Effects of pCa_i and pH_i on cell-to-cell coupling

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Summary. Internal longitudinal resistance (r_i), a determinant of cardiac conduction, is affected by changes in intracellular calcium and protons. However, the role and mechanism by which H^+ and Ca^{2+} may modulate r_i is uncertain. Cable analysis was performed in cardiac Purkinje fibers to measure r_i during various interventions. In some experiments, intracellular pH (pH_i) was recorded simultaneously to study the pH_i-r_i relation. Both intracellular Ca^{2+} and H^+ independently modified r_i. However, internal resistance of cardiac fibers was insensitive to pH_i changes compared to other tissues. A latent period preceded the pH_i-related changes in r_i and the amount of change depended upon methodology. The results suggest that direct action of protons on r_i may be subordinate to other regulatory processes. Ionic regulation of internal longitudinal resistance may occur by more than one mechanism: i) direct cationic binding to sites on junctional membrane proteins; and ii) H^+- or Ca^{2+}-dependent phosphorylation of junctional proteins.

Key words. Cell-to-cell coupling; intracellular pH; intracellular calcium; internal longitudinal resistance.

Presently, there seems to be little doubt that conduction of cardiac action potentials occurs via local ionic current flow from cell-to-cell through low resistance junctions[7,67-69]. Given that 1) internal longitudinal resistance (r_i) is important to the conduction process and 2) the longitudinal resistive path can be represented by two elements in series, myoplasmic resistance (r_{myo}) and junctional membrane resistance (r_j), the question arises as to what affects r_i and how is it controlled? This paper will focus upon intracellular protons and calcium ions as likely ionic regulators of electrical coupling in cardiac tissue. The results demonstrate that both H^+ and Ca^{2+} may modify r_i and thereby influence signal transmission. Some additional results will emphasize the interdependence of intracellular H^+ and Ca^{2+} – a factor which complicates assessment of the relative role of the two cations as independent regulatory substances. Finally, some potential sites of action for protons and calcium ions on r_i will be discussed and an hypothesis developed in which changes in H^+ and Ca^{2+} may be linked to or trigger part of a larger regulatory process.

Modifiers of internal longitudinal resistance

Modifiers of internal resistance have been described since 1954. Cooling not surprisingly increases R_i[12], a temperature effect similar to that known for the conductivity of KCl, a major constituent of myoplasm. Since the late 1960's and early 1970's, interest has grown in studying what factors alter cellular coupling and the precise mechanism for its control. Délèze and De Mello have made several observations on the 'healing-over' of injured myocardium that implicated a functional role for cellular junctions in electrically isolating a damaged region[14,15,17]. Essentially, it was noted that a high resistance barrier was re-established near a site of injury depending upon: i) the presence of certain divalent cations in the bathing solution (especially Ca^{2+} but also Sr^{2+}); ii) temperature; and iii) the rate of stimulation of the preparation. The results of Délèze[15] were particularly convincing since equations for the voltage distribution in a cable terminated by a short circuit quantitatively fit the spatial decay of electrotonic potentials near the site of injury. This result added perspective by linking the process of recovery from injury to cable theory.

Subsequently, a wide array of agents have been applied to cardiac tissue to investigate possible functional alterations in cable properties. It has become apparent that internal longitudinal resistance of cardiac tissue is a fairly stable quantity. For example, potassium ions[24,28], many of the common antiarryhthmic drugs[1,2,5,49], and beta-adrenergic antagonists[3,35] have little or no significant action to modify r_i. However, there are a series of conditions which do perturb the internal resistive path. Exposure to toxic concentrations of cardiac glycosides[13,70], metabolic uncouplers (dinitrophenol)[13,16,20], lysophosphatidylcholine[4], aliphatic alcohols (C6 to C8 chain length)[16], hypotonic media (isosmolar sucrose)[16,38], Sr^{2+}[44], and Ca^{2+}-free[29] and Ca^{2+}-loaded superfusates[49] all act to significantly increase r_i. Hypoxia[10,33,34,72,73] also is a potent stimulus to increase r_i provided glucose or other metabolic substrates are absent from the medium. Several common denominators to the development of high internal resistance emerge from this potpourri: i) contracture is a frequent coexisting event; ii) interruption of glycolytic or oxidative metabolism intensifies the effects; and iii) structural alteration of gap-junctional appearance (particle spacing and geometrical arrangement) seems to accompany the electrical changes[6,13,16,46,47,57]. The correlation of high internal resistance with ultrastructural changes at the junctional membrane level has been taken as corroborative evidence that closing of 'pores' in the 'low-resistance' junctions is the mediator of the electrical events. Unfortunately, alterations in gap junctional architecture may be a somewhat nonspecific response since it can occur after local cell 'injury'[6], exposure to divalent cations or protons[46,47,57], aliphatic alcohols[16], and even be a fixation artifact[16,45]. Thus, the study of gap-junctional ultrastructure alone is not sufficient to clarify the mechanism of intercellular transmission and may be misleading if utilized out of context.

Teleologically, it also seems logical that control of transmission within a multi-cellular structure would be found at cellular junctions which are obvious sites for control of charge or substance transfer. Recent direct observations support this line of reasoning: functional uncoupling can be induced by the binding of antibodies to the junctional membrane. Warner, Guthrie and Gilula[66] and Hertzberg, Spray and Bennett[32] both have shown that microinjection of polyclonal antibodies to gap-junctional protein selectively interrupts electrical coupling and intercellular dye transfer. Given the above evidence, one may infer that changes in total internal resistance (i.e. r_i) primarily arise from alterations in r_j. As such, functional relationships determined between r_i and, for example intracellular proton concentration ($[H^+]_i$), in essence may reflect the behavior of r_j to a change in $[H^+]_i$. One advantage of measuring r_i in multi-cellular structures is that both junctional and myoplasmic components are included and sometimes the latter (r_{myo}) may not be neglected.

