From *in vivo* plasma composition to *in vitro* cardiac electrophysiology and *in silico* virtual heart: the extracellular calcium enigma

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In spite of its potential impact on simulation results, the problem of setting the appropriate Ca$^{2+}$ concentration ([Ca$^{2+}$]$_o$) in computational cardiac models has not yet been properly considered. Usually [Ca$^{2+}$]$_o$ values are derived from *in vitro* electrophysiology. Unfortunately, [Ca$^{2+}$]$_o$ in the experiments is set significantly far (1.8 or 2 mM) from the physiological [Ca$^{2+}$] in blood (1.0–1.3 mM). We analysed the inconsistency of [Ca$^{2+}$]$_o$ among *in vivo*, *in vitro* and *in silico* studies and the dependence of cardiac action potential (AP) duration (APD) on [Ca$^{2+}$]$_o$. Laboratory measurements confirmed the difference between standard extracellular solutions and normal blood [Ca$^{2+}$]. Experimental data on human atrial cardiomyocytes confirmed literature data, demonstrating an inverse relationship between APD and [Ca$^{2+}$]$_o$. Sensitivity analysis of APD on [Ca$^{2+}$]$_o$ for five of the most used cardiac cell models was performed. Most of the models responded with AP prolongation to increases in [Ca$^{2+}$]$_o$, i.e. opposite to the AP shortening observed *in vitro* and *in vivo*. Modifications to the Ten Tusscher–Panfilov model were implemented to demonstrate that qualitative consistency among *in vivo*, *in vitro* and *in silico* studies can be achieved. The Courtemanche atrial model was used to test the effect of changing [Ca$^{2+}$]$_o$ on quantitative predictions about the effect of K$^+$ current blockade. The present analysis suggests that (i) [Ca$^{2+}$]$_o$ in cardiac AP models should be changed from 1.8 to 2 mM to approximately 1.15 mM in order to reproduce *in vivo* conditions, (ii) the sensitivity to [Ca$^{2+}$]$_o$ of ventricular AP models should be improved in order to simulate real conditions, (iii) modifications to the formulation of Ca$^{2+}$-dependent $I_{CaL}$ inactivation can make models more suitable to analyse AP when [Ca$^{2+}$]$_o$ is set to lower physiological values, and (iv) it could be misleading to use non-physiological high [Ca$^{2+}$]$_o$ when the quantitative analysis of *in vivo* pathophysiological mechanisms is the ultimate aim of simulation.

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1. Introduction

One of the main goals of the virtual physiological human (VPH) initiative and, more in general, of computational biology is the integration of knowledge coming from the wealth of data on individual physiological processes. Ultimately, pieces of information—separately analysed in vitro (e.g. the gating properties of each type of cardiac ionic channel)—will complement each other to predict the effects of alterations (e.g. mutations/drugs, etc.) on the whole organism in vivo (e.g. arrhythmic risk).

In this framework, researchers involved in computational modelling of pathophysiological processes are called to work on the border between different fields, methodologies and techniques. In this paper, we address a very specific but relevant example of how, along the ongoing trip towards VPH development, the computational approach could help in integrating the knowledge derived from in vitro experiments. At the same time, it will highlight some critical points in the extrapolation process to in vivo conditions and suggest possible solutions.

We focused on the problem of setting the appropriate electrolyte concentrations, particularly Ca\(^{2+}\), in computational cardiac cell models in order to correctly reproduce the physiological extracellular environment. In spite of its potential impact on simulation results, this aspect seems to be usually not appropriately considered in model formulation.

The problem is particularly relevant as far as the extracellular Ca\(^{2+}\) concentration, [Ca\(^{2+}\)]\(_{o}\), is concerned. In fact, cardiac computational models usually derive [Ca\(^{2+}\)]\(_{o}\) values from the experimental conditions supplying the data they are based on. Namely, extracellular electrolyte concentrations are usually set equal to those of the standard Tyrode’s solutions traditionally used in patch-clamp experiments. Unfortunately, the Tyrode’s Ca\(^{2+}\) content (1.8 or 2 mM) is significantly far from the physiological Ca\(^{2+}\) concentration in blood serum (normality range 1.0–1.3 mM; Gosling 1986; Tietz 1995) and, consequently, in the interstitial fluid, which represents the in vivo extracellular fluid.

It is obviously correct to simulate the electrical activity of cardiac cells by imposing the same extracellular concentration used in experimental protocols as far as the aim is a comparison with in vitro experimental data. On the contrary, it can be incorrect to use the same concentration when the analysis of in vivo pathophysiological mechanisms is the ultimate aim of simulation.

(a) The standardization of in vitro and in silico extracellular solutions: a brief review

The use of saline is believed to have originated during the cholera pandemic that swept across Europe and reached England in 1831. Here, the 22-year-old medical doctor William O’Shaughnessy proposed to treat patients ‘by the injection of highly oxygenated salts into the venous system’ (O’Shaughnessy 1831). The composition of salt solutions was intensively studied in the nineteenth century on isolated frog nerve and muscle by Sydney Ringer, leading to the development and subsequent modification of Ringer’s solution. He found that bathing the heart muscle in saline solution prepared with distilled water made the ventricle grow ‘weaker and weaker’, leading to cessation of contractility in
approximately 20 min. He concluded that ‘some of the inorganic constituents of the pipe water’ were essential for maintaining heart beat in vitro. Ringer’s solution was developed as a consequence of these observations by adding 6 g sodium chloride, 3.1 g sodium lactate, 300 mg potassium chloride and 200 mg calcium chloride (approx. 2 mM) to 1000 ml water (Ringer 1883).

