. 1993) and for left ventricular myocytes (VMs) (Stankovicova et al. 2000). After perfusing with 37 °C oxygenated Tyrode solution for 3–5 min to clear all blood from coronary vasculature, the perfusate was changed to a nominally Ca²⁺-free Tyrode solution for 30 min, after which the perfusate was exchanged with Ca²⁺-free solution containing the enzymes (collagenase A, 1.0–1.5 mg/ml, and protease XIV, 0.1 mg/ml, dissolved in Ca²⁺-free Tyrode solution). After 30–40 min, the tissue became swollen and opalescent, indicating digestion. At this time, free running Purkinje fibres were dissected and returned for further enzymatic digestion into a beaker positioned in a double-walled Petri dish, warmed to 37 °C and filled with fresh enzyme solution. All the following steps were the same as described above for Purkinje fibres from left ventricle.

Confocal microscopy and staining with di-8-ANNEPS

The cells were studied in a glass-bottomed perfused chamber placed on the stage of a Zeiss Axiovert 100M (Carl Zeiss Microscopy, Jena, Germany) inverted microscope with a $40 \times$ oil-immersion objective Plan-Neofluar (NA = 1.3). The cells were recognised and selected for further study by transmitted light imaging.

Purkinje cells were stained with the membrane-selective fluorescent dye di-8-ANNEPS, a voltage-sensitive dye, which is highly lipophilic and avidly stains the cell membrane. Cells were exposed to 20 μ mol/l dye for 1–3 min directly in experimental bath (1 ml volume, 1/1000 mol/l dilution of a 20 mmol/l stock solution in DMSO with 20% w.v. Pluronic). Confocal images were acquired and processed with a Zeiss LSM 510 confocal laser point scanning system (Carl Zeiss Microscopy). After washout with a normal Tyrode solution, stained cells were excited at a wavelength of 488 nm with a 25 mW Argon laser (output power 50%, intensity attenuation to 3–10%). Fluorescence emission was detected *via* a 505–530 nm band-pass filter.

In order to obtain a two-dimensional fluorescent confocal image of a selected cell, successive line scans were performed in the XY plane. For three-dimensional information of the cell, a series of XY-images along the Z axis was collected. The thickness of the optical slice on the Z axis was 1 μ m. The scanning speed varied between 1.5 and 3.8 ms/line. Scanning speed, excitation and amplification settings were kept constant during each experiment.

Electrophysiology data were recorded and analysed with pClamp (Ver. 8.2, Axon Instruments, USA).

Solutions and Drugs

The Tyrode solution used for perfusion contained (in mmol/l): NaCl 137; KCl 5.4; MgCl₂ 0.5; CaCl₂ 1.8; HEPES 11.8; glucose 10; adjusted with NaOH to pH 7.2. For cell isolation, the Ca²⁺-free Tyrode solution was used, which contained (in mmol/l): NaCl 130; KCl 5.4; KH₂PO₄ 1.2; MgSO₄ 1.2; HEPES 6; NaOH till pH 7.2. This solution could be supplemented with enzymes as described above. The low-Ca²⁺ Tyrode solution was made from the Ca²⁺-free Tyrode solution by adding 0.18 mmol/l CaCl₂. Di-8-ANNEPS was purchased from Molecular Probes (Europe BV, Leiden, Netherlands). All other chemicals were from Sigma (Bornem, Belgium) or Merck (Darmstadt, Germany) and were of analytical grade.

Results

Morphology

We first examined the cell morphology in partially digested Purkinje fibre bundles (after 40–50 min of digestion). At this time, the collagenous sheath lost its density, became more transparent and strings of elongated cells were visible inside the fibres



Figure 1. A. Transmitted light image of a partially digested large free-running Purkinje fibre dissected from porcine left ventricle. **B.** Detail from the area indicated in panel A showing Purkinje cells connected end-to-end and side-to-side. **C.** Same preparation after labelling of the surface membrane with the fluorescent dye di-8-ANEPPS, identifying individual cells organised in strings; the arrow shows a clear end-to-end connection.

(Fig. 1A,B). After an additional 30–60 min digestion Purkinje cell aggregated or individual single cells were found in the bath and these were then further examined.

A fraction of the cells exhibited spontaneous contractions, and some of these went into irreversible contracture, indicating membrane damage. In the transmitted light image, calcium-tolerant Purkinje cells kept a clear cylindrical shape only while connected end-to-end in the multicellular strands (Fig. 2A). Immediately after separation, individual cells changed their shape to a more elliptical geometry (Fig. 3A) and after several hours to "cardioballs". The cells markedly differed from VMs by lacking the typical staircase configuration and striations (Fig. 2A). Purkinje cells had smooth surface membranes, clear cytoplasm, and apparently contained little contractile material.