Many experiments have been directed at further understanding the cellular regulatory mechanism(s) for control of internal resistance. Using a functional approach, alteration of the internal ionic milieu by injection of a variety of cations has been found to induce cardiac cellular 'uncoupling' (decrease in dye transfer or in the amplitude ratio of electrotonic potentials between coupled cells). De Mello observed that intracellular injection of Ca^{2+} or Sr^{2+}[18], Na^+[19], Mn^{2+} or La^{3+}[21], or H^+[22] but not Mg^{2+}[18] led to such uncoupling. Un-

fortunately, it was not possible to identify what quantity of cation was necessary for uncoupling, or that microiontophoresis was entirely a specific perturbation nor even to exclude the possibility that some or all of the effects were due to changes in r_m, not r_i. Nevertheless, the findings seemed to suggest that cellular coupling might be controlled by the concentration of certain intracellular ions in the vicinity of junctional membranes. In addition, all were positively charged species of a certain radius and this seemed to imply the existence of a charged binding site where such ions could interact. This concept was bolstered by the experiments of Rose and Loewenstein[54]. They observed that uncoupling of salivary gland cells by injection of Ca^{2+} (monitored by the luminescence of aequorin) did not develop until the spread of aequorin luminescence reached the vicinity of the cell junctions. This result suggested that it was the local cytosolic concentration of Ca^{2+} that was responsible for the changes in junctional resistivity.

To date, several hypotheses have been formulated to explain the physiological basis for cell-to-cell transmission in the context of the aforementioned findings. Three primary concepts have been advanced which integrate ultrastructural data suggestive of aqueous junctional membrane channels with the functional behavior of the internal resistive path: i) Binding of Ca^{2+} to gap junctions regulates junctional resistivity ('Ca-hypothesis')[41,54,55]; ii) Direct binding of H^+ to a gap-junctional constituent (perhaps the purified junctional protein moieties known as 'connexons') regulates the state of junctional resistivity ('H^+-hypothesis')[59,60]; and/or iii) Intracellular cAMP, by increasing the number or functional permeability of connexons, regulates the resistivity of the intercellular path ('cAMP-hypothesis')[27,31]. The emergence of H^+, Ca^{2+}, and cAMP as likely intracellular control substances was a natural consequence of their known actions to modify r_i, their regulatory role in other cellular functions, and the knowledge that considerable energy was expended to maintain extremely low concentrations of these substances even against formidable electrochemical gradients.

Calcium ions as regulators of internal longitudinal resistance

Calcium ions as well as protons have been proposed as likely intracellular ionic regulators of longitudinal resistance and/or junctional membrane conductance[41,54,55]. The main controversy has centred around the relative importance and mechanism by which H^+ and Ca^{2+} carry out such regulation. This is partly because those agents which alter pH_i also affect $[Ca^{2+}]_i$ and vice versa. In support of the Ca-hypothesis, Loewenstein and co-workers[41] have observed that salivary gland epithelial cells electrically uncouple when Ca^{2+} enters the cytosol either by iontophoresis or leakage through an injured point in the cell membrane. In addition, uncoupling did not develop until Ca-ions (monitored by aequorin) reached the vicinity of the junctional membrane[54]. Furthermore, via simultaneous monitoring of pH_i (by ion-selective microelectrode) and $[Ca^{2+}]_i$ (by aequorin luminescence), Rose and Rick[55] found that electrical coupling of epithelial cells correlated closely in time with increased $[Ca^{2+}]_i$. Changes in electrical coupling did not seem related consistently to pH_i: i) changes in pH_i and coupling were out of phase during transitions; ii) changes in coupling did not develop upon lowering pH_i from 7.6 to 6.8 (by iontophoresis with a CaEGTA filled pipette); and iii) under special conditions, uncoupling occurred as pH_i increased > 7.8[55]. It is also difficult to ignore other indirect evidence in heart: i) Recovery of input resistance after injury, a change consistent with formation of a high resistance barrier near the short-circuit[15], depends upon Ca-ions; ii) Exposure to toxic concentrations of cardiac steroids, which are known to raise $[Ca^{2+}]_i$, leads to large increases in r_i[13,70]; iii) Intracellular injection of Ca^{2+} produces prompt and reversible electrical uncoupling[18]; and finally iv) Ca-ions produce changes in nexal ultrastructure of cardiac tissue that have been linked to uncoupled states[6,13,57].

In addition, there is biochemical evidence that Ca^{2+} bind specifically to isolated cardiac gap-junctional membranes. Nishiye, Mashima and Ishida[43] reported that binding of Ca^{2+} to isolated cardiac nexal membranes was sigmoidal with two apparent binding sites. Most of the binding (ca 75%) was associated with the second site at non-physiological $[Ca^{2+}]$. More recently, Spray et al.[60] have reported that gap-junctional resistance was an insensitive function of $[Ca^{2+}]_i$ with half-maximal changes around 0.1 mM. However, the data of Byerly and Moody[11] suggests that continuous variation of intracellular $[Ca^{2+}]$ may not be accomplished between 10^{-7} and 10^{-4} mol/l via a suction pipette method (monitored with a Ca^{2+}-selective microelectrode). Such results complicate the interpretation of Spray et al. studies of the r_j versus $[Ca^{2+}]_i$ relationship[60] since complete intracellular equilibration and buffering of Ca-ions was assumed to occur. An additional study[13] of the $r_i - [Ca^{2+}]_i$ relationship also can be criticized: i) the Ca-microelectrodes were not properly calibrated for $[Ca^{2+}]_i$ below 10^{-4} M; and ii) lipophillic substances were used to change $[Ca^{2+}]_i$ (e.g. dinitrophenol) which artifactually increase the Ca-signal (Pressler, unpublished observations). Such difficulties leave the role of Ca-ions in regulating junctional resistivity an open question, at least in cardiac tissue.

