M cells and transmural heterogeneity of action potential configuration in myocytes from the left ventricular wall of the pig heart

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Abstract

Objective: Heterogeneity of action potential configuration in the left ventricle (LV), and the contribution of M cells to it, has been observed in the human heart and is important for arrhythmogenesis. Whether the pig heart has similar properties remains a controversial but important issue as the pig heart is currently under study for use in xenotransplantation. Methods: Single myocytes were enzymatically isolated from the epicardium (EPI, n=29), midmyocardium (MID, n=38), and endocardium (ENDO, n=13) of the free LV wall (n=26, 14–22 weeks old, 55–80 kg), and studied at different stimulation rates during whole-cell recording (normal Tyrode’s solution, K-aspartate-based pipette solution, 50 μM K-fluo-3 as [Ca²⁺] indicator, 37°C). Standard six-lead ECGs were recorded from anesthetized pigs. Results: The action potential duration (APD) was not significantly different at 0.25 Hz vs. 2 Hz for the majority of cells in all three layers. However, a subpopulation of cells behaved like M cells and had a very steep frequency response (APD at 0.25 Hz 90±30 ms, vs. 337±9 ms at 2 Hz, P<0.05, n=22). These cells were found predominantly in the MID layer (34% of cells), but also (24%) in EPI. M cells had a more pronounced spike-and-dome configuration, with a significantly larger phase 1 magnitude and plateau voltage. The frequency response of these parameters was different from the other cell types. [Ca²⁺] transients tended to be larger in M cells. For the in vivo ECG of anesthetized pigs, the QT time was close to the APD of M cells, and J waves were seen in 7/12 recordings. Conclusions: In young adult pigs, M cells can be identified by a steep frequency response of the APD and by a spike-and-dome configuration. These cells are mostly, but not exclusively, found in the midmyocardium, and could contribute to the ECG characteristics. Their properties may however be different from those of other species, including humans. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Membrane potential; Myocytes; ECG

1. Introduction

The existence of heterogeneity of the electrical properties of the myocytes in the different layers of the left ventricle is now well established, and has been described for several animal species, as well as in the human heart [1–5]. In addition, differences between left and right ventricle, as well as variability in the apex to base regions, have been described [6–10]. Regional variation in remodeling during hypertrophy may contribute to increased and potentially arrhythmogenic heterogeneity [8,11]. Within the different layers of the free ventricular wall, i.e. epicardial, midmyocardial and endocardial, the midmyocardial layer has been described to contain cells which have action potentials with a particular configuration and frequency response, the so-called M cells [2,3,12]. These cells have a prominent spike and dome morphology, as also found in the epicardium, but the maximal rate of rise of the action potential upstroke is considerably greater than in either endocardium or epicardium. The hallmark of the M cells is the pronounced prolongation of action potential duration (APD) with deceleration of the stimulation rate, leading to a very steep rate dependence of the APD, compared to epicardium and endocardium. This feature of M cells resembles the APD rate relationships of...
Purkinje fibers, but M cells have no phase 4 depolarization [1]. The presence of M cells in the ventricular wall will contribute to the T wave morphology, the U wave and the J wave of the surface ECG [3,13–15]. In the normal heart in situ electronic coupling is expected to reduce heterogeneity, but M cells can still exert an important influence [16,17]. It is thought that M cells have a prominent role in arrhythmogenesis as differential response of these cells to e.g. drugs or ischemia may increase or decrease heterogeneity of conduction and refractoriness, thereby increasing/decreasing the dispersion and vulnerability of the ventricle to arrhythmias [1,18].

Recently there has been some controversy on whether M cells were present in the pig heart as well [19–21]. This question goes beyond the general relevance of comparative physiology, as the pig has proven to be an extremely useful animal for the study of a ischemic heart disease [22,23], as well as hypertrophy and failure [24,25], and is the animal currently being evaluated for xenotransplantation [26–28]. The size of the pig heart, and its coronary anatomy are indeed quite close those of the human heart, notwithstanding some important differences [29]. The transmembrane action potential of the pig has been studied during ischemia in vivo [30] and in isolated hearts [31] or during drug treatment of papillary muscles [32]. There are however few studies on isolated cells; changes in action potential duration and in calcium current during transition to heart failure have been reported [33,34]. Study of the characteristics of the pig ventricular action potential in different layers has been restricted to studies on transmural slices [19]. We therefore have studied properties of the action potential of single cells isolated from the different layers of the left ventricle. We also looked for evidence of electrical heterogeneity in the ECG waveform.