Tyrode’s solution, invented by Maurice Vejux Tyrode, an American physiologist, was a modification of Ringer–Locke’s solution also containing magnesium and buffered with bicarbonate (and later with HEPES) instead of lactate. This solution and countless further modifications represent the ‘standard’ extracellular milieu for electrophysiological experiments. It is worth noting that such conventional solutions used for in vitro experiments do not contain calcium buffers (only proton buffers), so that all the calcium is available in its free ionized form.

‘Calcium ions as carriers of charge have entered the scene but a few years ago...’ noted Weidmann in 1971. The note refers to the papers by R. Niedergerke and R. K. Orkand who first reported that ‘the overshoot of the action potential recorded from frog ventricles [...] is surprisingly sensitive to changes in the external calcium concentration’ (Orkand & Niedergerke 1964). Standard extracellular $[\text{Ca}^{2+}]$ used by Niedergerke & Orkand (1966) was 1 mM, although they tested the effect of calcium ions over a wide range of concentrations (0.1–10 mM). A year later, H. Reuter used 1.8 mM as a midpoint concentration to evaluate the dependence of ‘slow inward current’ ($I_{\text{si}}$, now $I_{\text{CaL}}$) on the extracellular calcium concentration of mammalian Purkinje fibres (Reuter 1967). $\text{Ca}^{2+}$ currents were long questioned as artefacts of inadequate voltage control (Johnson & Lieberman 1971), although Weidmann himself refers to Reuter’s evidence of Ca influx as rigorous (Weidmann 1971). Therefore, it is possible that, at that time, the ‘electrophysiological issue’ eclipsed the ‘biochemical issue’, i.e. the ionic composition of the extracellular milieu.

Soon after Hodgkin and Huxley laid the foundation for the use of integrative models of excitable cells, the first cardiac cell models were developed using a similar approach (Fitzhugh 1960; Noble 1962), but since $\text{Ca}^{2+}$ currents had not yet been discovered, $\text{Ca}^{2+}$ was totally absent from the scene. Aspects of intracellular $\text{Ca}^{2+}$ handling were introduced in the DiFrancesco–Noble Purkinje cell model (DiFrancesco & Noble 1985), and in more detail some years later in the Luo–Rudy ventricular cell model (Luo & Rudy 1994). Based on the most used standard solutions used in voltage-clamp experiments, extracellular $\text{Ca}^{2+}$ concentration was set in these models to 2 and 1.8 mM, respectively. Since then, almost all the models of cardiac action potentials (APs) have chosen between these two values. Among the several models with 2 mM $[\text{Ca}^{2+}]_o$ (Noble & Noble 1984; DiFrancesco & Noble 1985; Demir et al. 1994; Dokos et al. 1996; Priebe & Beuckelmann 1998; Winslow et al. 1999; Zhang et al. 2000; Fox et al. 2002; Kurata et al. 2002; Iyer et al. 2004; Ten Tusscher & Panfilov 2006), all those coming from Noble’s group and the human ventricular models by Priebe and Beuckelmann, Iyer and Ten Tusscher can be mentioned. On the contrary, all Rudy’s group models, the Courtemanche human atrial model and the Shannon rabbit ventricular model are among those with 1.8 mM $[\text{Ca}^{2+}]_o$ (Luo & Rudy 1994; Courtemanche et al. 1998; Jafri et al. 1998; Nygren et al. 1998; Matsuoka et al. 2003; Bondarenko et al. 2004; Shannon et al. 2004; Mahajan et al. 2008). Only in few models was $[\text{Ca}^{2+}]_o$ set to different values; in particular, in the
Lindblad model of rabbit atrial cell it was set to 2.5 mM (Lindblad et al. 1996), whereas in the Pandit model of rat ventricular myocyte it was set to 1.2 mM (Pandit et al. 2001). It is worth remarking that the assignment of this parameter has not been discussed in any of the published models, being implied that it should reflect the \( \text{Ca}^{2+} \) concentration used \textit{in vitro} during the experiments the model is based on. Thus, we aimed to examine the consistency among \textit{in vivo}, \textit{in vitro} and \textit{in silico} studies as far as the dependence of cardiac AP on extracellular calcium is concerned (figure 1).

2. Materials and methods

(a) Electrophysiology

Human atrial myocytes were enzymatically isolated from specimens of right atrial appendages. Detailed procedure for transport of atrial tissues and cell isolation has been previously published (Lonardo et al. 2004).

Cells were transferred to a recording chamber mounted on the stage of an inverted microscope and superfused by gravity with a six-line micropelusion system placed near the cell at a flow rate of 1 ml min\(^{-1}\) (temperature 36\(^\circ\)C). The experimental set-up used for patch-clamp recording in the whole-cell configuration was similar to that previously described (Pino et al. 1998; Lonardo et al. 2004). Cells were perfused with normal Tyrode’s solution with the following composition (in mM): D-(+)-glucose 10; HEPES 5.0; NaCl 140; KCl 5.4; MgCl\(_2\) 1.2; CaCl\(_2\) 1.2 or 2.0; and pH adjusted to 7.3 with NaOH. The perforated patch technique was used to record AP (pacing rate 1 Hz); amphotericin B (250 \(\mu\)g ml\(^{-1}\)) was diluted into the pipette solution containing (in mM) KMeSO\(_4\) 125, KCl 25, HEPES 5, EGTA 1 and pH= 7.2 with KOH.

