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DOI
10.1016/S0008-6363(96)00271-4
Publication date
1997
Document Version
Final published version
Published in
Cardiovascular research

ELSEVIER

Cardiovascular Research 33 (1997) 503–517

Cardiovascular Research

Review

# Differential electrophysiology of repolarisation from clone to clinic

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*Keywords:* Ischemia; Repolarisation; Refractoriness; Electrotonic interaction; Clones; Channels; Currents; Catecholamines; Species differences; Autonomic nervous system; Heart; Animal models; Patients

During the 18th Congress of the European Society of Cardiology in Birmingham (UK), a symposium dealing with the electrophysiological differences between various experimental models (ranging from clone to clinic) was organised by the Working Group on Cardiac Arrhythmias. The speakers have combined their efforts in this paper.

In biomedical research experimental conditions have to be vigorously controlled in order to permit unambiguous conclusions. Some questions simply cannot be answered in humans because of ethical matters. Animal research and studies on isolated organs, cells or even organelles generate information that needs to be extrapolated to the intact, healthy organism and from there to patients and groups of patients. Ideally, results from subcellular studies generate hypotheses that can be tested in more complex systems and in populations of patients. For example, a major breakthrough in the understanding of the inherited long QT syndrome has been made by the application of molecular biological techniques that identified four different aberrant genes encoding different proteins [1]. These four molecular abnormalities lead to the same clinical phenomenon but will require different treatment. However, the process of translation of results produced by well-controlled experiments to intact organisms is extremely difficult. For example, based on the electrophysiological effects of agents with class 1 action on isolated tissue [2] it cannot be easily perceived that arrhythmia suppression with these agents in a population of patients causes an increase in mortality. The paradoxical proarrhythmic effect underlying the failure of the Cardiac Arrhythmia Suppression Trial (CAST) [3] may be attributed to the fact that re-entry underlies arrhythmogenesis in these more complex models and that the agents used promote re-entry despite their efficacy in suppression of premature beats [4]. Therefore, the outcome of the CAST study is an illustration of 'model mismatch'.

This paper discusses some aspects of cardiac repolarisation studied at various levels ranging from clone to clinic. It underscores that on the one hand results from one model cannot always be extrapolated to another, and that on the other hand the differences between models may be used as a tool for the investigation of electrophysiological mechanisms.

## 1. Differences between clone and native ion channel *(Jan Tytgat)*

The successful cloning of several cardiac ion channel subunits has provoked much interest in determining which subunits contribute to the formation of the various types of native ion channels. To test the hypothesis that a specific clone encodes the channel for a specific myocyte current, the following criteria, summarised by Roberds et al. [5], should be evaluated: (1) the basic biophysical properties, such as kinetics, voltage-dependence, conductance, rectification, and ion selectivity should be in reasonable agreement; (2) the pharmacology of compounds known to interact directly with the channel pore should be similar [e.g., tetraethylammonium (TEA)]; (3) immunohistochemistry with isoform-specific antibodies made against cloned subunits should confirm that the channel protein is present in the cardiac myocytes; (4) affinity purification from native tissues should confirm the protein composition of the

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[1] Peter Taggart is supported by the BHF.

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PII S0008-6363(96)00271-4

native channel in terms of accessory subunits and multimer formation; (5) deletion of the cloned channel using in vitro anti-sense approaches or using transgenic animals should confirm the identity of the current that the gene supports. Since it may not always be possible to satisfy all of these criteria, differences between certain clones and native ion channels remain, some of which are addressed here.

Oocytes of *Xenopus laevis* have proven to be a useful and convenient transient expression system and are therefore most commonly used to compare the function and pharmacology of a cloned ion channel with an endogenous current [6,7]. While the large size of these oocytes allows easy manipulation and injection with channel mRNA, phenotypical differences between cardiac clone and native channel may exist because of: (1) minor differences in the voltage-dependence of channel gating when the surface charge of the cell membrane of the oocyte differs from that of the native myocyte, (2) different protein processing between the oocyte and the cardiac cell, (3) the presence of endogenous channel subunits in the oocyte, and (4) the charged hydrophobic yolk in the oocyte which may complicate drug equilibration [8].

The relationship between the various cloned $K^+$ channel subunits ($\alpha$- and $\beta$-types) and native $K^+$ channels in myocardial cells has been studied extensively because of the crucial involvement of cardiac $K^+$ currents in resting membrane potential, action potential duration and amplitude, automaticity and refractoriness. In addition, myocardial $K^+$ channels are the target of several antiarrhythmic drugs. Two basic types of native, voltage-dependent $K^+$ currents have been described based on different time- and voltage-dependent properties and pharmacological profile: (1) 4-aminopyridine (4-AP)-sensitive, rapidly activating and inactivating currents, referred to as $I_{to}$ (transient outward), and (2) TEA-sensitive, delayed, slowly inactivating currents, referred to as $I_K$ (delayed rectifier) [9]. The isoforms of voltage-dependent $K^+$ channels ('Kv') cloned from cardiac libraries originate from *Drosophila melanogaster* and include members of the *Shaker* subfamily (Kv1.1 to Kv1.6), *Shab* subfamily (Kv2.1 and Kv2.2), *Shaw* subfamily (Kv3.1 to Kv3.4), *Shal* subfamily (Kv4.1 and Kv4.2) and of the Kv5 (Kv5.1) and Kv6 (Kv6.1) subfamilies [8–10]. All these clones have been detected at the mRNA level in rat and/or ferret myocytes (atria, ventricles and/or SA nodal cells) [10,11], but protein expression in both rat and human atrial and ventricular myocytes was only confirmed for Kv1.5 [12,13], and in rat atrial and ventricular myocytes for Kv1.2, Kv2.1 and Kv4.2 [12]. In contrast, protein expression for Kv1.4 was not detected in rat heart [12]. This result does not tally with the suggestion, made 3 years earlier, that Kv1.4 is the molecular basis for $I_{to}$, although heterologous expression of Kv1.4 indeed gives rise to rapidly activating and inactivating $K^+$ currents, sensitive to 4-AP when studied in various heterologous expression systems [14,15]. Furthermore, heterologous expression of Kv4.2 also reveals $I_{to}$-like currents [16,17], prompting an investigation in depth of all the possible differences between the currents produced by Kv1.4, Kv4.2, and native $I_{to}$.

The current expressed from both rat and human Kv1.4 isoforms differs in several aspects from $I_{to}$ in the same species [18]. For example, Kv1.4 activates and inactivates at potentials 20–40 mV more negative than rat and human $I_{to}$. Furthermore, the half-maximal concentration for block of Kv1.4 by 4-AP ($K_d \approx 700$ $\mu$M) is lower than that observed for human $I_{to}$ ($K_d = 2$ mM) [19]. The most striking difference is the much slower recovery from steady-state inactivation of Kv1.4, and to a lesser extent of Kv4.2, than that of $I_{to}$: the time constant for Kv1.4 ranges from 3 to 8 s (at $-120$ mV), for Kv4.2 around 200 ms, but for native $I_{to}$ from 25 to 50 ms [20–22]. As a result Kv1.4 displays marked use-dependent accumulation of inactivation [23], as is seen for $I_{to}$ in rabbit but not in canine, rat or human myocytes. In addition, the recovery time constant of Kv1.4 displays little voltage dependence, whereas that of $I_{to}$ is steeply voltage-dependent [18]. The difference in recovery kinetics cannot be ignored and suggests that homotetrameric Kv1.4 channels do not underlie rat or human $I_{to}$.