Figure 1 shows analog records from sheep Purkinje fibers demonstrating changes in electrotonic potentials with alterations of extracellular calcium concentration ($[Ca^{2+}]_o$). A twenty-fold reduction in $[Ca^{2+}]_o$ resulted in a 46% decrease in r_i. Conversely, previous experiments in dog false tendons[49] showed that elevation of $[Ca^{2+}]_o$ increased r_i by 52%. The employment of several pharmacological agents in the course of cable analysis proved useful to distinguish whether the effects of Ca^{2+} on r_i were mediated extra- or intracellularly and to specify the likely path of Ca-action. One possible mode of action was Ca-influx via Ca-channels and a relatively specific Ca-blocker, verapamil, was used to investigate this possibility. Interestingly, verapamil (3 or 10 μM) had no direct effects on r_i (even though r_m increased by more than 50%) and had no significant blocking effect on $[Ca^{2+}]_o$-related changes in r_i[49]. In contrast, lanthanum (200 μM) com-

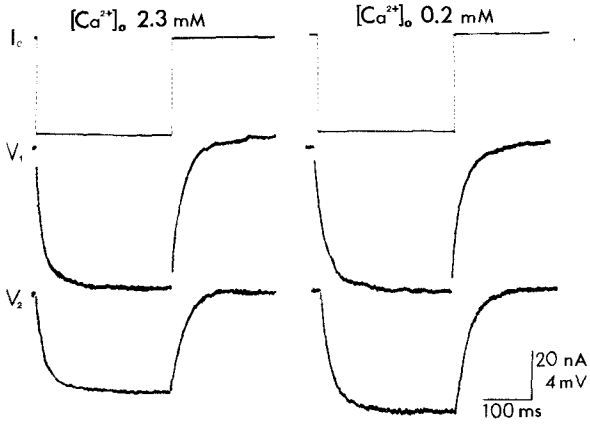

Figure 1. Cable analysis during changes in extracellular calcium concentration ($[Ca^{2+}]_o$) in a resting sheep Purkinje fiber. Abbreviations: I_o, intracellular current intensity; V_1, V_2, electrotonic potentials from proximal (site 1) and distal (site 2) recording locations. Lowering $[Ca^{2+}]_o$ by 20-fold lengthened the space constant (λ) without substantially altering the input resistance (R_{in}) of the fiber: λ increased from 2.73 mm to 4.62 mm at $[Ca^{2+}]_o$ 2.3 and 0.2 mM respectively; corresponding R_{in} values were 333 kΩ and 329 kΩ. Internal longitudinal resistance (r_i) decreased from 1.20 M Ω/cm to 0.65 M Ω/cm as $[Ca^{2+}]_o$ decreased from 2.3 to 0.2 mM. Exp. 16.8.82.

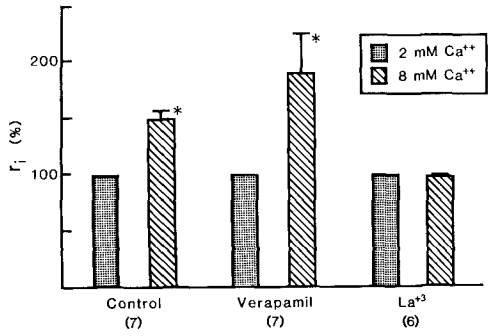

Figure 2. Effects of $[Ca^{2+}]_o$ on r_i in the absence and presence of Ca blockers. r_i was normalized to the value obtained at 2 mM $[Ca^{2+}]_o$. Raising $[Ca^{2+}]_o$ from 2 to 8 mM increased r_i both under control conditions and in the presence of verapamil (3 or 10 μM). La^{3+} ions (200 μM) prevented the $[Ca^{2+}]_o$-induced rise in r_i. Neither verapamil nor La^{3+} affected r_i per se. * $p < 0.005$. Numbers indicate number of experiments in dog Purkinje fibers. For abbreviations, see fig. 1. Reprinted with permission from Pressler et al.[49].

pletely blocked the effect of extracellular $[Ca^{2+}]$ to increase r_i despite no effects per se of La^{3+} on r_i (see fig. 2).

Some conclusions could be reached from these cumulative findings: i) Ca^{2+}-entry was required to alter r_i. If extracellular Ca^{2+}-diffusion to junctional membranes had brought about the changes in r_i, La^{3+} exposure per se should have mimicked the effects of Ca^{2+}, at least partially. Such was not the case for La^{3+} (200 μM). Lanthanum was especially useful in this regard since it does not cross the sarcolemma of normal cells[39] yet diffuses to the level of the gap junction[53]. ii) Ca channels were unlikely to be the means of this Ca-entry. Verapamil[40] and lanthanum[8,37] are both known Ca-channel blockers but verapamil more specifically targets Ca-channels whereas La^{3+} acts non-specifically to block all means of Ca-entry. By deduction, Ca-entry occurring primarily via Ca channels seemed unlikely as a mechanism for Ca_o-induced effects on r_i since La^{3+} blocked the effect and verapamil (during constant pacing) did not. iii) Ca-entry via Na^+/Ca^{2+}-exchange was the most probable path by which extracellular Ca^{2+} altered r_i. This conclusion was deduced on the basis of known effects of La^{3+} to inhibit Na^+/Ca^{2+}-exchange[8,39,52] and the lack of substantial effects by verapamil[8]. Unfortunately, there was no direct evidence that $[Ca^{2+}]_i$ actually increased nor whether the influx of Ca^{2+} directly or indirectly (perhaps by secondary changes in pH_i) mediated the observed changes in internal resistance.