2. Methods

2.1. Animal characteristics and in vivo ECG recordings

A total of 26 domestic pigs of either sex were used; 22 animals were between 14 and 17 weeks old (body weight 55–70 kg), four animals were 22 weeks old (70–80 kg). In this strain, breeding age is around 24 weeks, and these animals can be considered to be young adults. Animal care and treatment conformed with the Guide for the Care and Use of Laboratory Animals (N.I.H. Publication No. 85-23, revised 1996). In 12 animals, a standard six-lead ECG (Elema AB and Mingograph 720, Siemens) was recorded under ketamine (10 mg/kg i.v.) anesthesia, following premedication with Stresnil (40 mg/kg i.m.), with the animal in the supine position, and the artificial ventilation arrested for the time of the recording. QT time was measured at a paper speed of 25 mm/s in lead II and III, from the onset of the QRS complex to the end of the T wave. Biphasic T waves were included, but U waves were not included in the measurement. For cell isolation, the anesthetized animal was sacrificed with an intravenous bolus of 10 ml of 1 M KCl, to arrest the heartbeat. Then the heart was quickly removed through midsternal thoracotomy and placed in cold Ca$^{2+}$-free Tyrode’s solution.

2.2. Cell isolation procedure

Ventricular myocytes were enzymatically isolated from a wedge of the left ventricular free wall, supplied by the left anterior descending coronary artery, using the same method we have described for isolation of human myocytes [35]. The coronary artery was cannulated, and small leaking branches at the edges of the preparation were ligated. The tissue was perfused at constant pressure (−80 mmHg); all solutions were gassed with 100% O$_2$, and temperature was maintained at 37°C. First we perfused for 30 min with Ca$^{2+}$-free Tyrode’s solution (see below), next we added to this solution 1.4 mg/ml collagenase A (Boehringer Mannheim) and 0.1 mg/ml protease (type XIV, Sigma) for 30 min. The enzyme solution was washed out for 15 min with a low Ca$^{2+}$ Tyrode’s solution. The tissue was then sectioned perpendicular to the wall in slices of 2–4 mm thickness. From these slices the epicardial and endocardial parts (each 2–3 mm) were cut parallel to the surface and placed into separate dishes in low Ca$^{2+}$ (0.18 mmol/l) Tyrode’s solution; a central midmyocardial part of some 5 mm was also put in a dish. These preparations were minced, filtered through a nylon mesh, and the cells resuspended in low Ca$^{2+}$ Tyrode’s solution. After 20 min, the solution was slowly replaced with a normal Tyrode’s solution containing 1.8 mmol/l CaCl$_2$. Ca$^{2+}$-tolerant cells were stored at room temperature and treatment conformed with the Guide for the Care and Use of Laboratory Animals (N.I.H. Publication No. 85-23, revised 1996). In 12 animals, a standard six-lead ECG (Elema AB and Mingograph 720, Siemens) was recorded under ketamine (10 mg/kg i.v.) anesthesia, following premedication with Stresnil (40 mg/kg i.m.), with the animal in the supine position, and the artificial ventilation arrested for the time of the recording. QT time was measured at a paper speed of 25 mm/s in lead II and III, from the onset of the QRS complex to the end of the T wave. Biphasic T waves were included, but U waves were not included in the measurement. For cell isolation, the anesthetized animal was sacrificed with an intravenous bolus of 10 ml of 1 M KCl, to arrest the heartbeat. Then the heart was quickly removed through midsternal thoracotomy and placed in cold Ca$^{2+}$-free Tyrode’s solution.

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2.3. Solutions

The Ca$^{2+}$-free Tyrode’s solution used for cell isolation contained: (in mmol/l) NaCl 130, KCl 5.4, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, Na–Hepes 6.0, glucose 10 (pH 7.20). The composition of normal Tyrode’s solution used for recording of action potentials was: (in mmol/l) NaCl 137, KCl 5.4, MgCl$_2$ 0.5, CaCl$_2$ 1.8, Na–Hepes 11.8, glucose 10 (pH 7.40). [K$^+$]$_o$ in our study was comparable to what has been used for pig myocardium before [19], but slightly higher than in previous studies in dogs [12]. The standard pipette solution contained: (in mmol/l) K$^+$-aspartate 120, KCl 20, MgCl$_2$ 0.5, MgATP 5.0, K–Hepes 10, NaCl 10, K$_2$HPO$_4$ 3.05 (pH 7.20). With these solutions, a junction potential of +12 mV occurred, and was subtracted from the values of resting membrane potential, but other values were not corrected.
2.4. Membrane potential and \([Ca^{2+}]\) measurements