Figure 1. Effects of \([\text{Ca}^{2+}]_o\) on cardiac AP. (a) Superimposed recordings from the same guinea pig ventricular myocyte superfused with progressively raised \([\text{Ca}^{2+}]_o\) as indicated for each AP in mM (adapted with permission from Leitch & Brown (1996)). (b) Superimposed recordings from the same human atrial myocyte superfused with 1.2 mM (solid curve) and 2.0 mM (dashed curve) \([\text{Ca}^{2+}]_o\) (original data).
Cardiac AP models were implemented in SIMULINK (Mathworks Inc., Natick, MA, USA). A variable-order solver based on the numerical differentiation formulae was used to solve the model equations (ode15s; Shampine & Reichelt 1997). Matlab implementation of the Greenstein model was downloaded from www.ccbm.jhu.edu. Pacing at 1 Hz was maintained for 500 s, until a steady AP had been reached. AP duration (APD) was measured as the interval between the AP upstroke and the 90 per cent repolarization level (APD_{90}). Since the maximal L-type Ca^{2+} current (I_{CaL,max}) depends (nonlinearly) on the membrane potentials, $I_{CaL,max}$ values were reported in each figure (figures 2f–7f) for different levels of [Ca^{2+}]_o at the time of $I_{CaL}$ peak. For simulations shown in figure 8, we implemented the described model modifications and stimulation protocols (Tsujimae et al. 2007, 2008).

(c) Modifications to the $I_{CaL}$ formulation in the Ten Tusscher–Panfilov model

After $I_{CaL}$ activation, the L-type Ca^{2+} channel undergoes voltage and Ca^{2+}-dependent inactivation. In the Ten Tusscher–Panfilov model, $I_{CaL}$ kinetics is described by a voltage-dependent activation gate ($d$), two voltage-dependent inactivation gates ($f$ and $f_2$) and an inactivation gate ($f_{Cass}$) that depends on the calcium concentration in the dyadic space ([Ca^{2+}]_{ss}),

$$I_{CaL} = G_{CaL} d f_2 f_{Cass}^4 \left( \frac{V - 15}{RT} \right) \left( \frac{[Ca^{2+}]_o - 0.25[Ca^{2+}]_{ss} \exp \left( \frac{2(V-15)F}{RT} \right)}{1 - \exp \left( \frac{2(V-15)F}{RT} \right)} \right).$$

Because experimental data on Ca^{2+} dependence of inactivation (CDI) in human myocytes are unavailable, we modified the $f_2$ and $f_{Cass}$ formulations according to the following qualitative observations. (i) Experimental studies demonstrated that CDI of $I_{CaL}$ is dominant over the voltage-dependent process and its maximum inactivation level is higher than the 60 per cent included in the Ten Tusscher–Panfilov model (Peterson et al. 1999, 2000; Linz & Meyer 2000). (ii) During AP clamp experiments on guinea pig ventricular myocytes, $I_{CaL}$ inactivation ranged from 40 to 95 per cent in control conditions (Linz & Meyer 1998). It seems, therefore, unlikely that at [Ca^{2+}]_i equal to 300 nM CDI was completely exploited so that a 60 per cent degree of inactivation would be expected for any higher [Ca^{2+}]_i, as assumed by the Ten Tusscher–Panfilov formulation. (iii) During the same experiments, CDI persisted (and even increased) all along the APD. Taken together, these observations led to the following $f_{Cass,inf}$ formulation, in which the switch shape is smoothed, the threshold for inactivation is shifted towards higher [Ca^{2+}]_i, the level of inactivation beyond the threshold is higher with respect to the original model and CDI is kept constant during the AP plateau:

$$\frac{df_{Cass}}{dt} = k \frac{f_{Cass,inf} - f_{Cass}}{\tau_{f_{Cass}}} ,$$

$k = 0$ if $f_{Cass,inf} > f_{Cass}$ and $V > -60$ mV,

$k = 1$ otherwise,

$$f_{Cass,inf} = \frac{0.9}{1 + \exp \left( \frac{[Ca^{2+}]_{ss} - 1.95}{0.15} \right)} + 0.1.$$
Moreover, the maximum inactivation level achievable by the fast component of the voltage-dependent inactivation is decreased to 30 per cent,

\[ f_{2,\text{inf}} = \frac{0.3}{1 + \exp\left(\frac{V_m + 35}{7}\right)} + 0.7. \]

Figure 2. AP sensitivity to \([\text{Ca}^{2+}]_0\) of the Luo–Rudy model of guinea pig ventricular myocyte. (a) Examples of simulated ventricular APs with different \([\text{Ca}^{2+}]_0\); (b) dependence of APD_{90} on \([\text{Ca}^{2+}]_0\); (c) examples of simulated intracellular \(\text{Ca}^{2+}\) transients with different \([\text{Ca}^{2+}]_0\) levels; (d) dependence of systolic (peak) and diastolic intracellular \(\text{Ca}^{2+}\) on \([\text{Ca}^{2+}]_0\); (e) simulated L-type \(\text{Ca}^{2+}\) and (g) Na/Ca exchanger currents for different \([\text{Ca}^{2+}]_0\) levels; and (f) dependence of maximal \(I_{\text{CaL}}\) current and (h) intracellular \(\text{Na}^+\) concentration on \([\text{Ca}^{2+}]_0\).

\[ \tau_{f_{\text{Ca}}} = \frac{80}{1 + \left(\frac{[\text{Ca}^{2+}]_{88}}{0.05}\right)^2} + 1. \]
3. Results and Discussion

(a) In vivo $[Ca^{2+}]_o$: normality range and laboratory results

Serum level of calcium is closely regulated within a limited range in the human body. The physiological $Ca^{2+}$ concentration in blood serum is in the range 1.0–1.3 mM (Gosling 1986; Tietz 1995), which is remarkably far from Tyrode’s $Ca^{2+}$ content (1.8 or 2 mM). In order to rule out possible discrepancies in the

Figure 3. AP sensitivity to $[Ca^{2+}]_o$ of the Shannon model of rabbit ventricular myocyte. (a) Examples of simulated ventricular APs with different $[Ca^{2+}]_o$; (b) dependence of APD$_{90}$ on $[Ca^{2+}]_o$; (c) examples of simulated intracellular $Ca^{2+}$ transients with different $[Ca^{2+}]_o$ levels; (d) dependence of systolic (peak) and diastolic intracellular $Ca^{2+}$ on $[Ca^{2+}]_o$; (e) simulated L-type $Ca^{2+}$ and (g) Na/Ca exchanger currents for different $[Ca^{2+}]_o$ levels; and (f) dependence of maximal $I_{Ca,L}$ current and (h) intracellular Na$^+$ concentration on $[Ca^{2+}]_o$. 