The organization of conduction cells in the digested free running Purkinje fiber and the cell shape were confirmed and visualised more clearly after staining with di-8-ANEPPS. Images of the pre-digested strands revealed individual columns of conduction cells more tightly connected end-to-end than side-to-side (Fig. 1C, arrow). These connections could not be seen in the separated cell clusters or cells (Fig. 2B). Individual Purkinje cells showed a clear smooth surface membrane with-



Figure 2. A. Transmitted light image and B fluorescence image after staining with di-8-ANEPPS of Purkinje cells isolated from porcine right ventricle, illustrating the smooth surface membranes and the absence of transversal tubules. C. VM has a more scalloped appearance of the surface membrane and regularly spaced (2 μ m) staining in the inside of the cell due to the presence of T-tubules.



Figure 3. A. Transmitted light image, illustrating the sparse myofilaments below the surface membrane in the cytoplasm of Purkinje cells. B. Typical rod-shaped VM with well organised and clear cross striations.



out blebs or membrane disruptions. In contrast to VMs, Purkinje cells had no transversal tubules, which should be seen in the centre of the cells (Fig. 2B vs. C). Cells that had been identified as dead cells in the transmitted light image, based on the presence of rough, irregular membranes with blebs, and of coarse cytoplasmic granulations, did not exclude the dye and had irregular cytosolic staining, consistent with severe membrane damage (data not shown).

In the transmitted light image, striations inside the cytoplasm, indicative of sarcomeric organization of myofilaments, were only faintly identifiable, predominantly at the periphery in the subsarcolemmal area (Fig. 3A), in sharp contrast to the well-defined and recognizable sarcomeric pattern in VMs (Fig. 3B).

Whole cell patch clamp on both isolated Purkinje cells (Fig 3A) and VMs were performed (Fig 3B). The membrane capacitance was 109 ± 17 pF for Purkinje cells (n = 10) and 102 ± 6 pF for VMs (n = 41). Preliminary data indicate that the single Purkinje cells had Ca²⁺ currents, which could evoke Ca²⁺ transients (data not shown).

Cell dimensions

Purkinje cell suspensions included some single ventricular cells as well, and we compared cell morphology of the two cell types. Single Purkinje cells varied con-

Table 1. Cell dimensions

	${ m length} { m (\mu m)}$	$\substack{\text{width}\\(\mu\text{m})}$	${ m depth} { m (\mu m)}$
Purkinje cell $(n = 7)$	$62 \pm 9^*$	$32 \pm 3^*$	$41 \pm 4^*$
VM $(n = 26)$	156 ± 6	26 ± 1	17 ± 1

Purkinje cell vs. VM; * $p \leq 0.05$.

siderably in length (35–99 μ m) but their other two dimensions were less variable. Purkinje cell width varied between 22 to 45 μ m and Purkinje cell depth varied between 32 to 59 μ m. There were significant differences between the average dimensions of Purkinje cells and those of VMs (Table 1). Purkinje cells were shorter but somewhat wider compared to VMs, and their thickness was also larger.

We assumed that the Purkinje cell had the shape of an elliptical spheroid, and the VM was an elliptical cylinder, and calculated the cross-sectional area according to the formula

 $P=\pi ab$

where a is width/2; b is depth/2.

Data in Table 2 show that the cross-section of a Purkinje cell was approximately 3 times larger than the cross-section of a VM.

Based on the measured dimensions and on the cellular shape, an estimate of surface area and volume could be obtained. If we did not consider tubular membranes, the external cellular surface was calculated as

$$S_{\rm PC} = 4\pi ((abc)^2)^{1/3}$$

for Purkinje cells, where a and b are as above, and c is length/2, and for VM as

$$S_{\rm CM} = 2\pi ab + c[\pi(1.5(a+b)) - (ab)^{1/2}]$$

where a and b are as above, but c is length.

For calculation of volume, the following formulas were used:

$$V_{PC} = 4/3\pi abc$$

Table 2. Derived surface and volume dimensions

	cross-sectional area (μm^2)	$\mathrm{surface~area}\ (\mu\mathrm{m}^2)$	${ m volume} \ (\mu{ m m}^3)$	${ m surface/volume} \ (\mu { m m}^2/\mu { m m}^3)$
Purkinje cell $(n=7)$	$1 \ 048 \pm 167^*$	$5\ 999 \pm 1\ 135^*$	46968 ± 13997	$0.1467 \pm 0.0116^*$
$\overline{VM}_{(n=26)}$	345 ± 18	$11\ 091\pm719$	$52\ 722 \pm 4\ 237$	0.2164 ± 0.0038

Purkinje cell vs. VM; * $p \leq 0.05$.

where a and b are as above, and c is length/2,

 $V_{CM} = \pi abc$

where a and b are as above, but c is length.