If the above-mentioned discrepancy is caused by heteromultimer formation, then it is our task to elucidate the correct subunit combination or assembly. Coexpression of either Kv1.2 or Kv1.5 with Kv1.4 increases the rate of recovery, which suggests that functional $I_{to}$ channels might be heteromultimeric indeed. However, the recovery time constant is still around 700 ms [23]. Subsequent studies to investigate the possibility that association with $\beta$-subunits would normalize the recovery kinetics have also been performed. It turned out that neither Kv$\beta$1 nor Kv$\beta$3 subunits accelerate recovery from inactivation when coexpressed with Kv1.4 subunits [24,25].

Immunohistochemical experiments and Western blot analysis revealed that Kv1.4 protein is barely detectable in the membranes of adult rat ventricular myocytes, in contrast to Kv4.2 [12], supporting the hypothesis that Kv4.2 might be the molecular basis for $I_{to}$. For Kv4.2 expressed in cultured cells, the time constant for recovery from inactivation is approximately 160 ms at $-90$ mV at 20°C, which is not far away from that for $I_{to}$ [18]. Furthermore, Kv4.2 displayed little or no use-dependent inactivation during pulse trains up to 1 Hz from holding potentials of $-80$ mV or more negative, as is the case for $I_{to}$ in rat and human [26]. Consistent with the low sensitivity to flecainide for members of the *Shaker* Kv1 subfamily [27], Kv1.4 is only weakly sensitive to this class IC antiarrhythmic drug ($K_d > 60$ $\mu$M), while Kv4.2 is more sensitive ($K_d \approx 10$ $\mu$M) [Yeola and Snyders, unpublished results]. This pharmacological difference is quite interesting given the high flecainide sensitivity of rat and human $I_{to}$ [28,29]. Another independent piece of evidence in favour of Kv4.2 underlying $I_{to}$ can be found in the following observations. The distribution of $I_{to}$ is not homogeneous across the

ventricular wall: the density of $I_{to}$ increases from endo- to epicardial layers in rat, canine and human ventricle [30–32]. Interestingly, the distribution of Kv4.2 mRNA displays the same regional heterogeneity in rat ventricle, while no such gradient is observed for Kv1.4 [33]. It remains possible, however, that accessory β-subunits may be associated with Kv4.2 in vivo (or also some delayed rectifier-type clones), that contribute to narrowing the phenotypical differences between functional $I_{to}$ channels in the heart and myocardial Kv channels expressed heterologously.

In conclusion, it is tempting to conclude that Kv4.2 underlies $I_{to}$, at least in rat and human myocardium, rather than Kv1.4, as the latter clone displays too many points of discrepancies with native $I_{to}$ in these species. Nevertheless, the need to identify the Kv4.2 protein in other mammalian species, such as mouse, sheep and rabbit, as candidate clone underlying $I_{to}$ warrants additional experiments in the near future.

## 2. Differences between patch and myocyte *(Marieke Veldkamp)*

The development of two techniques proved to be essential for the progress made during the last decades in the investigation of membrane currents underlying cardiac electrical activity. First, in 1976, a procedure was developed to isolate viable, calcium-tolerant myocytes from adult mammalian hearts [34]. Previously, calcium-tolerant myocytes could only be isolated from fetal and neonatal hearts. Second, in the same period, the patch-clamp technique was introduced, which made it possible to reliably measure and characterise transmembrane ionic currents in these single cells [35,36]. The various configurations of the patch-clamp technique allow measurement of 'macroscopic' or whole-cell currents in the whole-cell configuration, and 'microscopic' or 'single channel' currents through individual channels in the cell-attached patch or excised patch configurations. Each of these configurations has its specific advantages and disadvantages and from each configuration specific data can be obtained. The differences between myocyte and patch in fact concern the differences between the whole-cell configuration and the various patch configurations which will be outlined and contrasted below.

### *2.1. Separation of current components*

A correct analysis of the properties of a particular type of ion channel requires that current through these channels can be reliably separated from current through other types of channels. In whole-cell voltage clamp, however, the current that is measured is the sum of currents through many different channel types. One way to eliminate current types is by means of the voltage protocol. A holding potential of −40 mV, for example, is often used to study the calcium current. At this potential the calcium channels are available for activation, whereas the sodium channels are inactivated. This method, however, is not always completely efficacious. Although the sodium current is eliminated, the delayed rectifier current, activated in the same voltage range as the calcium channels, is still present. Another tool for the isolation of a particular current component is the use of agents that block either the current that is investigated or the other currents that are active in the same voltage range. Either way, this requires that the blocking action of the agent(s) is specific for one channel type and is voltage-independent. Unfortunately, these conditions are not always satisfied.

In membrane patches, contrary to the whole-cell configuration, different channel types can more easily be distinguished from one another. In this configuration, openings and closures of individual channels can be seen as discrete steps in patch current and particular channel types can be recognised by their unitary current amplitude of openings, since each channel has its specific conductance and opening and closing characteristics. However, the conductance of many channels is small under physiological ionic conditions, and it may prove difficult to distinguish unitary currents from noise. In that case the use of a high concentration of the permeant ion often is a necessity to increase the single channel conductance.

### *2.2. Data interpretation*

Signals obtained in the various configurations differ in the type of information that can be deduced from them. In the voltage-clamp mode of the whole-cell configuration the sum of currents through many channels is recorded and thus the average population properties are studied. The current is the product of the number of channels, the channel open probability, and the single channel current amplitude. In voltage clamp the time course of the current is obvious and can be fitted with exponential functions of which the time constants give information on the behaviour of the underlying channels. The number of channels, open probability, and single channel current amplitude cannot, however, be directly deduced from the whole-cell current. Finally, in the current-clamp mode of the whole-cell configuration action potentials can be recorded, the resultant of the ensemble of the various membrane currents present in the myocyte.

Parameters that are not easily obtained in the whole-cell configuration are directly measured in the patch configurations: open and closed times of the channel, the open probability, and the single channel conductance. However, the stochastic behaviour of ion channels requires repeated measurements to obtain reliable mean values for these parameters. Lastly, the number of channels per patch can be estimated. This may give an indication of the total number of channels per cell when assumptions regarding the size of the patch membrane surface are made.

### 2.3. Environment

The behaviour of channels is not only regulated by transmembrane voltage, but also by intracellular factors. Large differences exist between the whole-cell configuration and the various patch configurations concerning the intracellular environment of the channels. In the conventional whole-cell configuration the cell is internally perfused with the electrode solution. This has the advantage that the composition of the intracellular medium can be controlled. On the other hand, intracellular factors essential for channel activation may be washed out and run-down of channel activity is the result. The recent introduction of the perforated patch-clamp technique, however, does offer the possibility of making whole-cell recordings without internal perfusion of the cell [37,38].

The intracellular environment of a patch completely depends on the configuration. In the cell-attached patch configuration the intracellular environment is completely intact and this offers the possibility of studying channels in their natural environment. In excised patches, channels are isolated from their natural intracellular environment and exposed to the bath solution. As in the whole-cell configuration, the intracellular medium can be completely controlled and, moreover, it can be quickly changed, which offers the possibility of studying channel regulation by 'intracellular' factors. However, also in this case, channel run down may occur.