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measurement methods usually used to evaluate Ca\(^{2+}\) concentration in clinical and basic research laboratories, we tested the Ca\(^{2+}\) concentration in two standard Tyrode’s solutions prepared in our laboratory. Two solutions nominally containing 2 and 1.2 mM CaCl\(_2\), respectively (see §2 for composition), were tested through a haemo-gas analyzer (GEM 3000, Instrumentation Laboratory), currently used in a clinical analysis laboratory (Bufalini Hospital, Cesena, Italy). As expected, the Ca\(^{2+}\) concentration of the two solutions was substantially equal.

Figure 4. AP sensitivity to [Ca\(^{2+}\)]\(_o\) of the Ten Tusscher model of human ventricular myocyte. (a) Examples of simulated ventricular APs with different [Ca\(^{2+}\)]\(_o\); (b) dependence of APD\(_{90}\) on [Ca\(^{2+}\)]\(_o\); (c) examples of simulated intracellular Ca\(^{2+}\) transients with different [Ca\(^{2+}\)]\(_o\) levels; (d) dependence of systolic (peak) and diastolic intracellular Ca\(^{2+}\) on [Ca\(^{2+}\)]\(_o\); (e) simulated L-type Ca\(^{2+}\) and (g) Na/Ca exchanger currents for different [Ca\(^{2+}\)]\(_o\) levels; and (f) dependence of maximal I\textsubscript{Ca,L} current and (h) intracellular Na\(^{+}\) concentration on [Ca\(^{2+}\)]\(_o\).
to the concentration of CaCl₂ (2.06 and 1.18 mM, respectively, average of two measures), hence all the calcium was in the free ionized form in the solutions. Such concentrations were compared with those of the routine ‘clinical’ blood samples analysed immediately before and after our solutions, which were in the normality range, with no difference between measurements on the plasma and the whole blood sample (1.10 ± 0.08 and 1.14 ± 0.06 mM, respectively, n = 14).

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The interstitial fluid represents the actual *in vivo* extracellular fluid. It could be questioned whether the plasma Ca\(^{2+}\) concentration is a reliable estimate of the interstitial one. Actually, it is usually accepted that Ca\(^{2+}\) concentration in the interstitial fluid equals that in plasma. Indeed, the distribution of free cations between vascular and interstitial compartments has been reported to agree with the Donnan theory (Gilanyi *et al.* 1988), which predicts a theoretical ratio between interstitial and plasma concentrations equal to 0.976 (Leeuwen 1964). In particular, the concentration of Ca\(^{2+}\) in the interstitial fluid was found

![Figure 6](http://rsta.royalsocietypublishing.org/)

Figure 6. AP sensitivity to [Ca\(^{2+}\)]\(_o\) of the Ten Tusscher model of human ventricular myocyte after modifications aimed to strengthen the Ca\(^{2+}\)-dependent inactivation of \(I_{CaL}\) (see §2). (a) Examples of simulated ventricular APs with different [Ca\(^{2+}\)]\(_o\); (b) dependence of APD\(_{90}\) on [Ca\(^{2+}\)]\(_o\); (c) examples of simulated intracellular Ca\(^{2+}\) transients with different [Ca\(^{2+}\)]\(_o\) levels; (d) dependence of systolic (peak) and diastolic intracellular Ca\(^{2+}\) on [Ca\(^{2+}\)]\(_o\); (e) simulated L-type Ca\(^{2+}\) and (g) Na/Ca exchanger currents for different [Ca\(^{2+}\)]\(_o\) levels; and (f) dependence of maximal \(I_{CaL}\) current and (h) intracellular Na\(^+\) concentration on [Ca\(^{2+}\)]\(_o\).
to be slightly decreased with respect to plasma (1.18 versus 1.26 mM; Fogh-Andersen et al. 1995); alike, such a result even overestimated the concentration of ionized calcium in interstitial fluid (Fogh-Andersen et al. 1995). The Ca\(^{2+}\) level in the interstitial fluid, which constitutes the extracellular environment in vivo, is therefore significantly lower than that of usually bathing cells in vitro during patch-clamp experiments.