More recently, the possibility has been investigated that changes in $[Ca^{2+}]_o$ may alter r_i by increasing $[H^+]_i$. Ten- to twenty-fold increases in $[Ca^{2+}]_o$ acidified the myoplasm but by quite insignificant amounts (0.06–0.08 log units, or ~ 5–6 nM change in $[H^+]_i$). For the r_i-pH_i relationship shown below (fig. 4), one would predict only a 1–2% increase in r_i as a result of such acidification by $[Ca^{2+}]_o$. This result in conjunction with the ~ 50% increase in r_i observed with raising $[Ca^{2+}]_o$[49] argues that calcium ions have an independent role in mediating changes in internal resistance. Unfortunately, such experiments still do not directly address whether cytosolic Ca^{2+} per se directly or indirectly alters r_i.

Protons as regulators of internal longitudinal resistance

The electrical conductance (conversely resistance) of the junctional membrane (assuming a constant cytosolic resistivity) can be assessed directly in two-cell preparations or indi-

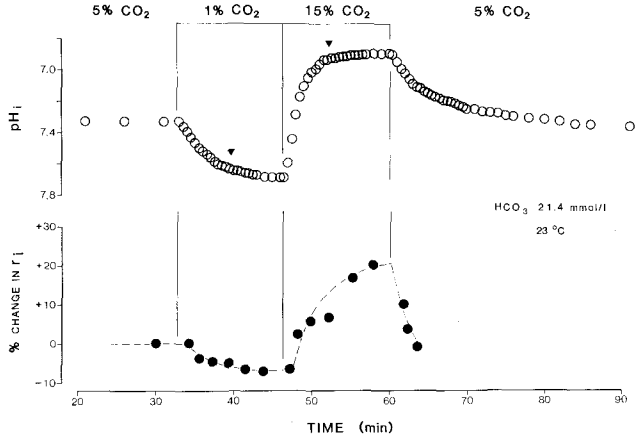

Figure 3. Alterations in r_i during CO_2-induced changes in intracellular pH (pH_i; open circles). Extracellular bicarbonate concentration was held constant while the percentage of CO_2 in the superfusate (noted at top) varied between 1 and 15%. R_i (filled circles) was normalized to the value measured in 5% CO_2 Tyrode. R_i decreased during intracellular alkalinization and more markedly increased during acidification. A latent period of 1.5 min preceded the initial change in r_i after the onset of changes in pH_i. This latency seemed to progressively decrease during repeated perturbations of pH_i. Arrowheads mark the times when 90% of the proton loss or gain had occurred. Each value of r_i was determined from the mean of several electrotonic potentials. Exp. 11.10.83.

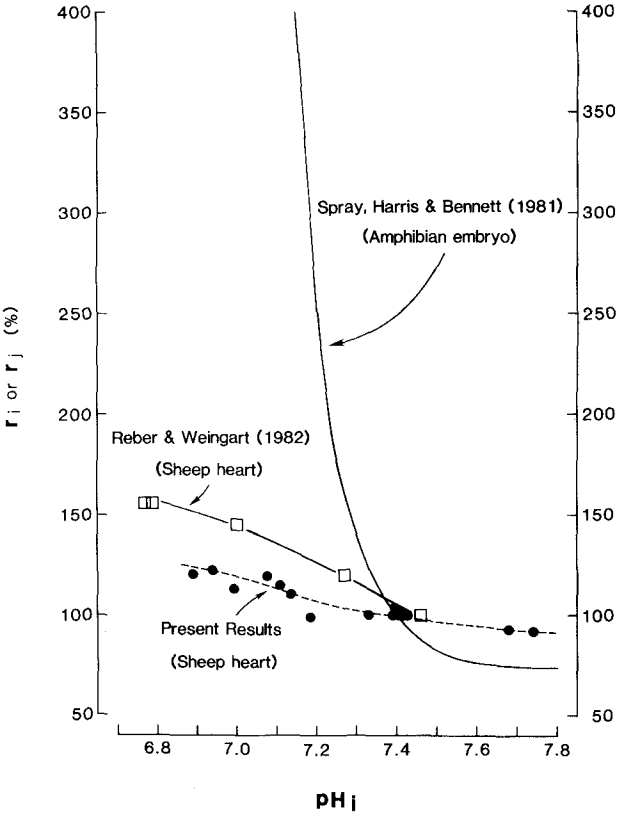

Figure 4. Summary of relationship between intracellular pH and internal longitudinal resistance (r_i) or junctional membrane resistance (r_j) in various tissues. Values of r_i were expressed as a percent of control measured in 5% CO_2 Tyrode solution ('Present results'); extracellular $[HCO_3]$ was constant as solution-CO_2 content varied. Data from Reber and Weingart[51] was re-scaled so that the r_i value at $pH_i = 7.46$ equalled 100%. Data from Spray, Harris and Bennett[59] was expressed in terms of r_j and re-scaled so that r_j value at $pH_i = 7.40$ equalled 100%. Both the present results and those of Reber and Weingart[51] show weak pH-dependence of r_i in cardiac tissue compared to the striking pH sensitivity of junctional membranes from amphibian embryos.

