

20% in nominally zero Ca_o ($n=5$). This Ca^{2+} -dependent uncoupling was demonstrated to be CaM-dependent by acute (10–15 min) pretreatment with 2 μM calmidazolium, wherein Cx43 g_j declined by $< 10\%$ within 10 min ($n=4$). To directly test for the involvement of the Cx43 amino acid residue #136–158 domain in this Ca^{2+} /CaM-dependent gap junction uncoupling process, 1 μM peptides were added to both whole cell patch pipettes and the 1 μM ionomycin/1.8 mM Ca_o perfusion experiments were repeated. The Cx43 #136–158 sequence mimetic peptide ($K_d(\text{CaM}) = 860 \text{ nM}$) effectively prevented the Cx43 g_j decline ($< 3\%$, $n=4$) whereas a scrambled sequence peptide control failed to prevent the Ca^{2+} -induced rundown of Cx43 g_j ($< 90\%$, $n=3$). These data unequivocally demonstrate that influx of external Ca^{2+} induces closure of Cx43 gap junctions in a CaM-dependent process involving the Cx43 residue #136–158 CL domain. This process has significant implications for the modulation of cardiac g_j by Ca_i and the “healing-over” of infarcted myocardium. Supported by NIH grants GM62999 & EY-05684 to JJY and HL-042220 to RDV.

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Cam Interaction and Binding Mode Study with Peptide from Intracellular Loop of Cx50

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 Connexin 50 (Cx50) is a member of the α family of gap junction proteins expressed in the lens of the eye where it has been shown to be essential for normal lens development. We have identified calmodulin (CaM) binding sites in the intracellular loop near to the 3rd transmembrane region of Connexin43 (Cx43) and Connexin44 (Cx44) which belong to the same α connexin family as Cx50. Sequence alignment of the candidate CaM binding regions of Cx43 and Cx44, with Cx50 identified a region encompassing residues 141–166 of Cx50 with a high predicted affinity for CaM. A peptide Cx50_{141–166} was synthesized to study the interaction of CaM with this domain of Cx50. Biophysical results indicate that in the presence of Ca^{2+} , Cx50_{141–166} binds with high affinity (0.14 μM) to CaM, as monitored by IAEDANS that was covalently attached to the C-terminal Cys of CaM. Electrophysiological data support the hypothesis that elevated intracellular Ca^{2+} concentration inhibits Cx50 gap junctions because omission of Ca^{2+} from the 1 μM ionomycin bath saline prevented the 95% decline in junctional conductance (g_j) observed in the presence of 1.8 mM CaCl_2 . This is likely CaM-mediated, because inclusion of a CaM inhibitor also prevented this Ca^{2+} -dependent inhibition of Cx50 gap junctions. The involvement of the Cx50 CaM binding domain in this Ca^{2+} /CaM-dependent regulation was further demonstrated by inclusion of the Cx50_{141–166} peptide in both whole cell patch pipettes, which effectively prevented the usual decrease in Cx50 g_j . These results demonstrate that the binding of Ca^{2+} -CaM to the intracellular loop of Cx50 is critical for mediating the Ca^{2+} -dependent inhibition of Cx50 gap junctions in the lens of the eye.

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Gating Modulation of Connexin45 Gap Junction Channels By Intracellular pH

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 Intracellular pH (pH_i) changes considerably during pathological conditions such as ischemia or epilepsy. Changes of pH_i affect the way cells communicate through gap junction (GJ) channels, and therefore disturb normal tissue function. To study pH_i -dependent modulation of GJ channels, we used HeLa cells expressing connexin45 (Cx45) or its fusion form with EGFP. The latter along with electrophysiological data allowed us to estimate the proportion of functional channels (N_F) within a junctional plaque (JP). We examined how junctional conductance (g_j) depends on pH_i and how pH_i affects voltage-gating properties of Cx45 homotypic and Cx45/Cx43-EGFP heterotypic GJs. Even at $\text{pH}_i \approx 8$, where the probability of the channels being fully open approximates 1, g_j was maximal but only 5 % of the channels were functional. Changes in pH_i from ~ 7.2 to ~ 8 increased g_j ~ 1.8 -fold in homotypic Cx45 GJs; g_j - pH_i dependence was sigmoidal with $\text{pK}_a \approx 7$. We used a stochastic four-state model of contingent gating to fit experimental g_j - V_j dependence, which allowed us to define parameters characterizing voltage-gating sensitivity (V_0 and A) and N_F . We found that alkalization increases g_j mainly by increasing V_0 , i.e., voltage at which open and closed states of hemichannel are at equilibrium. On the other hand, uncoupling by acidification was due to a decrease of both V_0 and N_F . In both cases, the constant A , characterizing the steepness of g_j changes over V_j remained stable. These results agree with data obtained from heterotypic Cx45/Cx43-EGFP GJs in which $\text{pK}_a \approx 6.7$, i.e., in between pK_a s of Cx43-EGFP and Cx45 homotypic GJs. In summary, pH_i modulates V_j -gating that largely explains observed pH-dependent changes of cell-cell coupling.