Average data are summarised in Table 2. The external membrane surface of Purkinje cells was approximately 0.54 times smaller than the external membrane surface of VMs (transverse tubular membrane excluded) but we could not detect a significant difference in volume between both cell types. The surface/volume ratio was significantly smaller in Purkinje cells. The contact area between the external membrane and intracellular structures of Purkinje cells was about 68% of the contact area in VMs.

Discussion

For isolation of porcine Purkinje cells, two isolation procedures were used. One was based on Langendorff perfusion *via* the coronary system of the isolated heart or part of the heart, i.e. ventricle, and the other was based on incubation of tissue chunks in an enzyme-containing solution. We successfully and equally obtained intact single cells *via* both approaches. The cells thus obtained had an ellipsoid shape, clear and smooth surface membranes, no T-tubules, and could sustain Ca^{2+} currents and Ca^{2+} transients.

Porcine Purkinje cells differed considerably in their appearance from the rabbit Purkinje cells studied previously (Sipido et al. 1993; Papp et al. 1995). Rabbit cells had clear striations consistent with a well-developed sarcomeric structure, and were smaller. These properties of the intact isolated porcine cells when compared with rabbit cells are consistent with the structural properties described for Purkinje cells in fixed, histological preparations of porcine and rabbit hearts described by Tranum-Jensen et al. (1991), who reported that cross sections of the ventricular wall in both animal species showed a thin superficial layer of large Purkinje cells, then a layer of small transitional cells, which made contacts between Purkinje cells and large ventricular muscle cells. In the pig, large Purkinje fibres penetrated full thickness of the left ventricular wall making intramural connections, whereas in the rabbit, Purkinje fibres were found only in the subendocardium. Porcine Purkinje cells were smaller than 100 μ m, and broader than 35 μ m without striations. This was in contrast to rabbit Purkinje cells characterised by a longer (approximately 150 μ m) and thinner shape, and by the presence of clear striations. Sommer and Johnson (1979) reviewed the differences between the morphology of Purkinje cells in small and larger mammals, concluding that the larger mammals typically had cells with less contractile material, and Purkinje fibres that were organised in packed small bundles, favouring the conduction needs of the larger hearts. Although their analysis did not include pig, our data indicate that this species follows that same paradigm.

Generally, Purkinje fibres in our study had a thick coat of fibrous tissue, in which the individual conduction cells were packed and organised into thin bundles. The composition of this fibrous tissue has been described during histological studies (Ohayon and Chadwick 1990). It has plentiful fibroblasts and matrix in which collagen, collagen struts (the isotropic components of the system), some elastic fibres and microfibrils surround conduction cells collected into bundles. The presence of such a thick fibrous sheath is a major difficulty for the enzymatic isolation procedure, as it increases the time needed to extract the Purkinje cells. Sheets et al. (1983) needed 4 hours for sheath digestion and revealing the columns of canine Purkinje cells. In the case of the porcine heart, 20 min in enzymatic solution allowed to confirm the typical organization of the individual cells in a chain-like arrangement in the partially digested Purkinje fibres, using transmitted light and confocal microscopy. However, much more time, up to 2 hours was needed to obtain single cells in solution.

Sommer and Johnson (1968) established that conduction cells have no transversal tubules. This has been shown to be true across all animal species (reviewed in Sommer and Johnson 1979). The absence of a T-tubular system may be one of the reasons why the more elongated shape, present in the multicellular preparation, cannot be maintained after loosening cell-to-cell contacts and constraint by the fibrous tissue. This transformation of Purkinje cells to elliptical spheroids or "cardiospheroids" and after several hours to "cardioballs" was also described for sheep Purkinje cells (Glitsch et al. 1989), and is reminescent of the atrial cardioballs (Bechem et al. 1983), observed in atrial cells that also lack the T-tubular system (Huser et al. 1996).