rectly through derivation of r_i from the cable properties of multicellular preparations. As discussed above, ionic regulation of junctional membrane conductance is an unsettled problem. In support of the H^+-hypothesis, a series of investigators recently have reported the effects of intracellular pH (pH_i) on several indices of cell-to-cell communication. Turin and Warner[63] observed that CO_2-induced intracellular acidosis (pH_i 6.30) led to electrical uncoupling of Xenopus embryonic cells from the 32-cell stage onwards. Reber and Weingart[51] observed similar effects in sheep cardiac Purkinje fibers wherein transient changes of pH_i (evoked by CO_2 or NH_4Cl exposure) were related to alterations of r_i. Specifically, these authors found that intracellular acidification of resting fibers was associated with an *increase* in r_i and conversely intracellular alkalosis with a *decrease* in r_i. The studies of Spray, Harris and Bennett[59,60] in amphibian and teleost embryonic preparations corroborated and extended the foregoing findings. Spray et al.[59] observed that junctional membrane conductance was directly proportional to the pH_i measured by a recessed-tip pH-microelectrode. Subsequent experiments by Spray, Harris, Bennett and co-workers[60,61] further implied that pH_i might be the primary regulatory factor for physiological control of gap junctional conductance (g_j) in that g_j was much more sensitive to changes in pH_i than $[Ca^{2+}]_i$. On a more general basis, these findings directly conflicted with previous results by Rose and Rick[55] in insect salivary gland cells. In salivary glands, alterations in cell coupling produced by H^+ were associated with changes in $[Ca^{2+}]_i$, as monitored by aequorin luminescence. $[Ca^{2+}]_i$ elevation either by direct injection or indirectly through changes in H^+ was observed to correlate directly with the development of uncoupling. Spray et al. in turn have argued that such apparent correlation might be an artifact of not measuring junctional resistivity directly[59,61]. Such concerns are legitimate given the knowledge that intracellular Ca^{2+} may also affect the resistance of the non-junctional membrane[42]. In addition, poor quantitation of actual $[Ca^{2+}]_i$ during changes in junctional resistivity has been a universal problem that has fueled the controversy.

Till recently[48] the time course of simultaneous changes in pH_i and r_i has not been well characterized so as to allow direct correlation between the development of alterations in H^+ and r_i. In addition, little has been known if active (i.e. stimulated) fibers behave differently from quiescent preparations. To address these issues, cable analysis and in some experiments, cable analysis + simultaneous pH_i recording, were performed in sheep Purkinje fibers during pH_i perturbations[48]. Intracellular pH was varied either by alterations in the CO_2/HCO_3 content of the bathing medium or by transient exposure to NH_4Cl. Sheep Purkinje fibers were superfused alternately by CO_2/HCO_3 Tyrode or HEPES-buffered Tyrode solution adjusted to a similar extracellular pH. Experiments were performed with a varying content of CO_2 (nominally '0'%, 5% and 15%) but a constant difference in HCO_3 (21.4 mM) between CO_2/HCO_3- and HEPES-Tyrode solutions. CO_2/HCO_3-withdrawal (intracellular alkalinization in HEPES-Tyrode) resulted in changes in λ, R_{in} and τ_m that depended on i) the amount of CO_2-loss; ii) time after solution exchange; and most strikingly iii) the presence or absence of cellular excitation during the perturbation. Internal longitudinal resistance was consistently affected by the CO_2/HCO_3-perturbation whereas no significant effects of CO_2/HCO_3 were noted on r_m.

The results showed that the change in r_i grew progressively larger as the CO_2-gradient between solutions increased. No significant changes of the cable constants were apparent with HCO_3- loss alone when nominally a '0' gradient of CO_2 existed between HCO_3- and HEPES-Tyrode. However, a surprising finding was that the CO_2-induced changes in r_i were *opposite* (even in the same fiber) when the cells were active versus when they were quiescent. CO_2/HCO_3-withdrawal (intracellular alkalinization) *increased* r_i in paced fibers yet the same maneuver *decreased* r_i when the preparations were quiescent. Furthermore, the early CO_2-dependent decrease in r_i dissipated in the steady-state (after 40–60 min) for resting fibers but the increase in r_i was maintained in the paced preparations.

Such apparently anomalous findings with respect to the H^+-hypothesis could easily be rectified if the withdrawal of CO_2/HCO_3 induced different intracellular pH-changes in the resting and active states. To investigate this possibility, changes in pH_i consequent to the same alterations in the bathing media were measured with pH-microelectrodes in alternately resting and paced preparations (again no directional bias). However, the results from a series of 7 experiments demonstrated that the cellular pH_i response to H^+ loss or gain through diffusion of CO_2/HCO_3 was affected little by the presence of action potentials. The direction of pH_i-change was identical and the magnitude of the change similar in quiescence and during pacing. Using transient exposure of NH_4Cl to vary pH_i likewise revealed little activity-dependence to H^+ handling intracellularly.

Two additional experiments employed NH_4Cl-exposure (15–20 mM) during cable analysis to further test the above findings. NH_4Cl-exposure was performed in the same fibers alternately paced at 2 Hz or resting for a long interval. The behavior of r_i again was activity-dependent even though the means of altering pH_i was different. During pacing, NH_4Cl-exposure (intracellular alkalinization) was associated with a delayed and blunted oscillation of r_i around control (or perhaps a slight increase early in the time course) whereas the same maneuver at rest evoked a monophasic 15–18% decrease in r_i. Internal longitudinal resistance increased in both resting and paced fibers during washout of NH_4Cl. The similar direction of such effects made it difficult to be certain of true activity-dependent differences during this 'recovery' phase (intracellular acidification). Nevertheless, the experiments in both preparations exhibited similar findings which corroborate the observation that the effects of pH_i on r_i may be activity-dependent, particularly in the alkaline direction.

To test the hypothesis that protons directly regulate r_i, cable analysis and pH-microelectrode techniques were combined during alterations of CO_2-content. Experiments were performed at room temperature and confined to studies of the time course 10–20 min after solution transitions. Unidimensional cable theory was used to analyze the electrotonic potentials and once again, r_i was the most significant CO_2-induced alteration of the cable properties. Changes in CO_2-content between 1 and 15% produced reversible alterations of pH_i that reached a quasi steady-state after 12–14 min; pH_i returned to control in 5% CO_2-Tyrode over a more prolonged period of 18–24 min. Figure 3 shows that r_i decreased during intracellular alkalinization and increased during acidification; r_i returned to its initial value (within 2 to 4%) as pH_i returned towards control. The pH_i-associated changes in r_i were disproportionate in that a smaller decrease in r_i was observed with alkalinization and a larger increase during acidification. Interestingly, the changes in r_i were *delayed* in onset despite the nearly immediate onset of CO_2-induced changes in pH_i ($\sim$ 10–15 s). In figure 3, a latency of 1–2 min elapsed after the onset of changes in pH_i before changes of r_i commenced. In two other experiments (not shown), a particularly striking latency of $\sim$ 5 min was observed before changes in r_i developed. A time lag of variable duration preceded the pH_i-related changes in r_i in each of 6 experiments during the first alteration of pH_i. A pattern was noted that during subsequent pH_i-alterations, the latent period diminished or was not readily detectable. The delay was evident no matter whether r_i initially increased or decreased. Furthermore, no correlation was observed between the