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Single Channel Connexin43 Plaque Formation

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Connexin43 (Cx43) is one of the most ubiquitous gap junction proteins in the human body and plays an essential role in cell-to-cell communication for a variety of organs and organ systems. Gap junction hemichannels are composed of six, often identical subunits, which can range from 26 kD to 60 kD, which assemble into water tight ion conduits which bridge the extracellular space between opposing cells and allow transfer of electrical impulses and small solutes up to 1 kD. Single hemichannels have been shown to remain functional in a cell membrane even when unopposed and have been linked to propagation of intercellular calcium waves, release of NAD^+ and ATP, neuronal signaling, and the activation of many different kinase cascades. Here, we explore the electrophysiological properties of single Cx43 and Cx43eGFP hemichannels and their interactions during plaque formation in a planar lipid membrane (BLM). The average conductance of a Cx43 channel was found to be $753 \pm 31 \text{ pS}$ ($n = 30$) for a 500 mM KCl buffer. Cx43eGFP exhibited an average conductance of $783 \pm 53 \text{ pS}$ ($n = 30$). Unlike in-vivo patch clamp experiments, Cx43 was purified and isolated from other membrane constituents, producing a system capable of probing both connexon electrophysiology and the roles of several well known gap junction blockers, namely: lanthanum, carbenoxalone and lindane. We also use single channel BLM to examine the critical number of hemichannels required for plaque formation and the emergent electrical properties therein.

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New Classes of Gap Junction Channel Blockers for Cx43 and Cx50

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In many tissues gap junction channels as well as hemichannels play important roles in intercellular electrical and biochemical coupling, cell synchronization, differentiation, growth and metabolic coordination. Therefore they have been proposed as potential new targets for the treatment of diseases such as epilepsy, cardiac arrhythmia and cancer. However, highly specific and potent pharmacological tools to further study their physiological as well as pathophysiological role are missing. The existing gap junction channel modulators are either of low potency, cross-react with other ion channels or exhibit no subtype specificity. To identify potent and selective gap junction blockers we screened a small library of compounds containing ion channel modulating pharmacophores. We identified five small molecule chemotypes including quinolines and triarylmethanes (TRAMs) that inhibited intercellular coupling via Cx43 or Cx50 in the lower micromolar range.

The triarylmethane derivatives, e.g. T66 (*N*-[(2-chlorophenyl)(diphenyl)methyl]-*N*-(1,3-thiazol-2-yl)amine) (IC_{50} 3 μM), blocked Cx50 currents with IC_{50} values in the range of 1–10 μM while having only small or no effects on other gap junction channel subtypes such as Cx32, Cx36 and Cx46. The quinoline derivative SB002 (4-(4-phenoxybutoxy)quinoline) inhibited Cx50 (IC_{50} 3 μM) as well as Cx43 (IC_{50} 8.3 μM). We currently are exploring the structure-activity relationship (SAR) to increase potency and for the quinoline derivatives to shift the subtype selectivity profile towards Cx43.

We propose quinolines as well as triarylmethanes as new pharmacological tool compounds to further elucidate the physiological roles of Cx43 and Cx50 and to study their contribution to disease pathogenesis.

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Heteromultimeric Gap-Junction Channel Permeance: Directional Fluxes Simulated Using a Brownian Dynamics Model

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Permeance for distinct connexin channels has been hard to predict. Heterotypic connexin combinations yield preferential directional fluxes of fluorescent molecules with small influence from particle charge, suggesting regulation through differences in pore's shape. We have simulated particles' movement across a 3D geometric pore's representation from X-ray crystallography following a Brownian Dynamics Model (BDM). A central prolate represents the pore's vestibule; a cone and a cylinder represents the pore's mouths for Cell 1 (C_1) and Cell 2 (C_2) respectively. Lucifer yellow molecules ($e=0$ or $-2e$, Stokes radius of 4.9 Å) were represented as spheres. Particle-channel charge interactions were simulated placing a charged ring near the channel's mouth. BDM described closely particle behavior where the displacement vector (dx) was calculated using particles' diffusion matrix, net force and a random vector from

Gaussian distributions after every time step ($dt=10ps$). Unidirectional particle fluxes were determined by counting particles that crossed the channel (particle gradient 4 to 0).