In order to reproduce an ‘average’ virtual physiological setting, we chose the central value of the normality range (1.15 mM) as average Ca\(^{2+}\) concentration for the simulations described in §3d. More sophisticated applications might require some level of patient-specific modelling; in this case, electrolyte

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**Figure 7.** AP sensitivity to [Ca\(^{2+}\)]\(_o\) of the Courtemanche model of human atrial myocyte. (a) Examples of simulated ventricular APs with different [Ca\(^{2+}\)]\(_o\); (b) dependence of APD\(_{90}\) on [Ca\(^{2+}\)]\(_o\); (c) examples of simulated intracellular Ca\(^{2+}\) transients with different [Ca\(^{2+}\)]\(_o\) levels; (d) dependence of systolic (peak) and diastolic intracellular Ca\(^{2+}\) on [Ca\(^{2+}\)]\(_o\); (e) simulated L-type Ca\(^{2+}\) and (g) Na/Ca exchanger currents for different [Ca\(^{2+}\)]\(_o\) levels; and (f) dependence of maximal I\(_{Ca,L}\) current and (h) intracellular Na\(^{+}\) concentration on [Ca\(^{2+}\)]\(_o\).
concentrations directly measured on the patient should be used as a model parameter. This is not always feasible, but there are some clinical cases where such values are strictly monitored. As a relevant example, in silico analysis using patient-based electrolyte levels proved to be a valuable tool to gain insights into the impact of haemodialysis treatment on cardiac cellular electrophysiology, where abrupt interstitial $\left[ \text{Ca}^{2+} \right]_o$ changes can cause prolongation of APD and hence arrhythmias (Severi et al. 2008).

Some species specificity could exist in blood electrolyte concentrations. Higher Ca$^{2+}$ plasma concentration than in other species has been reported for rabbits (Harcourt-Brown 2002), whereas values more similar to those in humans have been reported for guinea pigs (e.g. Rummens et al. 2000). Nevertheless, even when using animal model-based AP models, the computational analysis of cardiac cell electrophysiology aims to clarify some pathophysiological mechanism in humans. Therefore, we focused our analysis on the physiological Ca$^{2+}$ plasma concentration in humans.

Figure 8. Modulation by different $\left[ \text{Ca}^{2+} \right]_o$ levels (1.8 mM thin lines, 1.15 mM thick lines) of the effects of potassium current blockage on human atrial (Courtemanche model) APD. (a) Frequency dependency of APD under control conditions (dashed lines) and after 90% reduction of maximum conductance of the rapidly activating K$^+$ current (IKr) (continuous lines). (b) The percent APD$_{-70 \text{mV}}$ prolongation after 90% reduction of maximum IKr conductance is also shown; values were normalized to 500 ms CL. (c, d) AP prolongation by block of IKur in normal and atrial fibrillation (AF)-remodelled atrial myocytes. (c(i),(ii)) Simulated APs resulting from the reduction of the conductance of $I_{\text{Kur}}$ to 90, 70, 50, 30 and 10% (thin lines, reading from left to right) of the control level (shown by the thick line) in the AF-remodelled atrial myocyte model. (d) The relationship between APD (APD$_{-70 \text{mV}}$) and the fraction of blocked IKur in the normal atrial myocyte (dashed lines) and in the AF-remodelled myocyte (continuous lines) at $[\text{Ca}^{2+}]_o=1.8$ mM (thin lines) and 1.15 mM (thick lines).
(b) Effects of $[\text{Ca}^{2+}]_o$ on in vivo electrocardiogram and in vitro cardiac action potential

It is well known that changes in serum calcium influence the cardiac electrical activity, particularly affecting ventricular repolarization (Braunwald et al. 1992; Fisher et al. 2001; Diercks et al. 2004). The primary electrocardiographic manifestation of hypocalcaemia is QTc (corrected Q–T interval) prolongation (Surackwicz 1995; Huang et al. 2000), which is associated with increased risk of early afterdepolarizations and triggered arrhythmias. On the other hand, hypercalcaemia exerts an opposite effect on the electrocardiogram with the hallmark of abnormal shortening of the QTc interval (Surackwicz 1995). Ca$^{2+}$ dependency of repolarization is particularly relevant in the setting of haemodialysis, when plasma Ca$^{2+}$ levels may vary widely according to the protocol used. In such a setting, QTc was found to inversely correlate with plasma Ca$^{2+}$ levels by several authors (Nappi et al. 2000; Covic et al. 2002; Genovesi et al. 2003; Severi et al. 2008). Experimental studies in isolated cells demonstrated that the duration of cardiac AP is sensitive to the external calcium concentration. As a rule, elevated concentrations shorten the AP and reduced concentrations lengthen it. Such a behaviour has been observed since the 1950s in dog cardiomyocytes (Hoffman & Suckling 1956) and later in calf ventricular muscles (Kass & Tsien 1976). Since $[\text{Ca}^{2+}]_o$ variations usually tested were extreme (e.g. 0.2 versus 2.7 versus 8 mM; Hoffman & Suckling 1956), it could be questioned whether smaller ones (i.e. from 1.2 to 2 mM) can have significant effects. Upon changing $[\text{Ca}^{2+}]_o$ from 2.7 to 1.35 mM, Temte & Davis (1967) found an increase in APD from 344 to 373 ms in calf Purkinje fibres. In guinea pig ventricular myocytes, Bai et al. (2005) found an APD decrease by increasing $[\text{Ca}^{2+}]_o$ from 1.8 to 2.4 mM. Even slighter Ca$^{2+}$ variations were found to affect APD by Leitch & Brown (1996) (figure 1a).

Data on the effects of hypo- and hypercalcaemia on human APs are not available, but the consistency between QTc and ventricular APD changes suggests that the same phenomenon occurs in the clinical and experimental settings. We recorded APs in human atrial myocytes superfused with 1.2 and 2 mM $[\text{Ca}^{2+}]_o$; preliminary results confirmed the inverse relationship between APD and $[\text{Ca}^{2+}]_o$ (figure 1b). Therefore, since experimental evidence in vitro suggests that even a small deviation of $[\text{Ca}^{2+}]_o$ from physiological range may affect electrophysiological properties, we decided to analyse how different $[\text{Ca}^{2+}]_o$ values can affect the predictions of the currently used computational models of cardiac cells.