magnitude of delay and diameter of the preparation. This, along with the waning of the delay with repeated pH_i perturbations, suggested that the latency was not simply a diffusional effect. In 6 experiments, the time delay (minimum estimate) averaged 2.5 min before r_i changed $\geq 2\%$ from control; during the second alteration of r_i, the delay averaged only 0.6 min. During the latent period, approximately 56% of the gain or loss in proton concentration occurred before r_i initially began to change. Unfortunately, the measurements of the delay were no more precise than $\pm$ 1 min; this was approximately the interval needed to acquire a set of 3 or 4 electrotonic potentials.

Figure 4 summarizes the individual r_i data in relation to simultaneously measured pH_i (labeled 'Present results'). There was no statistically significant difference between mean r_i in 5% CO_2-Tyrode during control and recovery periods (100% versus 98.8 $\pm$ 5.3%, respectively). The values of r_i shown were those measured at the steady pH_i reached 10–20 min after a change in CO_2-content. A shallow curvilinear relation described the variation in r_i with pH_i-changes (at constant $[HCO_3]$) between 6.9–7.7 (fig. 4). The total change in r_i over this pH_i interval was $\sim$ 25–30%.

It might be expected from the H^+-hypothesis that the same proportionate response in resistance would occur for a given change in $[H^+]_i$, irrespective of the means of altering proton content. Of course, this also presumes that protons are the sole or predominant regulatory substance. Two additional cable-analysis experiments have been performed in which pH_i was varied by a slightly different methodology (CO_2/HCO_3 alterations at constant pH_o). The magnitude of change in r_i was found to be dependent upon the method of pH_i-perturbation even when activity was not a consideration. The decrease in r_i during cellular CO_2 loss if extracellular HCO_3 also varied (not shown) was several-fold greater than previously observed when $[HCO_3]_o$ was constant (fig. 4, 'Present results'). Over the pH_i interval from 7.13 to 7.76, the summary data in figure 4 ('Present results', $[HCO_3]$ constant, pH_o varying) would have predicted a 15% decrease in r_i during CO_2/HCO_3 withdrawal; the mean decrease observed for the same pH_i-interval under different conditions was 43% (pH_o constant, $[HCO_3]$ varying) – a *2.9-fold* greater change. This was approximately the magnitude of change Reber and Weingart reported previously (see fig. 4). However, it is also evident in figure 4 that junctional resistance from other preparations (amphibian embryo) may be much more sensitive to alterations in pH_i than cardiac tissue.

It is highly likely that CO_2-associated alterations in r_i did not develop through the action of an extracellular mediator (e.g. pH_o). Changes in r_i occurred even under conditions where there were no significant changes in pH_o (NH_4Cl-exposure, CO_2/HCO_3-withdrawal effected by transient superfusion with PIPES-Tyrode at the same pH_o). Furthermore, extracellular diffusion of impermeant cations (presumably to the level of gap junctional membranes) does not affect r_i (e.g. La^{3+})[49] even though intracellular injection of these cations produces prompt uncoupling.[21] Both results strongly argue that regulation of r_i occurs via alteration of some cytosolic constituent or enzymatic process.

The aforementioned results in Purkinje fibers, though preliminary, conflict with the H^+-hypothesis by several lines of evidence. i) Changes in r_i were activity-dependent despite no profound activity-dependence in associated changes in pH_i. Internal longitudinal resistance decreased with CO_2/HCO_3 withdrawal (alkalinization) at rest but paradoxically increased with the same perturbation during activity. ii) A variable latency period preceded the pH_i-associated changes in r_i which could not be explained by diffusion alone. iii) The magnitude of the pH_i-associated changes in r_i demonstrated methodological-dependence. Alterations in r_i were two to three-fold larger for the same pH_i-range if HCO_3 also was absent from the medium than when $[HCO_3]_o$ was constant. These observations, albeit deductive, support alternative hypotheses wherein either i) another intracellular regulator predominates over the direct effects of H^+ on r_i; and/or ii) the effects of H^+ on r_i are mediated indirectly via some interposed substance or reaction.

Cytosolic H^+-Ca^{2+} interaction

Calcium ions and protons have many possible sites for interdependence within the cytosol. The degree of protonation of intracellular proteins, phospholipids and carbohydrates influences the number of divalent cations bound to such sites. Fabiato and Fabiato[26] have described one such example, namely the reduced Ca^{2+}-sensitivity of the myofilaments during acidosis. In addition, the Ca-channel is pH-dependent and the magnitude of the inward Ca-current is decreased during intracellular alkalinization by NH_4^+[50]. Calcium transport into mitochondria is coupled with the extrusion into the cytosol of two protons[65] but the kinetics of Ca^{2+}-uptake by isolated mitochondria are too slow for them to be important in normal Ca-cycling in contractile tissues. More importantly, a H^+-gradient develops across the sarcoplasmic reticulum (SR) membrane during active Ca^{2+}-uptake which is then dissipated during SR Ca^{2+}-release[58]. Intracellular pH and $[Ca^{2+}]_i$ are maintained both via intracellular buffering and several ion transport processes that are interlinked (e.g. Na^+/H^+-exchange, Na^+/Ca^{2+}-exchange). Net changes in pCa_i from changes in pH_i appear to depend upon the experimental conditions under which the changes are measured. Several workers[64] have observed that raising $[Ca^{2+}]_o$ during rest (presumably increasing $[Ca^{2+}]_i$) acidifies the myoplasm. However, Weingart and co-workers[71] have noted that acidification of the sarcoplasm decreases $[Ca^{2+}]_i$ and conversely alkalinization increases $[Ca^{2+}]_i$. In contrast, Bers and Ellis[9] found the opposite effects on pCa_i using NH_4Cl to change pH_i; alkalinization decreased $[Ca^{2+}]_i$ and acidification increased $[Ca^{2+}]_i$. The reason for the interest in such H^+-Ca^{2+}-interdependence is two-fold: i) Determination of the relative role of these ions in directly regulating r_i (i.e. the predomin-