All experiments were done at 36–37°C. Action potentials were measured in current clamp mode (Axopatch-1D, Axon Instruments) during whole-cell recording; patch pipettes typically had tip resistances of 2–3 MOhm. Cells were stimulated via the pipette with brief depolarizing current pulses, at different frequencies, each recorded repeatedly and in random order. Action potentials were analyzed after a steady state was reached, usually after 10 to 15 stimuli. Signals were sampled at 2 kHz on three channels, with an effective time resolution of 1.5 ms per signal, precluding a reliable measurement of the rate of rise of the action potential, \(V_{\text{max}}\), which was therefore not included in the present analysis. Cell capacitance was determined in voltage clamp mode, by integrating the capacitive transient during 5 mV hyperpolarizing steps from \(-70\) mV. Values obtained with this method correlated well with values obtained by fitting of the decay phase.

The setup for \([Ca^{2+}]\), measurements was as described before [36]. The fluorescence signals obtained with fluo-3 were corrected for the background recorded after seal formation, and normalized for baseline values at rest before stimulation. The pseudo-ratios thus obtained provide a reasonable estimate of \([Ca^{2+}]\), and have previously been used for group comparisons [37].

2.5. Data analysis

Group data are shown as mean±S.E.M., the number of cells used is given. One-way ANOVA was used to compare morphological parameters of different cell types, followed by Fischer’s test if appropriate. ANOVA for repeated measurements was used to analyze frequency dependence of action potential parameters, followed by Bonferroni/Dunn’s test to further identify differences between groups. \(P\) values <0.05 were considered significant.

3. Results

3.1. Action potential configuration and frequency response of isolated cells

M cells have previously been characterized by their steep increase in action potential duration (APD) at low frequency. We therefore first analyzed the frequency response for cells in the different anatomical layers. Fig. 1 shows the APD at 90% repolarization (APD$_{90}$) at stimulation frequencies of 0.25 and 2 Hz for all cells. In all three layers, the majority of cells had a rather shallow frequency dependence (see below). However, a subpopulation of cells had a pronounced increase in APD$_{90}$ at the low frequency.

![Fig. 1. Action potential duration at 90% repolarization (APD$_{90}$) at 0.25- and 2-Hz stimulation frequency of all cells, shown according to their anatomical layer of origin. The dashed line indicates the cut-off of 420 ms. This cut-off was chosen as all cells with an APD$_{90}$ of more than 420 ms at 0.25 Hz have a steep frequency dependence; they were designated M cells. The prevalence of M cells in the layers is shown below.](https://academic.oup.com/cardiovascres/article-abstract/45/4/952/300717)
This was most prominent in the midmyocardial layer, less so in the epicardial layers, and practically absent in the endocardial layer. To further analyze the properties of these cells we decided to group all cells with a \( \text{APD}_{90} \) of more than 420 ms at 0.25 Hz, as this group included exclusively cells with the steep frequency response. These cells we called M cells; cells that not fulfilled this criteria are further referred to according to their anatomical region of origin. Based on these criteria, the midmyocardial layer contained 34% M cells, the epicardial layer 24%, and the endocardial layer 15%.

Fig. 2 shows typical examples of the action potential configuration at different frequencies for each cell type. Following the action potential upstroke, all cell types had a clear phase 1 repolarization. This phase 1 repolarization could be followed by a small second depolarization, giving rise to the so-called spike-and-dome configuration. This pattern was almost completely restricted to the M cells, as it was observed in 18/22 M cells, versus 2/11 endo cells, 3/25 midmyocardial cells, and 5/22 epi cells. The action potential plateau declined slowly (phase 2) followed by a rapid phase 3 repolarization. We never observed phase 4 depolarization in any cell type.

Mean resting membrane potential was comparable for all the cell types (endo cells \(-77 \pm 1 \text{ mV}, \) midmyocardial cells \(-78 \pm 1 \text{ mV}, \) epicardial cells \(-77 \pm 1 \text{ mV}, \) and M cells \(-79 \pm 1 \text{ mV} \). Phase 0 amplitude was also not different between the different types. The phase 1 magnitude decreased with frequency in all cell types, except in the M cells where it increased (Fig. 3, upper panel). At 1 and 2 Hz, phase 1 magnitude was significantly larger in M cells than in MID or ENDO cells. To analyze the prevalence of a ‘dome’ in M cells, we measured the value of the membrane potential at 70 ms, as the peak of the dome typically occurred at 70–80 ms after the stimulus (Fig. 3, lower panel). In M cells, this value was significantly larger than in the other groups, and declined with increasing frequency, in contrast to the increase observed in the other cell types.