### 2.4. Whole-cell and patch experiments

In the most favourable situation, information obtained from whole-cell and patch experiments will be partly overlapping and partly complementary, thereby contributing to a better understanding of channel function. Fig. 1 shows an example of an experiment in which two pipettes were placed on the same spontaneously beating sino-atrial (SA) node cell. One pipette is used to record whole-cell currents (voltage-clamp mode) or action potentials (current-clamp mode), and a second pipette is used to record delayed rectifier ($I_K$) channel activity from a cell-attached patch at the same time. Both pipettes were filled with a 150 mM KCl solution. The experiment in Fig. 1A and B was performed to compare properties of the channels in the patch membrane with those underlying the whole-cell $I_K$ current. Via the whole-cell pipette the cell was depolarised to +30 mV from a holding potential of −70 mV. In the upper trace, showing the whole-cell current, an inward peak current is seen which is caused by a rapidly activating calcium current ($I_{Ca}$). The following change in outward direction is caused by inactivation of $I_{Ca}$ and activation of $I_K$. On repolarisation $I_K$ deactivates again, seen as a slow decrease in current. The four lower traces show simultaneously recorded patch currents with $K^+$ channel activity. During depolarisation no channel openings can be observed, probably due to the small driving

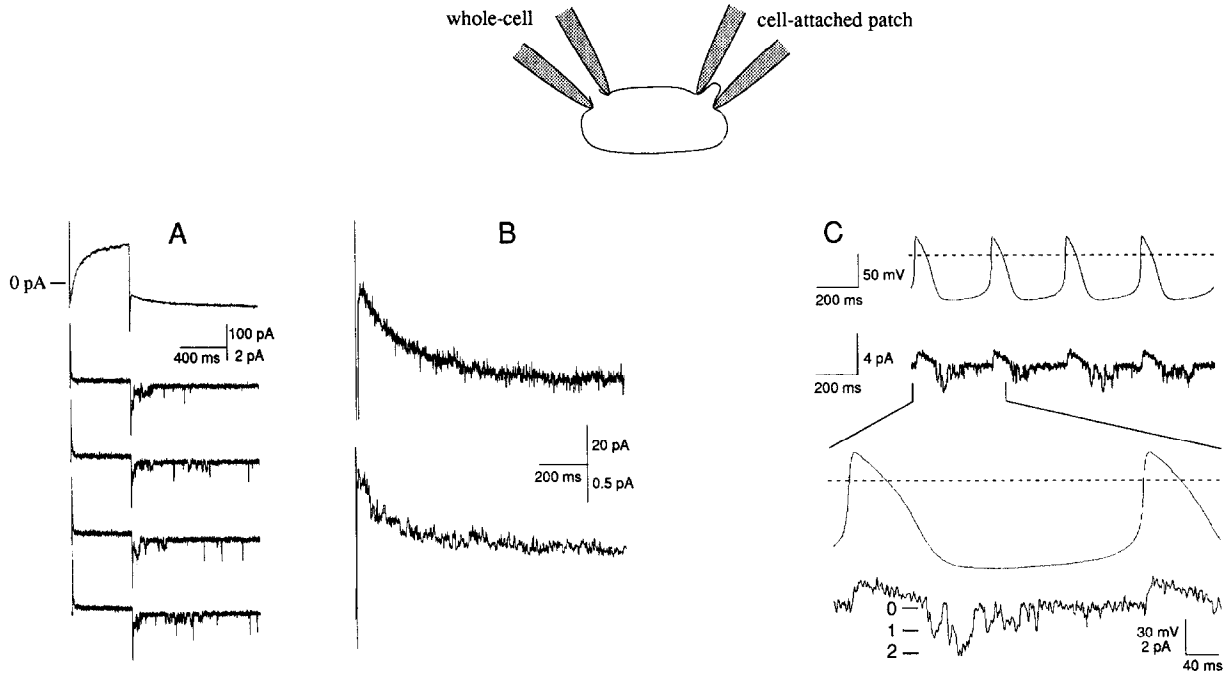


Fig. 1. Delayed rectifier ($I_K$) channel activity during spontaneous action potential generation in the SA node. Top: Schematic drawing of rabbit SA node cell with a whole-cell and a cell-attached pipette. (A) The whole-cell $I_K$ current (upper trace) and simultaneously recorded single $I_K$ currents (lower traces). Channel openings are seen as downward deflections. $I_K$ was activated by a 500 ms step to +30 mV from a holding potential of −70 mV. (B) Time course of current decay on repolarisation to −70 mV for the whole-cell $I_K$ (upper trace) and for the ensemble averaged current (lower trace). The ensemble averaged current (reversed polarity) was constructed from 43 patch current traces. (C) Action potentials (upper trace) and simultaneously recorded single $I_K$ channel activity (lower trace). *Inset:* Channel openings are seen as downward deflections. The levels 0, 1, and 2 correspond to number of $I_K$ channels in the open state.

force for potassium at this potential ($E_K$ for the patch is 0 mV) and fast inactivation of the channel. On repolarisation, however, a burst of channel openings is seen which disappears with time, due to deactivation. Fig. 1B shows the enlarged whole-cell tail current (upper trace) and the ensemble averaged patch current, shown with reversed polarity to make comparison easier (lower trace). The time course of deactivation of both is the same indicating that these channels underlie the whole-cell $I_K$. This enabled us to study their role in action potential generation in the SA node. Fig. 1C shows action potentials (upper trace) and $I_K$ channel activity (lower trace) simultaneously recorded from the same cell. $I_K$ channel openings only occur during the later part of repolarisation and persist during the first phase of diastolic depolarisation (inset). Subsequently, channel activity decreases during the course of diastolic depolarisation. These data suggest a role for $I_K$ in final repolarisation of the action potential and in diastolic depolarisation.

Data obtained from whole-cell and patch measurements are not always in agreement. Fig. 2 shows an experiment in which the whole-cell data and the patch data seem to be incompatible. Again, two pipettes were used on the same rabbit ventricular myocyte, one for whole-cell recording of action potentials and one for cell-attached patch recording of ATP-regulated potassium ($K_{ATP}$) channel activity. Both pipettes were filled with a 150 mM KCl solution. Several studies have shown that the outward current responsible for the action potential shortening during metabolic inhibition is carried by $K_{ATP}$ channels [39–42]. This experiment was performed to establish the relation between action potential shortening during metabolic inhibition and the opening of $K_{ATP}$ channels. Energy metabolism of the cells was depressed by superfusion with Tyrode's solution containing 0.2 mM 2,4-dinitrophenol (DNP). In Fig. 2A the action potential duration versus time is plotted and in Fig. 2B the simultaneous single channel recording from a membrane patch of the same cell is shown on the same time scale. Shortly after switching to the DNP-containing solution, a rapid decline in action potential duration was observed and eventually the cell became unexcitable. Simultaneous recordings of a membrane patch of the same cell in the control condition (Fig. 2B) shows activity of 6 inward rectifier ($I_{K1}$) channels. During the period in which the action potential shortened, $I_{K1}$ channel activity persisted, but no $K_{ATP}$ channel events could be detected in the cell-attached patch. Shortly after cell inexcitability the first $K_{ATP}$ channel activity was observed, characterised by infrequent, short spike-like openings (arrow 1, see inset). Much later, $K_{ATP}$ channel behaviour abruptly changed to long-lasting openings with flickering behaviour, seen as a large increase in current (arrow 2, see inset). Wash-out of DNP resulted in the closure of $K_{ATP}$ channels and partial restoration of the action potential duration. Contrary to what is expected, action potential shortening does *not* occur at the same time as the opening of $K_{ATP}$ channels, but is followed by it. For reasons unknown, it appears that $K_{ATP}$ channels in the patch membrane behave differently from the $K_{ATP}$ channels in the whole-cell membrane.