(c) Effects of $[\text{Ca}^{2+}]_o$ on in silico action potential models

We performed a sensitivity analysis on extracellular calcium concentration in the range 1–3 mM using three among the most used ventricular cell models: the Luo–Rudy guinea pig (Faber & Rudy 2000); the Shannon rabbit (Shannon et al. 2004); and the Ten Tusscher–Panfilov human (Ten Tusscher & Panfilov 2006).

The results (figures 2–4) clearly highlighted that $[\text{Ca}^{2+}]_o$ markedly affected both the intracellular Ca$^{2+}$ kinetics (figures 2c,d,4c,d) and the AP morphology and duration (figures 2a,b,4a,b). This is not surprising since the intracellular calcium loading strictly depends on membrane calcium currents ($I_{\text{CaL}}, I_{\text{NaCa}}$ and $I_{\text{ibCa}}$)
that are driven, at least in part, by the \([\text{Ca}^{2+}]\) gradient across the membrane. In turn, \([\text{Ca}^{2+}]_i\) modulates the Na/Ca exchanger, and therefore the intracellular Na\(^+\) concentration, which indeed increased in all the three models when increasing \([\text{Ca}^{2+}]_o\) (figures 2\(h\)–4\(h\)). Overall, a change in \([\text{Ca}^{2+}]_o\) may affect many electrogentic transport mechanisms across the membrane.

The surprising result of our analysis was that all three ventricular models responded with APD prolongation to \([\text{Ca}^{2+}]_o\) increase (figures 2\(b\)–4\(b\)), i.e. opposite to the APD shortening observed in vitro and in vivo when extracellular or plasma calcium concentration is increased. To some extent, this oversight can be justified when considering that the extracellular environment is usually kept fixed, both in the in vitro experiments and in the predictions for which models are used. So, the sensitivity on extracellular electrolyte concentrations has not usually been deeply addressed in the development of models, more attention being devoted to the faithful description of a wealth of physiological processes.

A possible explanation for the observed discrepancy consists in underestimating the effects of CDI on \(I_{\text{CaL}}\) in the AP models. In fact, experimental studies demonstrated that CDI is largely dominant over the voltage-dependent inactivation process (Peterson et al. 1999, 2000; Linz & Meyer 2000) and constitutes an important feedback mechanism to limit the \(\text{Ca}^{2+}\) influx when \([\text{Ca}^{2+}]_i\) increases. In turn, a decrease in \(I_{\text{CaL}}\) should reduce the duration of the AP plateau phase, but this was not observed in the computational ventricle AP models, in which the amplitude of \(I_{\text{CaL}}\) during AP phase 2 increases when increasing \([\text{Ca}^{2+}]_o\) from 1 to 3 mM (figures 2\(e\)–4\(e\)). In fact, the increase in \([\text{Ca}^{2+}]_o\) affects \(I_{\text{CaL}}\) in two opposite directions: it increases the driving force, thus allowing a larger \(\text{Ca}^{2+}\) influx, and enhances the CDI. The first led to an increase in maximal \(I_{\text{CaL}}\) (i.e. the resulting current when all the channels are open; figures 2\(f\)–4\(f\)), an effect that overcame CDI-induced reduction in all the analysed models. Thus, the outcome was a net current increase during AP phase 2 and consequent APD prolongation.

The deficiency with respect to the description of CDI in ventricular AP models has been addressed before by Greenstein et al. (2006). They claim that their model of ventricular cardiomyocyte has an experimentally based balance between voltage- and \(\text{Ca}^{2+}\)-dependent inactivation. This construction suggested us to test this model to check whether a stronger CDI could rectify the relationship between APD and \([\text{Ca}^{2+}]_o\) (figure 5). In the range from 1.6 to 3 mM, the model correctly showed decreasing APD for increasing \([\text{Ca}^{2+}]_o\), and the amplitude of \(I_{\text{CaL}}\) decreased during a large part of the AP when going from 2 to 3 mM \([\text{Ca}^{2+}]_o\). However, for \([\text{Ca}^{2+}]_o=1.4\) mM, the APD was very long (figure 5\(a,b\)) and at lower, yet physiological \(\text{Ca}^{2+}\) concentrations, traces showed abnormal oscillatory patterns with alternans between domeless and long APs (figure 5\(b\), inset).

The important issue of the mechanisms underlying the modulation of APD by \([\text{Ca}^{2+}]_o\) has also been thoroughly investigated in a recent theoretical study (Grandi et al. 2008) based on the Ten Tusscher et al. model (Ten Tusscher et al. 2004). Similar to the above-mentioned AP models, this model also responded with APD shortening to decrease in \([\text{Ca}^{2+}]_o\), i.e. in the opposite direction with respect to the experimental observations. Therefore, the mechanisms of APD modulation by \([\text{Ca}^{2+}]_o\) in human cardiomyocytes were evaluated in more detail.
Incorporation of Ca\(^{2+}\) dependency of K\(^{+}\) currents could not reproduce the inverse relationship between APD and \([\text{Ca}^{2+}]_o\). Only when \(I_{\text{CaL}}\) inactivation process was strengthened, as indicated by experimental studies, did simulations predict APD prolongation at lower \([\text{Ca}^{2+}]_o\). Taken together, these results support the hypothesis of CDI as the mechanism primarily responsible for the dependency of APD on \([\text{Ca}^{2+}]_o\).