Morphology of isolated cells described so far varies considerably between animal species. Glitsch et al. (1989) described the freshly isolated sheep Purkinje cells as brick-shaped, with some cross striations, transforming later on to more spherical "cardioballs" with a diameter of 40–70 μ m without deep invaginations of the cell membrane. Single canine cardiac Purkinje cells were described to have well-defined striations, cell borders (Sheets et al. 1983), and fine finger-like processes at either end (Boyden et al. 1989) with averaged dimensions $164 \pm 41 \ \mu$ m by $35 \pm 4 \ \mu$ m (at 22°C, Sheets et al. 1983), $125 \pm 26 \ \mu$ m by $32 \pm 11 \ \mu$ m (at 37°C, Boyden et al. 1989), or $157-314 \ \mu$ m by $35-36 \ \mu$ m (Boyden et al. 2000). The canine Purkinje cells possessed sparse myofilaments, and a more spherical shape compared to rabbit Purkinje cells. The rabbit Purkinje cells are 130–140 μ m long and 10–15 μ m wide (Cordeiro et al. 1998), with clear cross-striations. Our data are most reminiscent of the data on sheep and dog Purkinje cells.

We performed morphological measurements that served as a basis to estimate the cross-sectional area, surface and volume of isolated cells. The changes in shape after isolation of Purkinje cells creates difficulties in extrapolating the measured cell length and width to the dimensions of length and width in the intact fibre. However, the derived values of volume and surface area are not invalid, as there is no indication that there is a loss of membrane or cell contents, which would alter surface area or volume. Despite this limitation, and allowing for an overestimation of the width of the Purkinje cells, the data suggest that the morphology of Purkinje cells is consistent with their function as a conduction cell. Action potential propagation through Purkinje fibres becomes very rapid (2–4 m/s) and is slower through ventricular muscle (0.3–1 m/s) (Bers 2001). The organization of thin bundles of cells in the Purkinje fibres (Sommer and Johnson 1979), and the number and conductance of the junctions are of major importance for conduction speed as well.

The volume of cardiac myocytes from different mammalian species was about 30 pl for rabbits, 32 pl for ferrets, and 34 pl for rats, as estimated by Satoh et al. (1996). The myocyte sizes from all species were smaller than those of VMs isolated from porcine heart (see also Heinzel et al. 2002). This fact together with the higher precision of measurement by three-dimensional volume rendering of the confocal images could explain the higher volume value approximated in our experiments. Bers (2001) summarised some quantitative ultrastructural data of sarcolemmal components in cardiac cells. In mammalian cardiac tissue the described surface/volume ratio of VMs was between 0.24–0.56 μ m⁻¹ and is close to our calculated data for porcine VMs. The sarcolemmal area of porcine Purkinje cells was somewhat larger than the sarcolemmal area of canine Purkinje cells.

Cordeiro et al. (1998) measured the capacitance of rabbit Purkinje cells. The mean capacitance was 66.5 ± 2.5 pF (n = 65), compared to 86.7 ± 8.9 pF for the ventricular cells. The larger capacitance of the rabbit VMs may reflect the presence of the T-tubular system, but the contribution of differences in size was not studied. The capacitance of porcine Purkinje cells in this study is not significantly different from that of VMs, despite the fact that the ventricular cells have T-tubules and the Purkinje cells do not. This apparent discrepancy with the larger surface area of VMs in the morphologic analysis can be partly explained by the fact that these are different samples, and that the range of capacities in the Purkinje cells was wide (48–164 pF) which may have led to some overestimation for the average. Moreover, we have recently demonstrated that the T-tubular system in VMs is only sparsely developed in the pig (Heinzel et al. 2002). The exact contribution of these factors needs further study in combined surface/volume measurements of larger samples as described by Satoh et al. (1996).

The absence of T-tubules in Purkinje cells implies that only the external membrane surface can make contact with the sarcoplasmic reticulum (SR) membrane, implying that activation of SR Ca^{2+} release channels by Ca^{2+} influx *via* sarcolemmal Ca^{2+} channels will occur predominantly in this area. This has important consequences for Ca^{2+} handling, and indeed, small and spatially inhomogeneous transients were demonstrated for rabbit and canine Purkinje cells (Sipido et al. 1993; Cordeiro et al. 1998; Boyden et al. 2000); our own preliminary data on porcine Purkinje cells are consistent with this. It could be hypothesised that this arrangement is more suited to the primary electrical conduction role of the Purkinje cells, in which contraction is only of minor importance. Acknowledgements. We thank Fons Verdonck for useful comments and suggestions during the experimental work, Patricia Holemans for technical assistance, and Alexandra Zahradnikova for help during data analysis. This work was supported by grants of the FWO, the Flanders Fund for Scientific Research (KRS, KM), and by grants No. 1/8220/01 and No. 1/0522/03 of the VEGA SR (TS).

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