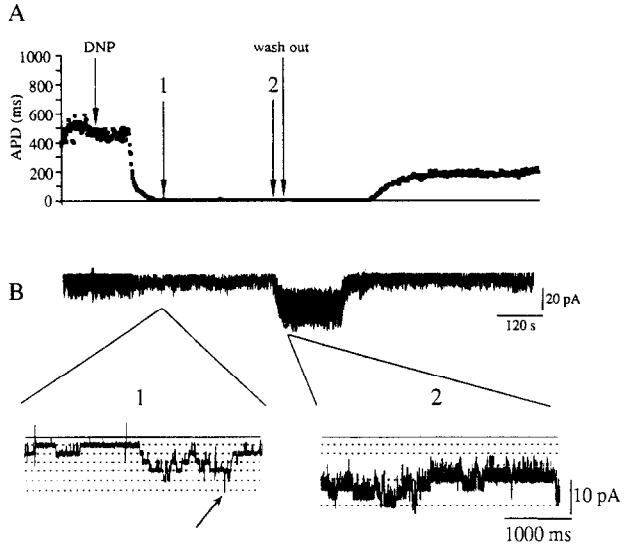


Fig. 2. Effects of metabolic inhibition on action potential duration and $K_{ATP}$ channel activity in a rabbit ventricular myocyte. (A) Action potential duration as a function of time. Addition and wash-out of DNP are indicated with arrows. (B) Simultaneous recording of single channel activity in a membrane patch of the same cell. The potential of the cell-attached patch pipette was held at 0 mV. Channel openings are seen as downward deflections. *Inset 1:* Single channel activity recorded at time indicated by arrow 1 in panel A. Solid line indicates the level at which all channels are closed, dotted lines indicate the unitary current level of the inward rectifier channels. The vertical lines at regular intervals on top of the current trace are stimulus artefacts. The first short opening of a $K_{ATP}$ channel is indicated by an arrow. *Inset 2:* Single channel activity recorded at time indicated by arrow 2 in panel A. Solid line indicates the level at which all channels are closed. First two dotted lines indicate the unitary current level of the inward rectifier channels; third and fourth dotted line indicate the unitary current level of the $K_{ATP}$ channel.

Except for the obvious differences between patch and whole-cell described above, the experiment in Fig. 2 suggests that there are also less obvious differences which may be the cause of the discrepancy. For example, the type of glass, whether soft or hard, has an influence on channel kinetics [43]. Also, the gating properties of the ATP-sensitive K channel depend on the electromotive force for potassium ions [44]. The use of unphysiologically high concentrations of the permeant ion to make single channel currents visible may therefore affect channel kinetics. Even more elusive factors may be involved: the gating kinetics of the cardiac sodium channel and the $K_{ATP}$ channel are regulated by the cytoskeleton [45,46]. It could be speculated that the cytoskeleton in the various patch configurations is distorted or disrupted from the cell membrane which is far up in the pipette [47], thereby influencing channel kinetics.

In conclusion, measurements performed in the whole-cell configuration give information about the average channel population properties whereas measurements performed in a patch configuration give information about the

properties of individual channels. Because different kinds of information are obtained from these different configurations, they should be used complementarily. When data from whole-cell and patch are incompatible, the cause of the discrepancies may provide insight into the function and regulation of ion channels.

## 3. Differences between myocytes and the intact heart *(Ruben Coronel)*

### *3.1. Electrotonic interaction*

It was only in 1954 that Sjöstrand and Andersson first provided definite electron-micrographic proof that the myocardium is not a syncytium and that the cells are separated from each other along their entire circumference, even at the intercalated disks [48]. This is surprising because as early as 1877 Engelmann described electrophysiological experiments from which he inferred that "[...] although the cells can transmit the excitation process to each other during life they die solitary." [49] During these experiments the heart was injured with a knife and the potential difference between the injured and the uninjured myocardium was measured. The potential difference declined in the course of minutes, a phenomenon we now refer to as 'healing over'. At present we know that the closing of gap junctions underlies this process and that these specialised regions of the sarcolemma play a prime role in the intercellular exchange of matter and current.

The importance of the interaction between cells for electrophysiologic behaviour is elegantly demonstrated by the experiments of Tan et al. [50] In these experiments the current generated by a guinea-pig cell was injected into a model cell through a variable resistor and vice versa. With the resistor set at a particular, critical, value the action potential generated in the guinea-pig myocyte became grossly distorted and demonstrated, depending on the potential of the model cell, action potential shortening or prolongation. This was also shown in experiments by Rook et al. in which two neonatal rat myocytes were brought into contact and were allowed to form gap junctions [51]. Within seconds after the establishment of contact the action potential generated in one of the two cells led to a small depolarisation in the other, quiescent cell, and to changes in the action potential configuration in the stimulated cell. After 16 seconds of contact the action potential was shown to propagate to the previously quiescent cell, be it with a long delay. Indeed, within this short time frame gap junctions had formed.

### *3.2. In cells, not in heart*

Heterogeneities in action potential duration and refractoriness favour the initiation of re-entrant arrhythmias [52]. Large epi- to endocardial differences in action potential duration are known to exist [53]. Sicouri et al. have recently described a region with long action potentials in transmural tissue slices from the ventricular myocardium of dog hearts [54]. This zone was named the 'M-cell region'. The tissue slices were produced by 'shaving' a transmural section of the ventricle and essentially the same results were obtained in single cells isolated from the different portions of the ventricular wall [55]. The prolongation of the action potential as a response to heart rate slowing is much more pronounced in the M-cell region than in the subepicardial or subendocardial layer especially during ischaemia or after exposure to antiarrhythmic drugs.

These findings may be particularly relevant for arrhythmogenesis caused by triggered activity following early afterdepolarisations. Alternatively, re-entry based on electrophysiological inhomogeneities in refractoriness may be promoted by the transmural differences in action potential duration. The question remains, however, whether these large transmural differences also exist in the intact heart. Gordon Moe tells us that he once thought that large action potential differences may cause a current to flow from the site with the longer towards the site with the shorter action potential and thus induce premature beats [56]. In fact, he wondered why closely-coupled premature beats were not much more common. After having made acquaintance with Mendez he became aware that the action potential differences would disappear as a result of the electrotonic interaction and he "..was forced to abandon an attractive hypothesis..." [56].

In the intact dog heart the transmural differences in refractory period following a large pause in the pacing cycle are not larger than after a regularly driven rhythm [57]. In another canine model the existence of spatial dispersion of repolarisation and refractoriness was recently demonstrated in a three-dimensional mapping study [58]. Although there is a clearly longer activation–recovery interval and refractory period in the middle part of the left ventricular wall [58] than at the endocardial and epicardial sites, the transmural differences are much less than expected based on the observations made on single cells at the same cycle length [55]. In another study no significant transmural differences in repolarisation among the different myocardial layers were detected in a syncytial preparation [59]. Therefore, in the intact preparation, action potential differences based on prolongation of repolarisation in the M-cell region are probably too small [60] to account for arrhythmogenesis unless the cells are partially uncoupled [51].