Uncharged particle flux was not affected by the pore's mouth shape. When $e=-2e^-$, particle flux depended on pore's shape ($C_{1to2}=1.6 \pm 0.04 \times 10^6$ particles/sec/channel [p/s/ch]; $C_{2to1}=0.75 \pm 0.03 \times 10^6$ p/s/ch). A negative charged ring reduced/blocked particle flux and pore-particle interactions. A positive charged ring doubled particle flux for C_{2to1} only, highlighting the mechanism's complexity.

Particle number at steady-state (SS) during bidirectional fluxes was calculated after placing 8 particles on either side. The average number of particles per cell remained close to 4 at SS and did not vary significantly ($<6\%$; $n=15$) over $6\mu s$; the time to reach steady state for C_{1to2} was smaller ($1.5\mu s$ and $2.1\mu s$ for C_{2to1}). Our mathematical model explains preferential directional fluxes by differences in pore mouth's shape. Identical number of particles at SS indicates thermodynamics compliance.

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Propagation Mechanisms of Calcium Waves Arising During Arterial Vasomotion

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Vasomotion consists in cyclic arterial diameter variations induced by synchronous contractions and relaxations of smooth muscle cells (SMCs) present in the arterial wall. These contractions have been shown to be due to an increase in the intracellular cytosolic calcium concentration. However, the arteries do not contract simultaneously on macroscopic distances and a propagation of the diameter variations can be observed. Our aim was to investigate this propagation. We stimulated endothelium denuded rat mesenteric arterial strips with the vasoconstrictor, phenylephrine (PE) to obtain vasomotion and observed that the contraction waves are linked to intercellular calcium waves through the SMCs. A velocity of about $100 \mu m/s$ was measured for the two kinds of waves which could propagate either from the distal to the proximal side of the artery or in the opposite direction. To investigate the calcium wave propagation mechanisms, we used a method allowing a PE stimulation of a small area of the strip. No calcium propagation could be induced by this local stimulation when the strip was in its resting state. However, if a low PE concentration was added to the whole strip, local PE stimulations induced calcium waves spreading over finite distances. The calcium wave velocity induced by local stimulation was identical to the velocity observed during vasomotion. This suggests that the propagation mechanisms are similar in the two cases. Using inhibitors of gap junctions and of voltage operated calcium channels, we showed that the locally induced calcium propagation likely depends on the propagation of the SMCs depolarization. Finally, from the experimental data gathered, it is possible to propose a model of the mechanisms underlying the propagation.

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Edema: A Missing Link in the Conduction Velocity-Gap Junction Relationship

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Introduction: Myocardial edema i.e., increased extracellular volume (V_{ECS}) occurs in diverse pathologies. Often these states are also linked with sudden cardiac death and conduction slowing, sometimes linked to gap junction (Gj) remodeling. Yet, edema's effect on the conduction velocity (CV)-Gj relationship remains unknown. We hypothesized that edema modulates the CV-Gj relationship.

Methods: CV was quantified by optical mapping in Langendorff-perfused guinea pig ventricles while pacing from the anterior epicardium. Tissue water content was estimated by the wet weight (WW) to dry weight (DW; after $24h \cong 60^\circ C$) ratio. V_{ECS} was modulated by perfusion of mannitol (26.1 g/l) or albumin (4 g/l). Gap junctions were uncoupled using carbenoxolone (10, 13 and $50 \mu M$).

Results: Mannitol caused edema, increasing WW/DW ratio relative to control ($10.7 \pm 6\%$, $p<0.05$, $n=6$). It decreased transverse CV (CV_T) by $24 \pm 4\%$ and longitudinal CV (CV_L) by $9 \pm 2\%$ ($p<0.05$, $n=4$). Consequently, edema increased anisotropy of conduction ($AR_{CV}=CV_L/CV_T$) from 2.30 ± 0.16 to 2.75 ± 0.19 ($p<0.05$). Albumin had the opposite effects. It dehydrated the heart, decreasing WW/DW relative to control ($11.3 \pm 5\%$, $p<0.05$, $n=6$). Dehydration increased CV_T by $71 \pm 10\%$ ($p<0.05$, $n=4$) without significantly affecting CV_L . Thus, dehydration reduced AR_{CV} to 1.84 ± 0.07 ($p<0.05$, $n=4$). Under control conditions, 10 and $13 \mu M$ carbenoxolone did not significantly affect