The most striking characteristic of the M cells, the frequency dependence of the action potential duration is illustrated in Fig. 4. The \( \text{APD}_{90} \) and \( \text{APD}_{50} \) of M cells was significantly larger than of other cell types at all frequencies. Whereas M cells had a steep frequency dependence, the APD did not vary significantly with frequency in the other cells.

3.2. Characteristics of the body surface ECG of the anesthetized pig

We looked for evidence of heterogeneity in repolarization on the ventricular wall by analyzing QT(U) morphology of the body surface ECG. Since we were not able to reliably record ECG in awake animals, ECGs were recorded during anesthesia. In 12 animals, the mean heart rate was 112 ± 35 beats per min (range 63–176). The mean QRS duration was 38 ± 1 ms. The QT interval as a function of heart rate is shown in Fig. 5A (solid circles, shown with the linear regression line). For the longer QT intervals, biphasic T waves, or U waves could be observed (data points marked with a cross). An example of such a recording is shown in Fig. 5B. In this same recording a
Fig. 3. (A) Phase 1 magnitude, i.e. the difference between peak and notch, measured at different frequencies, including after 1-min rest (data points close to 0). ANOVA for cell*frequency was, P < 0.001, # indicates P < 0.05 for M cells vs. all other cell types, * indicates P < 0.05 for M cells vs. MID and ENDO after applying the Bonferroni/Dunn test; at 2 Hz, EPI is also different from MID and ENDO. Average values for 21 EPI cells, 25 MID cells, 11 ENDO cells, and 23 M cells. Frequency dependence was significantly different for M cells vs. other cell types. (B) Membrane potential of the dome or plateau, measured as the potential at the Ca transient immediately after the upstroke. Overall ANOVA P < 0.0001, # indicates P < 0.001 for M cells vs. all other cell types with Bonferroni/Dunn test. Decrease with frequency for M cells, and increase for the other cell types was significant for all groups (P < 0.01 for frequency up to 1 Hz, NS for 2 Hz).

small J wave can be seen as well as in lead III. Such a J wave was seen in 7/12 recordings. The Q–J interval was between 30 and 54 ms, close to the range of the time to the notch (between 29 and 35 ms for M cells with spike-and-dome configuration).

3.3. [Ca^{2+}]_i transients of M cells

Because of the pronounced differences in APD, we looked for differences in the behavior of the [Ca^{2+}]_i transient. The frequency behavior of the [Ca^{2+}]_i transient is illustrated for a M cell Fig. 6A, with average values for cells of all layers shown in Fig. 6B. After 1-min rest, M cells had small [Ca^{2+}]_i transients and the amplitude increased with increasing frequency of stimulation. This behavior was significantly different from that of the other cell types, where the amplitude of the [Ca^{2+}]_i transient varied very little with frequency. Comparing Fig. 6B to Fig. 3A, it can be seen that the frequency dependence of the [Ca^{2+}]_i transients closely resembles the frequency dependence of the phase 1 magnitude.

3.4. M cell morphology

The general appearance of M cells with regular transmitted light was not different from any other cell type. In particular, they did not have the smooth, elongated appearance of single Purkinje myocytes, and they had conspicuous edges at the intercalated disc areas. Average cell capacity was not different between the groups, although it tended to be less for M cells (P = NS), whereas cell length was slightly larger for M cells (P < 0.05, Table 1).

4. Discussion

In this study of single myocytes isolated from the different layers of the left ventricular wall of the pig heart, we found a subpopulation of cells with characteristics...
similar to the ones of M cells described previously in other adult mammalian species [1, 2], including humans [3]. These M cells had a steep frequency response with long action potentials at low frequencies; most of them had a prominent spike-and-dome action potential configuration. The majority of these cells were found in the midmyocardial layer. Our findings support the idea put forward by Antzelevitch [20], that the absence of M cells in the pig heart reported earlier [19], may have been related to the young age of the pigs under study. We can at present not rule out that a difference in strain may also contribute to the contrasting findings. The distribution of the cells we find in the pig, is not completely similar to those of M cells previously described. A major difference is the fact that they are not confined to the midmyocardial layer, but are also present in the epicardial layer. One possible explanation is that our sampling of the epicardial layer should have been restricted to a more superficial layer. Another difference is the fact that a substantial portion of the midmyocardial layer consists of cells which lack the typical M cell features. This is in contrast to observations in transmural slices of the dog, where M cell behaviour was homogenous in the midmyocardial layer [9], although in isolated canine cells behaviour was more variable and transitional cells were observed [38].