### *3.3. In heart, not in cells*

Although regional differences in action potential duration in a normal syncytial preparation are mitigated by electrotonic interaction it has been hypothesised that the transmembrane potential differences that occur in acute regional ischaemic myocardium may cause premature ven-

tricular beats that in turn may initiate ventricular fibrillation [61,62]. The regional differences in resting membrane potential of ischaemic myocardium are reflected by local depression of the TQ-segment and, indeed, ventricular arrhythmias resulting from acute ischaemia are closely associated with heterogeneities in TQ-potential [63]. ST-segment and TQ-segment changes in locally recorded DC-electrograms are caused by electrotonic interaction or flow of 'injury' current between normal and depolarised myocardium. Of course, for this current to flow, an intact intercellular communication is a prerequisite.

Fig. 3 shows two conditions in which the injury current may be relevant to arrhythmogenesis. During diastole, the current flows intracellularly from the depolarised to the normal myocardium, thereby tending to depolarise the latter. This may lead to a decrease in the local current requirement to attain stimulation threshold. Secondly, during early diastole, activation in the ischaemic part of the muscle may be delayed, causing a large potential difference between ischaemic and normal myocardium. The current flows in the same direction as in the previous condition. The potential difference is reflected by a large negative T-wave of the electrogram recorded from the tissue with delayed activation. The hypothesis of the arrhythmogenic role of the injury current is supported by the observation that ventricular premature beats during early ischaemia (usually between 2 and 4 min after occlusion) are closely associated with these deeply negative T-waves and that they originate from the normal side of the ischaemic border [61].

The idea that normal myocardium close to the ischaemic border is influenced by the flow of injury current was tested by measuring diastolic stimulation threshold at these sites [64]. The position of the stimulation electrodes relative to the ischaemic border was verified by local extracellular potassium concentration. This was measured at the same site with the use of miniature potassium sensitive electrodes. Sites where potassium did not rise following the production of regional ischaemia were defined as normal. Fig. 4 shows a graph of the change of the diastolic stimulation threshold at a normal site close to the ischaemic border [64]. After production of ischaemia in the adjacent vascular bed a decrease in the diastolic stimulation threshold by about 15% was measured. Similar observations were made up to 5 mm from the electrophysiological border. These findings indicate that a band of normal tissue with increased excitability surrounds the ischaemic myocardium. The potential gradient across the ischaemic border during a deeply negative T-wave is about 5 times as high as during mid-diastole [63]. This indicates that the injury current may bring the normal myocardium to excitation threshold especially if the safety factor of normal tissue is taken into account. Computer simulation studies have indicated that the electrotonic interaction may cause premature activation especially if an 'ischaemic' cell is coupled to a normal cell through the interposition of an unexcitable cell [65].

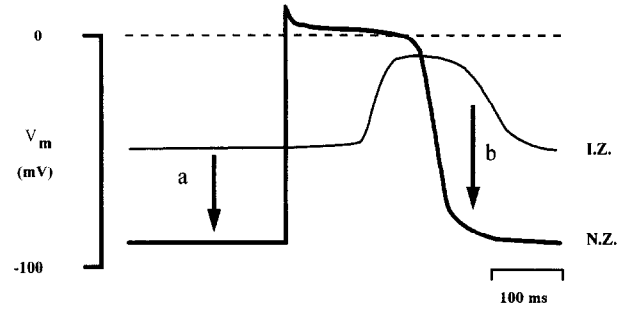


Fig. 3. Diagram illustrating the flow of electrotonic currents during regional myocardial ischaemia. Schematic action potentials recorded from the ischaemic zone (I.Z.) and from the normal zone (N.Z.) are shown. The potential difference between the two action potentials (mid diastolic and early diastolic, indicated at *a* and *b*, respectively) generate 'injury' currents with potential effects on arrhythmogenesis.

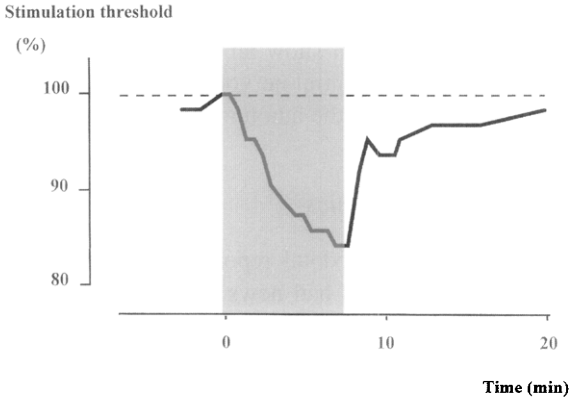


Fig. 4. Recording of the diastolic stimulation threshold of normal myocardium close to the ischaemic border. A period of ischaemia (shaded area) was produced by cross-clamping the left anterior descending artery. Relative to the pre-ischaemic control value a decrease of the stimulation threshold by about 15% was recorded which returned to normal following reperfusion.

Thus, during the acute phase of myocardial ischaemia myocardium with increased excitability surrounds ischaemic myocardium with decreased excitability. As a result, ventricular premature beats may occur exactly when large heterogeneities in refractoriness exist within the ischaemic tissue. Therefore, the 'trigger' and the 'substrate' for the initiation of ventricular fibrillation are closely associated in time and space. Electrotonic interaction is a major determinant of the electrophysiologic behaviour of myocardium especially under conditions where large inhomogeneities exist and/or where cells are partially uncoupled.

## 4. Differences between the isolated heart and the in vivo heart *(Tobias Opthof)*

The heart in an animal is subjected to more complicated influences than when it is isolated and not working. In this

section we will focus on the effects of the autonomic nervous system. We will show an example of arrhythmogenesis for which the central nervous system is a causative factor and one in which the autonomic nervous system acts as a modulating factor.

### 4.1. Stress and arrhythmias

There are many anecdotal reports on sudden death of persons having received bad news with huge impact [66]. Of course, the causes of these deaths cannot be determined with certainty, but it suggests that emotions can trigger fatal events. There are cases of sudden death caused by ventricular fibrillation without signs of necrosis [67]. A short period of acute ischaemia, not long enough to cause necrosis, may have caused sudden death in these cases. Alternatively, psychological stress may be so intense that it may predispose to fatal arrhythmias in the absence of acute ischaemia [68]. Stress is very hard to mimic in experimental conditions. It is not easily quantified because it does not affect the organism homogeneously. Although catecholamines are released as a result of psychological stress, one cannot extract the degree of stress from (nor)adrenaline levels in blood or urine [69]. Also, the induction of autonomic reflexes by hyperventilation, carotid sinus massage or the Valsalva manoeuvre does not predispose to ventricular arrhythmias as does psychological stress [70].

Theoretically there are three different modes by which stress may produce harmful effects. It may: (1) provoke fatal arrhythmias directly without any involvement of the coronary circulation; (2) cause ischaemia and associated arrhythmias that would be absent without stress; (3) exacerbate existing ischaemia. There is support in the literature for all three kinds of interaction, but we will discuss an example of the first mode.

### 4.2. Brain stimulation

The recognition that the heart is able to beat without a neural input [71] does not imply that the influence of the autonomic nervous system on the heart is irrelevant. Although controversies about the efficacy of all kinds of anti-arrhythmic agents are abundant, there is consensus on only one fact: β-blockers protect survivors of myocardial infarction from sudden cardiac death [72]. Agreement on the beneficial effect of β-blockade is, at present, best explained by the assumption that interference between the sympathetic nervous system and β-receptors in the heart is deleterious at least in patients with a healed myocardial infarction. The dichotomy of the autonomic nervous system in the parasympathetic and sympathetic limbs is at least valid for the efferent system. Also, separate afferent autonomic nerves have been described [73]. Preganglionic sympathetic fibres meet postganglionic fibres in the stellate ganglia or the midcervical ganglia. Vagal preganglionic fibres connect to postganglionic fibres within the heart.