Here, we applied a similar strengthening of CDI to the Ten Tusscher–Panfilov model in order to test whether this correction could be sufficient to enable the model to reproduce the relationship between APD and \([\text{Ca}^{2+}]_o\) changes towards the physiological range of 1.15 mM. After modifying the model, as described in §2, simulations were in agreement with the experimental observations, APD being reduced when increasing \([\text{Ca}^{2+}]_o\) (figure 6). Although APD dependency on \([\text{Ca}^{2+}]_o\) was probably underestimated (note the different scale in figure 6b with respect to figure 4b), the negative sign of \(d(\text{APD}_{90})/d[\text{Ca}^{2+}]_o\) observed experimentally was correctly predicted by the modified version of the model. Altogether, these results allow one to infer that the proposed solution advances consistency among in vivo, in vitro and in silico measurements.

Besides \(I_{\text{CaL}}\), the Na/Ca exchanger could also play a role. Simulations show quite different patterns of \(I_{\text{NaCa}}\) during the AP plateau in the different models: the current is positive (i.e. repolarizing) in the Luo–Rudy model, negative (depolarizing) in the Shannon model and almost null in the Ten Tusscher and Greenstein models (figures 2g–5g). Increasing \([\text{Ca}^{2+}]_o\) may have multiple effects on \(I_{\text{NaCa}}\), since the exchanger is sensitive to \([\text{Ca}^{2+}]_o\), \([\text{Ca}^{2+}]_i\) and also \([\text{Na}^{+}]_i\), which in turn depends on the overall net sodium flux due to \(I_{\text{NaCa}}\) during both the systolic and diastolic phases. The direct effect of a \([\text{Ca}^{2+}]_o\) increase is likely to overwhelm the indirect effect of the consequent \([\text{Ca}^{2+}]_i\) increase, at least during the plateau phase when the positive membrane potential favours sodium outflow and calcium inflow through the exchanger. The slight \([\text{Na}^{+}]_i\) increase observed in all the models (figures 2h–5h) should also favour sodium outflow, promoting a more repolarizing contribution of \(I_{\text{NaCa}}\) following \([\text{Ca}^{2+}]_o\) increase. In fact, when increasing \([\text{Ca}^{2+}]_o\) from 1 to 3 mM, \(I_{\text{NaCa}}\) becomes more repolarizing or is kept overall substantially equal in most of the analysed models (figures 2g–5g), so it seems not to underlie the observed non-physiological AP prolongations. However, the actual role of \(I_{\text{NaCa}}\) in modulating APD is far from being fully understood and deserves a more detailed analysis based on a thorough review of available experimental data, also taking into account the strong dependence of \(I_{\text{NaCa}}\) on the pacing rate.

We performed the sensitivity analysis on \([\text{Ca}^{2+}]_o\) also with the Courtemanche model of human atrial cardiomyocyte (Courtemanche et al. 1998). In contrast to the previously analysed ventricular models, the atrial AP showed a very slight dependence of its duration on \([\text{Ca}^{2+}]_o\), at least in the range from 1 to 2 mM (figure 7a,b). The sign of this dependence was coherent with the experimental observations since APD decreases when \([\text{Ca}^{2+}]_o\) increases. However, this was probably the result of a more approximated rather than detailed description of \([\text{Ca}^{2+}]_o\) influence on ionic transports through the cell membrane. In fact, in the Courtemanche formulation, the maximal \(I_{\text{CaL}}\) conductance and its reversal potential are kept fixed without any dependence on \([\text{Ca}^{2+}]_o\). As a consequence, the maximal current did not depend on \([\text{Ca}^{2+}]_o\). The extracellular calcium enigma

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(figure 7f), and the increase in \([\text{Ca}^{2+}]_o\) affected \(I_{\text{CaL}}\) only by enhancing the CDI (figure 7e). The resulting decrease in current amplitude was, in turn, the main cause of APD decrease, whereas \(I_{\text{NaCa}}\) was only slightly affected by \([\text{Ca}^{2+}]_o\). The small variations of systolic intracellular calcium even for large variations in \([\text{Ca}^{2+}]_o\), which could be questioned as being physiological or not, produced, in turn, negligible variations in \([\text{Na}^+]_i\).

In several cardiac AP models (e.g. Luo–Rudy or Courtemanche), a bulk myoplasmic \([\text{Ca}^{2+}]_i\) is assumed to control CDI. These models fail to capture the local aspects of \(I_{\text{CaL}}\) \(\text{Ca}^{2+}\)-dependent inactivation. In fact, \(\text{Ca}^{2+}\) channels sense a much higher concentration in the dyadic space. More recent models (e.g. Shannon et al. 2004; Ten Tusscher & Panfilov 2006) have incorporated a cleft subspace, whose \(\text{Ca}^{2+}\) concentration controls the dynamics of the L-type \(\text{Ca}^{2+}\) channels and the ryanodine receptors. Also, this description of calcium-induced calcium release is based on the assumption of a ‘common pool’, i.e. the release of calcium from the sarcoplasmic reticulum (SR) is controlled by calcium concentration in the same pool into which the calcium from both the sarcoplasmic reticulum (SR) and the sarcolemmal calcium current enter. Stern (1992) demonstrated the limitations of any such model in describing graded \(\text{Ca}^{2+}\) release from the SR. Moreover, ongoing work has shown extremely intricate signalling mechanisms in CDI (Tadross et al. 2008) which are yet to be incorporated in cardiac models, in which the representation of CDI is mainly phenomenological rather than mechanistic. Nevertheless, it is worth noting that we found correct APD dependence on \([\text{Ca}^{2+}]_o\), both in the local control model of Greenstein et al. (at least in the ‘stable’ range of \([\text{Ca}^{2+}]_o\)) and in the common pool models of Courtemanche et al. and Ten Tusscher et al. as modified by Grandi et al. (2008). Therefore, the issue of \(\text{Ca}^{2+}\) compartmentation does not seem crucial for describing the phenomenon under study.