The contribution of M cells to regional heterogeneity of repolarization in the intact heart has recently been the subject of some debate (for review see [39, 50]). Indeed, in vivo with intact gap-junctional coupling, the differences in APD of the cell types are expected to be eliminated to a large extent through electrotonic interactions [40]. Our results cannot directly contribute to the resolution of this issue. The longer QT time at lower heart rates is compatible with an important contribution of cells with clear rate-dependent changes in APD, a feature we most clearly observed in M cells. Analysis of the ECG recordings of the anesthetized pigs suggested the presence of some heterogeneity of action potential configuration and repolarization by the presence of biphasic T waves and J waves [13–15], although this was observed only in some animals. We can at present not know whether this related to an effect of the anesthetic agents, but the observation indicates the presence and influence of myocardium with a more pro-
nounced notch and longer action potential duration in these conditions. Our experiments on isolated cells offer most direct evidence for the existence of M cells, which could be a substrate for heterogeneity and pronounced rate-dependence of repolarization. To identify whether indeed these cells are responsible for the observed ECG characteristics, a combined measurement of electrograms across a larger multicellular preparation and transmembrane action potentials is needed (see e.g Ref. [41]).

Previous studies have pointed out that M cells share common characteristics with Purkinje cells [1]. Purkinje cells have a rather distinct appearance, with less striations than ventricular cells, and smooth edges, as described for dog [42], sheep [43], and rabbit heart [44]. In the present study, M cells had no distinct appearance or cell size, although on average they tended to be slightly longer. Their presence in the midmyocardial and epicardial layer, makes it further unlikely that they would represent intramyocardial Purkinje cells.

The ionic basis for the typical action potential configuration, and frequency response of M cells has been studied extensively in the dog. Several repolarizing currents are quantitatively different for M cells. They have a higher density of the transient outward K⁺ current, I_{to}, compared to endocardial, but not to epicardial cells, and the (slow) delayed rectifier current, I_{Kr}, is smaller in M cells than in epicardial cells [1,38,45]. In the human heart as well, regional differences in I_{to} have been reported [46–48]. In the current study, the action potential of cells from all layers has a conspicuous phase 1 repolarization, reminiscent of action potentials in the dog, or human, where a transient outward current is known to be responsible for this phase. However, in these pig cells this phase seems to be smaller, as also noted by Rodriguez-Sinovas et al. [19]. In addition, the phase 1 repolarization is slower than e.g. in canine M cells, where we measured a time to the notch of 19±2 ms (n_cells=7, K.R. Sipido and P.G.A. Volders, unpublished), versus 31±2 ms in pig M cells in this study, using identical recording conditions. The frequency dependent behavior of the notch in M cells is also different, as the phase 1 magnitude tends to increase rather than decrease with increasing frequency. These characteristics suggest that the underlying outward current may be different from the ‘classic’ outward K⁺ current in other species. In a recent study it was indeed reported that the current density of the transient outward K⁺ current in pig myocytes was very low (cells studied with intracellular Ca²⁺ buffering [49]). The absence of a transient outward K⁺ current may help to explain the differences in frequency dependent behaviour of early repolarization in the pig, compared to other species. The identity of other currents implicated in the regional heterogeneity of action potential configuration remains to be established. Since Ca²⁺ handling may also be different for M cells in the pig, Ca²⁺-dependent currents may play a role. The differences in APD and its frequency-dependent behaviour in M cells, are likely to be related to differences in delayed K⁺ currents, and/or in inward currents contributing to the plateau, such as the Ca²⁺ current, window Na⁺ current, or Na/Ca exchange current.

In conclusion, in single cells from the left ventricular wall of the young adult pig, we found a population of cells closely resembling M cells described in other species. These cells could contribute to the overall behaviour of the heart in vivo. However, pig myocardium also carries some distinctions, which may be important to take into account when extrapolating data from pig studies of electrical remodeling, or when evaluating the pig heart for xenotransplantation.

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