The vasomotor centre of the heart is located in the medulla oblongata. Stimulation of higher centres in the brain also influences the heart as was already shown by Danilewsky in 1875 [74] and more directly by Karpus and Kreidl in 1909 [75]. Reviews on this topic have been published by Engel in 1978 [76] and by Mauck and Hockman in 1967 [77]. Stimulation of the hypothalamus in cats under anaesthesia provokes arrhythmias [78,79]. Bilateral cooling of the vagi or bilateral stellectomy prevented the occurrence of arrhythmias elicited by brain stimulation, and these arrhythmias could be mimicked by simultaneous stimulation of both limbs of the autonomic nervous system [79]. These data suggest that a loss of the interplay between the vagal and the sympathetic limbs of the autonomic nervous system is involved in arrhythmogenesis [76,80]. Arrhythmias elicited by brain stimulation may result from direct disturbance of electrical activity within the heart [81,82] or from indirect disturbance brought about by ischaemia. In dogs in the post-anger state reduction in coronary flow has been demonstrated [83] and ventricular fibrillation can be initiated by hypothalamic stimulation both in normal hearts and after coronary occlusion [84]. Blockade of the frontocortical–brainstem pathway by cooling prevents ventricular fibrillation after occlusion of the left anterior descending coronary artery in the pig heart [85]. Coronary occlusions in the same animals without cryo-blockade resulted in 100% incidence of ventricular fibrillation. Electrical stimulation of the same cortical areas that, if cooled, *prevented* ischaemia induced ventricular fibrillation, *caused* arrhythmias in hearts not subjected to coronary occlusion [85]. Thus, an intervention in the brain can abolish the fatal outcome of acute coronary occlusion in the pig and conversely stimulation of the same site results in arrhythmias in the absence of ischaemia. Thus, a pharmacological agent targeted at the same brain area must in theory be capable of preventing ischaemia-induced arrhythmias [85,86]. Indeed, infusion of l-propranolol into the lateral cerebral ventricles prevents or postpones ventricular fibrillation after a 15 min coronary artery occlusion in pigs unadapted to laboratory circumstances [87].

### 4.3. The long QT syndrome

Cardiac arrhythmias may occur when a 'trigger' interferes with a suitable electrophysiological 'substrate' in the presence of 'modulating factors' [88]. The long QT syndrome (LQTS) has been defined as an "unique example of non-coronary neurally mediated sudden cardiac death" [89]. This suggests that an aberration in the autonomic nervous system underlies the fatal arrhythmias. There are acquired, but also congenital, forms of LQTS. Typical clinical examples of congenital LQTS are the occurrence of syncope or cardiac arrest related to emotion, exercise or rest in young persons with a prolonged QT interval [90]. The responsiveness of those arrhythmias to factors that are related to the autonomic balance has led to the formulation

of the 'sympathetic imbalance hypothesis'. This hypothesis assumed that the right sympathetic innervation to the heart is deficient, particularly in its supposed inhibiting action on the left (sympathetic) stellate ganglion [89–91]. The clinical significance of this hypothesis has been suggested by the efficacy of left cervicothoracic stellectomy for the abolition of recurrent ventricular arrhythmias in a patient with LQTS [92] and 20 years later in a larger group of similar patients in which β-adrenoceptor blockade did not prevent syncope or cardiac arrest [93]. Interestingly, the 5-year survival rate was 94%, which is extreme in such a high-risk group, despite the fact that the corrected QT interval was only slightly shortened by the procedure: it was still abnormally prolonged [93]. The question remained, however, whether LQTS is primarily a disease of the autonomic nervous system or an intrinsic disease of the heart. The 'sympathetic imbalance hypothesis' leaned heavily on the observation that right stellectomy shortens ventricular refractoriness, whereas left stellectomy prolongs ventricular refractoriness in vagotomised dogs [94]. However, scrutiny of these data demonstrates that the refractory periods after the interventions were similar and that their control values in the groups subjected to either right or left stellectomy differed more than the magnitude of the effects ascribed to stellectomy (Fig. 1A and 1B in Ref. [94]). Moreover, there are conflicting reports describing prolongation of refractory periods after both right and left stellectomy [95,96]. One consequence of the 'sympathetic imbalance hypothesis' would be that right stellate stimulation may be as effective as left stellectomy and that right stellectomy may be as harmful as left stellate stimulation. However, chronic right stellectomy proves very effective in suppression of early ischaemia-induced ventricular tachycardia or fibrillation [97]. Moreover, data on acute right stellectomy [98] also do not support pro-arrhythmic effects of right stellectomy as reported by others [99]. The 'sympathetic imbalance hypothesis' assumed that the autonomic nervous system provided both the trigger and the substrate of the arrhythmias. The hypothesis was also difficult to combine with the observation that autotransplantation of the heart is not able to correct an abnormally prolonged QT interval [100] as reported previously by Zipes [101].

The more recent exciting data on gene mutations have settled this formerly vivid cardiovascular controversy. The congenital form of LQTS is an intrinsic disease of the heart based on one or more aberrant membrane currents relevant to ventricular repolarisation [102,103]. This intrinsic abnormality presents the substrate for the inherited form of LQTS. The role of the sympathetic nervous system may still be important in LQTS, because it may deliver the 'trigger' for the onset of lethal arrhythmias and it may act as an important modulating factor as well.

Thus, the autonomic nervous system may both directly cause arrhythmias and modulate other arrhythmogenic mechanisms.

## 5. Differences between animal models and patients *(Peter Taggart)*

In addition to electrophysiological differences between cellular, multicellular and whole animal models there are many well-known differences between one animal species and another [104]. Therefore, extrapolation of experimental results obtained from animals to the clinical situation is hindered by uncertainty as to which species may be truly representative. For example, studies on regional ischaemia are complicated by the wide variation in collateral flow in different animals. In one comparative study in which collateral flow to an area of regional ischaemia was expressed as a percent of flow to the normal area, reported values were: pig 0.6%, rabbit 2.0%, ferret 2.4%, rat 6.1%, cat 11.8%, dog 15.9% and guinea-pig estimated as up to 100% [105]. In canine models considerable variability is present between individual dogs and the incidence of ventricular fibrillation following coronary occlusion has been reported to vary between 0 and 100% depending on whether collateralisation is present [106]. It is likely that patients may occupy virtually any position in the spectrum from minimal collateral flow in childhood to substantial in older people and to extensive in older patients with coronary heart disease.

In the context of ischaemia and arrhythmogenesis several other factors may be relevant to differences between species and between animal studies and patients. Following coronary occlusion in pigs and dogs, two separate phases of ventricular arrhythmias occur [104]. An early phase occurs between 2–10 min following ligation (type 1A) and a second early phase occurs between about 15–30 min (type 1B). Type 1A are probably re-entrant. The mechanism underlying type 1B is not yet elucidated. In the hearts of smaller species (rats, guinea pigs, cats and rabbits) (see Ref. [107] for references) a single unimodal rather than a bimodal distribution is observed occurring at about 6–15 min following ligation, possibly corresponding to type 1B in larger species. Whether type 1A or 1B occurs in patients is at present unknown.

Heart size may be relevant for the occurrence of arrhythmias. There is some experimental evidence that sustained ventricular fibrillation is uncommon in small animal hearts [108] and it is a clinical observation that ventricular fibrillation is rare in human neonates [J. Deanfield, personal communication], in line with the theoretical concept of a minimum size required to sustain multiple wavelet re-entry [104].