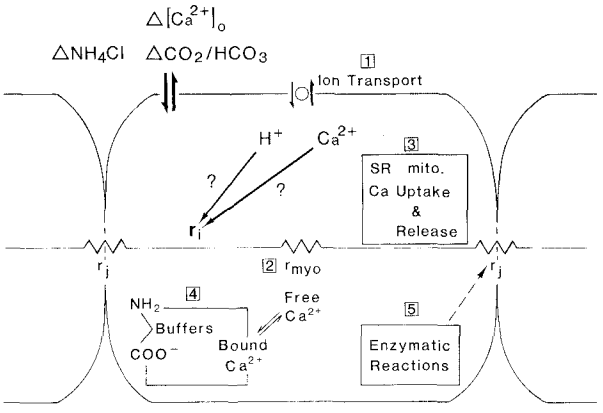

Figure 5. Schematic diagram of cellular proton and calcium interdependence and potential sites of action on internal longitudinal resistance. Both H^+ or Ca^{2+} seem to modify r_i but their putative roles remain uncertain. Either junctional membrane resistance (r_j) and/or myoplasmic resistance (r_{myo}) could be altered by H^+ and Ca^{2+} and thereby affect r_i. Cellular control mechanisms interlink $[H^+]_i$ and $[Ca^{2+}]_i$ and hence complicate attempts to determine whether H^+ or Ca^{2+} independently act to modulate r_i. Broad categories for H^+/Ca^{2+}-interdependence are: (1) Sarcolemmal ion transport: (3) co-transport by sarcoplasmic reticulum (SR) and/or mitochondria (mito); (4) buffering by intracellular proteins. In addition, it seems likely that enzymatic processes (e.g. phosphorylation) may modify r_i at the junctional membrane level (5) and either H^+, Ca^{2+} or both could serve as regulatory substances for such reactions.

ance of the H- or Ca-hypothesis) may depend upon understanding their interdependence within the sarcoplasm. Protons might affect r_i through changes in $[Ca^{2+}]_i$ and vice versa. ii) activity-dependent behavior of H^+-induced changes in r_i may be related to H^+-mediated changes in $[Ca^{2+}]_i$ during activity. Hence, elucidating the ionic regulation of r_i may well depend upon understanding pH-mediated changes in Ca-current, Ca^{2+}-uptake and release from the SR, or any activity-dependence of ion-transport processes (e.g. Na^+/Ca^{2+}-exchange). Experiments already suggest (Pressler, unpublished observations) that cellular handling of H^+-loads or losses is not activity-dependent.

Other potential regulators of internal longitudinal resistance

Given the above evidence, it seems indisputable that both H^+ and Ca^{2+} can modify r_i. However, it is also clear from the above results that the effects of protons on r_i may be mediated indirectly. Although Ca-ions may serve as such an intermediate, it also may be true as well that Ca^{2+} do not act directly to modify r_i. Consequently, another hypothesis for regulation of gap-junctional resistance is that changes in intracellular H^+ or Ca^{2+} merely act to trigger or modify an intermediate substance or reaction which per se affects the resistance of the nexal connections. A calcium-binding protein might be one example of such an intermediate wherein changes in either $[H^+]_i$ or $[Ca^{2+}]_i$ lead to changes in the free concentration of the other. One possible protein in this regard, calmodulin, can be virtually excluded since there is minimal alteration in the Ca^{2+}-affinity of the protein with pH alterations between 5.5 and 7.5[30]. Perhaps other pH-dependent intracellular Ca^{2+}-binding substances (e.g. parvalbumin) act differently but this is supposition.

A more likely alternative than H^+-Ca^{2+}-interaction via Ca-binding proteins may be that H^+ and/or Ca^{2+}-influence junctional protein phosphorylation. It seems pertinent to indicate that the profound changes in $[H^+]_i$ (and perhaps also $[Ca^{2+}]_i$) necessary to alter r_i may be applicable only to the pathological process of cellular uncoupling. Hence, it is conceivable that a different regulatory mechanism (? phosphorylation of junctional proteins) pertains to physiological modulation of r_i in contrast to the 'disaster control' of injury or cell death-related uncoupling (? protease effect). Particularly under physiological circumstances one can envision that fine regulation of gap-junctional resistance might be linked to those low molecular weight substrates, e.g. cAMP, inositol triphosphate, diacylglycerol, which act within the cell to transduce and amplify receptor inputs. Cyclic-AMP is known to diffuse from cell to cell[62] and also to modulate the behavior of sarcolemmal channels.[36] In this regard, Ewald, Williams and Levitan[25] have reported recently that the conductance of a non-junctional membrane channel in an artificial lipid bilayer (Ca^{2+}-dependent K^+-channel) was modified by exposure to a cAMP-dependent protein kinase. Interestingly, Flagg-Newton, Dahl and Loewenstein[27] in cultured mammalian cells and De Mello[23] in heart tissue have observed modulation ('up-regulation') of junctional permeability and cell-to-cell coupling that correlated with intracellular levels of cAMP. This contrasts with the lack of direct effects of dibutyryl cAMP and 8-bromo-cGMP on r_i within cat ventricular muscle observed by Wocjtczak.[73] In addition, Spray, Bennett, and co-workers[56] recently have demonstrated that a cAMP-dependent protein kinase is able to phosphorylate the 27 kDa polypeptide associated with gap junctions. The functional correlates to this phosphorylation event are still being investigated and unknown at this juncture.