\(\text{(d)}\) Can changes in \([\text{Ca}^{2+}]_o\) affect in silico quantitative predictions?

An example on drug effect analysis

The last step of this analysis would have been to assess whether changing \([\text{Ca}^{2+}]_o\) from the value currently used in each model to the ‘physiological’ value of 1.15 mM could affect the quantitative predictions made by the model, e.g. on drug effects on APD. However, this purpose turned out to be meaningless after realizing that such models respond with a non-physiological increase of APD upon decreasing \([\text{Ca}^{2+}]_o\). It is evident that—owing to the poor reliability of tested models—new predictions based on physiological \([\text{Ca}^{2+}]_o\) could not be considered as more realistic as those obtained at higher \([\text{Ca}^{2+}]_o\). Moreover, the results of our analysis suggest that an improvement in the model formulation, not simply a change in a model parameter (i.e. \([\text{Ca}^{2+}]_o\)), is needed in order to correctly reproduce AP features in physiological conditions.

We therefore focused a demonstrative test on the atrial AP model. The Courtemanche model has been recently used in a couple of papers by Tsujimae et al. (2007, 2008) to analyse the effects of various blockers of the rapid and ultrarapid delayed rectifying potassium currents (\(I_{\text{Kr}}\) and \(I_{\text{Kur}}\), respectively) on atrial APD in normal conditions and after atrial fibrillation (AF)-induced electrical remodelling. We reproduced their simulation results by implementing all the modifications to the original Courtemanche formulation as reported by

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the authors, in particular to reproduce the AF-induced electrical remodelling. Then, the simulations were repeated in the same conditions apart from changing the \([\text{Ca}^{2+}]_o\) parameter value to 1.15 mM, in order to test whether this change alone could significantly affect model predictions. Some substantial differences in the simulated effects of blockage were found both for \(I_{Kr}\) and \(I_{Kur}\) (figure 8).

While the frequency dependence of APD was only slightly affected by the change in \([\text{Ca}^{2+}]_o\) in control conditions, the prolonging effect of 90 per cent blockage of \(I_{Kr}\) had a markedly greater effect when \([\text{Ca}^{2+}]_o\) was set to 1.15 mM than when it was set at 1.8 mM (figure 8a). Similarly, in normal conditions, the \([\text{Ca}^{2+}]_o\) level affected only slightly the effects of \(I_{Kur}\) blockage on APD, whereas in AF remodelling conditions the AP prolongation due to \(I_{Kur}\) blockage was enhanced at lower \([\text{Ca}^{2+}]_o\) (figure 8a). These simulation results—although not further investigated at this time—point out that changes in \([\text{Ca}^{2+}]_o\) can affect \textit{in silico} quantitative predictions. This makes the choice of \([\text{Ca}^{2+}]_o\) critical when aiming at reproducing \textit{in vivo} conditions and supports our indication of using a physiological concentration value instead of the standard Tyrode one.

The observation that using physiological calcium concentrations may somehow amplify the effect of \(I_{Kr}\) blockers deserves some comments. Discrepancies between preclinical markers of torsadogenic potency and clinical findings have been clearly identified for several drugs (Gintant 2008). For example, verapamil is free from QT prolongation in humans despite blocking \(I_{Kr}\) at concentrations close to the therapeutic range. Oppositely, the occurrence of torsades de pointes has been reported for ciprofloxacin, fluoxetine and other drugs blocking \(I_{Kr}\) at concentrations more than 100 times the therapeutic range. For verapamil, and to some extent for amiodarone, the inconsistency between \textit{in vitro} results and clinical findings has been attributed to the concomitant block of other channels such as \(I_{CaL}\) that may tone down hERG block. Since \textit{Ca}^{2+}\)-dependent inactivation on \(I_{CaL}\) and possibly other mechanisms, deeply influenced by extracellular calcium concentrations, are apparently disregarded in the AP models, the prediction of drug effects on APD may result as inaccurate. Needless to say, AP modelling has an increasing regard in the field of drug development and prediction of cardiovascular safety; therefore, the implementation of computational models should pay particular attention to a correct description of all the mechanisms potentially involved in AP prolongation.

4. Conclusions

The conclusions that can be drawn based on the presented analysis are the following. (i) \([\text{Ca}^{2+}]\) usually used \textit{in vitro} is markedly far from that \textit{in vivo} and should be changed from 1.8 to 2 mM to approximately 1.15 mM. This observation is obviously relevant not only for the computational applications, but also, first of all, for the electrophysiological experiments and deserves to be further considered and discussed by the physiology scientific community. (ii) \textit{In vitro} results suggest that a variation of \textit{Ca}^{2+}\) from 2 to 1.2 mM has significant effects on APD both in the ventricle and the atrium. (iii) Several of the most popular computational models of cardiac AP respond with APD shortening to decrease in \([\text{Ca}^{2+}]_o\), i.e. opposite to the experimental observations. (iv) Modifications to \textit{Ca}^{2+}\)-dependent
$I_{\text{Cal}}$ inactivation formulation succeed in making a ventricular model suitable for AP analysis when $[\text{Ca}^{2+}]_o$ is set to low physiological values.

We are aware of the fact that countless discrepancies between in vivo cardiac cell physiology and in silico modelling are insurmountable (basal neurohumoral stimuli, intercellular communications with cardiac and non-cardiac cells and with the extracellular matrix, etc.) and are beyond the scope of this paper. However, we feel that eliminating or just addressing a minor discrepant point as extracellular calcium concentration may help to bring the two worlds closer. To this aim, using non-physiological high $\text{Ca}^{2+}$ concentration can be misleading, especially when the prediction of in vivo pathophysiological mechanisms is the ultimate aim of simulation.

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