A number of other factors may be important. Heart rate influences action potential duration and refractoriness and may influence metabolic parameters such as the severity of ischaemia [109]. The location of a coronary artery occlusion may influence the size of an ischaemic area and hence the incidence of arrhythmias. The presence and level of anaesthesia and stress may be relevant as described above. There is substantial evidence that in the context of acute,

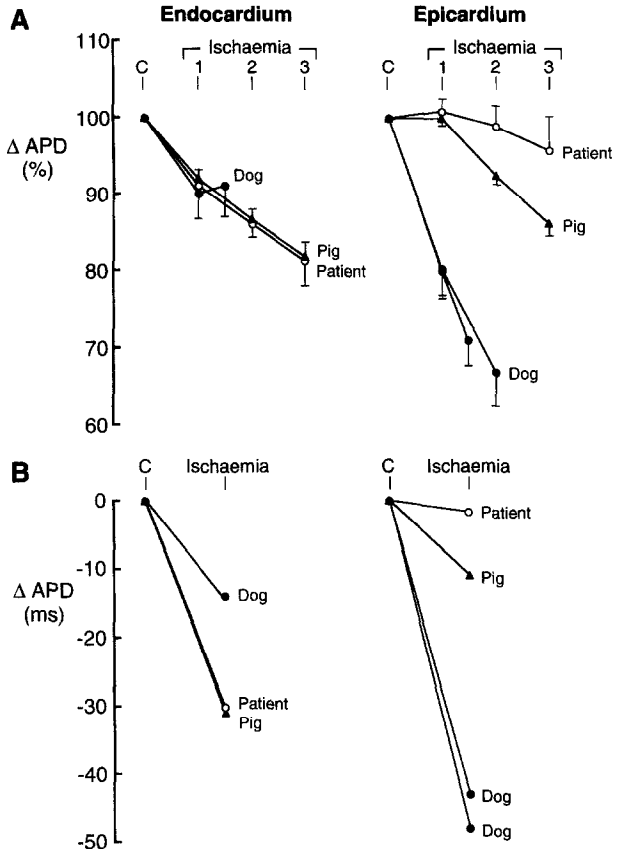


Fig. 5. (A) Change in APD as percent of control values (C) after 1, 2 and 3 min of ischaemia on endocardium and epicardium in patients, pig and dog (mean ± s.e.m.). (B) Change in APD in milliseconds after 90 s ischaemia.

subacute or chronic models of infarction and ischaemia the autonomic nervous system plays a major role [110], over and above effects mediated by an effect on heart rate [111]. In addition to the foregoing there are well-known electrophysiological differences at the cellular level in terms of the existence of different currents and current densities, and the functional characteristics of many of these in the intact human ventricle have yet to be determined. Furthermore, pathological conditions may alter the electrophysiology.

It is not always possible to accurately simulate the diseased human heart in animal models, and the most appropriate model may be man himself. Despite inherent difficulties and limitations data are now becoming available by integrating basic electrophysiological techniques into routine clinical procedures. Here, some specific comparisons will be made between the rather limited available data derived from patients and those from animal experiments during the first few minutes of ischaemia. In Fig. 5, changes in (monophasic) action potential duration during the first 3 min of ischaemia are shown for patients, pig and dog. The human endocardial data were derived from the area of the right ventricular septum rendered ischaemic during angioplasty of a left anterior descending coronary artery stenosis [112]. The human epicardial data were obtained during a 3-min period of global ischaemia in patients on cardiopulmonary bypass by cross-clamping the aorta between the inflow from the pump oxygenator and the coronary arteries [113] (this has been shown to have a cardioprotective effect during a subsequent period of ischaemia [114]). The pig data were obtained during a 3-min left anterior descending coronary artery occlusion in an open chest model [115]. Dog data were obtained during a 90-s left anterior descending occlusion in one study [116] and from epicardium only during the first 2 min of a 3-min occlusion in another [117]. On the endocardium action potential duration (APD) (normalised relative to control) shortens in a similar manner for all three 'species' (Fig. 5A). On the epicardium a wide variability in the response is seen. The dog data show a more rapid and pronounced APD shortening compared to the pig and the patients. In Fig. 5B the data are shown as change of APD in msec. The same overall relationship is apparent despite the differences in heart rate between the different species. The greater APD shortening in dog subepicardium tissue compared to dog subendocardium is consistent with canine cellular studies using simulated ischaemia in which the endocardial/epicardial differences were attributed to a greater density of $I_{to}$ in epicardium compared to endocardium (approximately 5-fold) [118]. A similar greater $I_{to}$ on epicardium compared to endocardium (approximately 4-fold) has been shown in human ventricular myocytes [119,120]. On this basis the slower APD shortening in human epicardium is opposite to what might be expected. Epicardial cooling may be one explanation, since cooling lengthens APD [121]. Cooling may be more pronounced in epicardial tissue due to exposure of the epicardium to the ambient temperature in open-chest preparations. The presence of $I_{to}$ in the pig heart remains to be evaluated.

Examination of the individual data points for patients and pig during ischaemia shows that although the overall pattern is similar, the scatter of the data points is considerably greater for the human data (Fig. 6). Several factors mentioned earlier may contribute to the greater coefficient

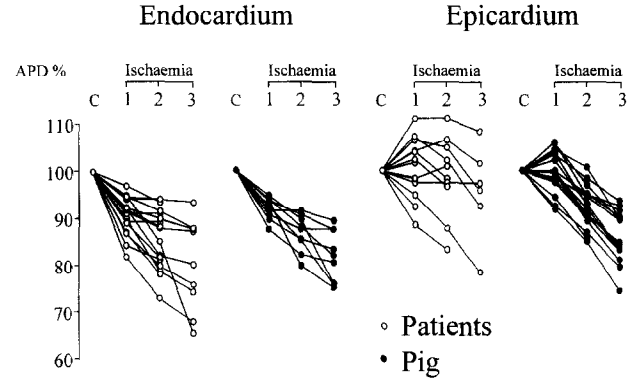


Fig. 6. Individual data points for change in APD in patients and pig during 3 min ischaemia (1, 2, 3) on endocardium and epicardium.

of variation in patients including differences in collateral flow. The pig heart has almost zero collateral flow. The patients in these studies, on the other hand, all had coronary heart disease of variable extent but sufficient to bring them to surgery and would therefore be likely to have developed varying degrees of collateralisation.