It seems cogent to indicate that initiation of such enzymatic processes might demand a certain time interval for full expression – which in turn might account for the variable latent period between changes in pH_i and changes in internal resistance as observed. The failure of previous workers[51] to measure such a time delay may have been a consequence of simply the temperature at which the measurements were performed (i.e. 35 °C). If such an enzymatic event is temperature-dependent, low temperature may facilitate the detection of such a latent interval between changes in $[H^+]_i$ and r_i. It would be consistent with such a phosphorylation hypothesis that a latent period should also be detectable for $[Ca^{2+}]_i$-induced alterations in r_i if such an enzymatic process serves as an intermediate.

Conclusions and model for cell-to-cell transmission in heart

Evidence has been presented that r_i is an important determinant of conduction and may be affected both by changes in intracellular proton and calcium concentrations. What is uncertain is the role H^+ and Ca^{2+} may serve in regulating r_i and whether such regulation is by direct interaction or indirect through some intermediate substance (e.g. cAMP) or reaction. Some tentative conclusions:

a) Internal longitudinal resistance of the cardiac conduction system is modified independently by changes in cytosolic protons and/or calcium ions. However, r_i seems to be relatively insensitive to pH_i changes in the heart.

b) The direct action of protons on r_i is subordinate to other mediators or regulatory processes. Part of the pH-related alteration in resistance may be mediated by changes in $[Ca^{2+}]_i$.

c) Ionic regulation of junctional resistance seems likely to occur by more than one mechanism: i) direct binding of cations to anionic sites on the membrane proteins; and ii) phosphorylation of junctional membrane proteins by a H^+- or Ca^{2+}-dependent mechanism. Figure 5 shows a schematic diagram of the possible cellular sites of action and interactions of H^+ and Ca^{2+} on the internal resistive path.

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Cell-to-cell coupling studied in isolated ventricular cell pairs

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Summary. Cell pairs isolated from adult rat and guinea pig ventricles were used to study the electrical properties of the nexal membrane. Each cell of a pair was connected to a voltage-clamp system so as to enable whole-cell, tight-seal recording. The current-voltage relationship of the nexal membrane was found to be linear, revealing a resistance r_n of 2–4 MΩ. r_n was insensitive to the sarcolemmal membrane potential (range: −90 to +30 mV), and exerted no time-dependent gating behavior (range: 0.1 to 10 s). Lowering pH_i yielded a small increase in r_n. Vigorous elevations in $[Ca^{2+}]_i$ gave rise to an increase in r_n which was associated with a cell shortening. Uncoupling caused by aliphatic alcohols or halothane did not produce cell shortening. Cell pairs were also used to study action potential transfer.
Key words. Myocytes; electrical coupling; cell-to-cell coupling; nexus.

Introduction

Since the pioneering work of Weidmann[15], cable analysis has been used extensively in cardiac tissue to study the electrical properties involved in intercellular coupling[4]. This approach revealed quantitative information about the overall intracellular resistance pathway (r_i) which consists of the repetitive arrangement of two resistive elements in series, the cytoplasm (r_c), and the nexal membrane (r_n). However, a major limitation of cable analysis has been that the structure responsible for intercellular communication, i.e. the nexal membrane, is not accessible *directly* to a functional investigation.

With the introduction of novel methods such as enzymatic procedures for isolating cells, and recording techniques suitable for small cells[11], the situation changed fundamentally. Utilizing isolated cardiac cell pairs in conjunction with patch-clamp pipettes, it became feasible to explore the electrical properties of the nexal membrane itself.

Methods

Figure 1A shows the experimental arrangement[17] adopted. Each cell of a cell pair was connected to a patch-clamp pipette so as to enable tight-seal, whole-cell recording. A double voltage-clamp method was employed which allowed one to control the membrane potential of cell 1 (V_1) and cell 2 (V_2) individually. The associated currents flowing through each pipette were measured separately (I_1 and I_2). Figure 1B shows the equivalent circuit used for the analysis. It includes three resistive elements: $r_{m,1}$ and $r_{m,2}$, the resistances of the sarcolemmal membrane of cell 1 and cell 2; and r_n, the resistance of the nexal membrane.

According to this model, current injected into a pipette flows via either of two pathways, a) directly through r_m of the injected cell, or b) via r_n through r_m of the other cell. Thus, in general the pipette current represents the sum of two current components, which, however, under appropriate conditions may be separated. This is possible when voltage-clamp pulses are applied to one of the cells, while the other cell remains at the common holding potential, V_H. In this case, the voltage

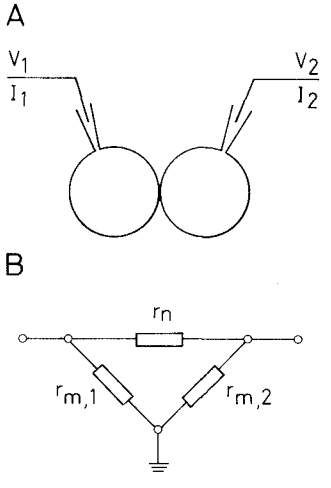

Figure 1. *A* Diagram of the experimental arrangement. Each cell of a cell pair was connected to a patch-clamp pipette. Separate voltage-clamp circuits enabled us to apply voltage steps (V_1, V_2) to each cell and to measure the resulting currents (I_1, I_2) individually. *B* Equivalent circuit used to analyze the data. It consists of $r_{m,1}$ and $r_{m,2}$, the resistances of the sarcolemmal membranes of cell 1 and cell 2, and r_n, the resistance of the nexal membrane. From Weingart[17].