Arrhythmias are commonly initiated following an abrupt alteration in cycle length. Several electrophysiological parameters including APD are strongly cycle-length-dependent [104]. Therefore, the slope of the electrical restitution curve may be relevant to arrhythmogenesis [122]. For example, the steeper the slope, the greater will be dispersion of repolarisation during propagation of an early premature beat. Differences in the slope between neighbouring regions of myocardium as a result of a pathological process would be expected to influence the repolarisation gradients between these regions during interpolated beats of short or long cycles. However, direct extrapolation from animal data to the clinical situation may not be straightforward. For example, Lucas and Antzelevitch [118] have shown in canine right ventricular slices that simulated ischaemia flattened the time course of electrical restitution on the endocardium. On the epicardium at cycle lengths longer than 340 ms simulated ischaemia resulted in a negative slope with progressively shorter APD at longer coupling intervals (Fig. 7A,B). The latter was attributed to the presence of a strong $I_{to}$ in dog epicardium compared to endocardium and its slow recovery kinetics [118]. Recent studies have been performed during ischaemia in humans. The construction of complete electrical restitution curves takes time. Therefore, in order to examine the effect of the first few minutes of ischaemia in patients, an abbreviated electrical restitution curve was constructed using just 5 points on the curve [111]. On the endocardium ischaemia flattened the electrical restitution curve in line with in vivo pig endocardium [123] and in vitro canine data [118]. Noteworthy was the finding that in the human heart the flattening of the curve occurred within the first 2 min (Fig. 7C). In preliminary observations on the epicardium in patients, the effect of ischaemia was less pronounced than on the endocardium and the negative slope seen in Fig. 7B was not observed [113], being more in line with the pig. Experiments on the effect of small changes in temperature on the restitution curve in ferret single cells suggest that the reported influence of temperature on the electrical restitution curve is unlikely to influence comparisons using normalised data [113]. An alternative explanation could be intrinsic differences in the kinetics of $I_{to}$ between the canine (in which recovery is slow) and the human heart (in which $I_{to}$ recovery is rapid).

Experimental studies have implicated increased dispersion of refractoriness as a major cause of arrhythmia during the early phase of ischaemia [104]. Since it is not possible to measure refractory period simultaneously at more than one site, a method has been developed which utilises the interval between the activation moments of

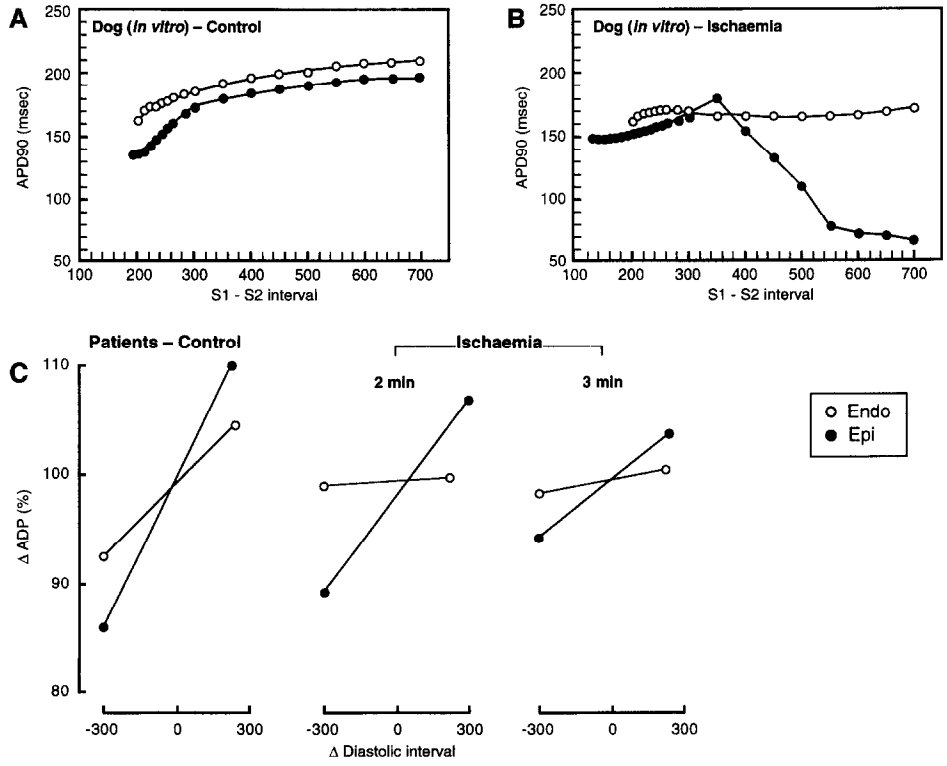


Fig. 7. Electrical restitution curves for in vitro canine ventricular muscle preparations shown separately for endocardium and epicardium under control conditions (panel A) and during simulated ischaemia (panel B) (redrawn from Ref. [118]). Panel C shows the slope of abbreviated electrical restitution curves obtained from patients under control conditions and after 2 and 3 min of ischaemia (see text).

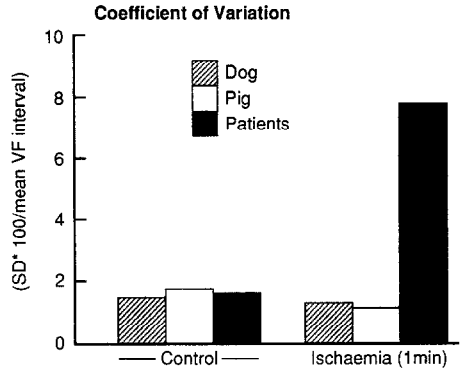


Fig. 8. Coefficient of variation of ventricular fibrillation intervals in dog, pig and patients. Under control conditions the values are similar. After 1 min of ischaemia the values are much greater in patients than in pig and dog.

ventricular fibrillation signals as a reliable index of refractoriness [124]. This technique has been adapted to recordings during a 3-min period of global ischaemia in patients with coronary heart disease on cardiopulmonary bypass. Preliminary results have shown that under control conditions the ventricular fibrillation interval on human epicardium is about 165 ms [125], a value slightly shorter than in human endocardium in another study [126], but much longer than in the dog [124,127] and pig [128]. By using a grid of electrodes it is possible to determine the mean value of the VF intervals at each electrode site, and the coefficient of variation of these mean values as an index of dispersion of refractoriness. Early results on humans show that the coefficient of variation increases rapidly during the early phase of ischaemia in marked contrast to the lesser increase in the canine and pig heart [125,128,129] (Fig. 8).

## 6. Conclusion

The several examples mentioned above illustrate that even within one animal species, extrapolation from studies on the subcellular level to more complex systems is by no means straightforward. Studies on characterisation of a repolarising current in a clone may yield information that is difficult to reconcile with the repolarisation process in an isolated myocyte, let alone with repolarisation in an intact heart in an intact organism. This emphasizes the need for studies on a particular problem on many levels, preferably within the same laboratory or in close collaboration between different laboratories. In addition, species differences deserve more attention. For example, repolarising currents in the rat are different from those in many other species in terms of magnitude ($I_{to}$) and kinetics ($I_{kur}$) and the rat may not be the most appropriate model for the study of cardiac repolarisation. This becomes important in evaluating animal models mimicking human disease. Thus, the rat is often used in studies on cardiac hypertrophy and heart failure (e.g., Ref. [130]), processes accompanied by alterations in repolarisation. A greater awareness of species differences, on the one hand, and of the differences between investigations at different levels of complexity within one species, on the other hand, is relevant to bridge the gap between animal laboratory and clinic.

## Acknowledgements

We thank Dirk Snyders for providing unpublished data and helpful information (J.T.) Studies on explanted human hearts were made possible by the Transplant Team of the University Hospital of Utrecht (AZU). Cell isolation was performed by Berend de Jonge and Jan Bourier (M.V.). Max Lab is acknowledged for access to data from his laboratory (P.T.). The acquisition of basic electrophysiological data from the hearts of patients was made possible by the close cooperation between cardiologists, cardiac surgeons and physiologists including Dr Peter Sutton, Dr Howard Swanton and Mr Wilfred Pugsley (P.T.). Experiments on isolated hearts could not have been performed without the support of Francien Wilms-Schopman, Charly Belterman and Wim ter Smitte (R.C.). Dr Arthur Wilde is thanked for fruitful discussions (T.O.). The authors thank Prof. Dr. Michiel J. Janse for organising the symposium.

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