CV while $50 \mu M$ carbenoxolone slowed CV_T by $25 \pm 1\%$ ($p<0.05$, $n=3$) but had no effect on CV_L . During edema, $13 \mu M$ carbenoxolone significantly slowed CV_T by $38 \pm 9\%$ ($p<0.05$, $n=4$) but had no effect on CV_L . Conduction was completely abolished by $50 \mu M$ carbenoxolone and mannitol.

Conclusions: These data demonstrate that edema slows conduction and unmasks a steeper CV-Gj relationship. Therefore, these data may explain conduction differences between nearly identical models of Cx43 down-regulation. Specifically, V_{ECS} determines whether Gj uncoupling slows conduction.

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Increased Intercellular Communication in Activated Cardiac Fibroblasts

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Rationale: A critical event in the development of cardiac fibrosis is the transformation of fibroblasts into myofibroblasts; however the electrophysiological consequences of this phenotypic switch remain largely unknown. **Hypothesis:** Fibroblast activation results in a distinct electrophysiological phenotype that makes the heart more susceptible to arrhythmias. **Methods:** Ventricular fibroblasts were isolated from the ventricles of normal (Fb) and infarcted (MI-Fb) adult rat hearts. Fb and MI-Fb were plated onto myocyte monolayers at densities of 200, 400 and 600 cells/mm². Cultures were optically mapped with a voltage sensitive dye and conduction velocity (CV) and action potential duration (APD_{70}) were obtained. Functional intercellular communication, Cx43 expression levels and distribution were determined. **Results:** At 200 cells/mm², MI-Fb significantly increased (22.0 ± 0.6 cm/s) CV compared to homocellular myocyte cultures (Myo; 17.8 ± 0.4 cm/s). Fb (145.0 ± 3.9 ms) and MI-Fb (131.1 ± 3.7 ms) significantly reduced APD_{70} compared to Myo (158.6 ± 2.5 ms), and APD_{70} was significantly shorter with MI-Fb compared to Fb. At higher fibroblast densities CVs were slower compared to Myo (Fb: 13.8 ± 0.4 and 11.4 ± 0.3 cm/s; MI-Fb: 10.6 ± 0.3 and 9.5 ± 0.3 cm/s, at 400 and 600 cells/mm², respectively). Fb and MI-Fb CVs were significantly different at all plating densities. At higher densities APD_{70} was significantly longer for Fb compared to Myo (171.0 ± 2.1 and 165.7 ± 1.8 ms at 400 and 600 cells/mm², respectively). MI-Fb values were significantly longer at 400 cells/mm² (175.8 ± 2.7 ms). Cx43 staining was present in contact areas between myocytes and fibroblasts from both sources. Immunoblotting showed a significant increase of Cx43 levels in MI-Fb compared to Fb. Intercellular coupling evaluated with gap-FRAP was significantly increased between myocytes and MI-Fb compared to Fb. **Conclusions:** These data demonstrate fibroblast activation results in important electrophysiological changes that could contribute to the greater incidence of arrhythmias observed in fibrotic hearts. These observations highlight the fibroblast activation process as a potentially new therapeutic target for arrhythmia prevention.

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Astrocytes Control Breathing Through pH-Dependent Vesicular Release of Atp

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Extracellular signalling mediated by ATP has been associated with central chemosensory function. It has been shown that increase in pCO_2 in the arterial blood triggers an immediate release of ATP from the chemosensitive regions located on the ventral surface of the medulla oblongata (VMS).

Using in vitro and in vivo preparations and novel genetically encoded Ca^{2+} indicator based on cyclically permuted GFP - Case 12, we show that astrocytes in the ventral regions of the medulla oblongata are highly sensitive to changes in pH. Decrease in extracellular pH from 7.4 to 7.2 induced transient increases in $[Ca^{2+}]_i$ in astrocytes from dissociated neuro-glia cultures prepared from the VMS, in ventral astrocytes of organotypic brainstem slice cultures and in acute horizontal brainstem slices of adult rats, as well as on the VMS in anaesthetized and artificially ventilated rats. ATP receptor antagonists such as MRS2179, PPADS and TNP-ATP effectively blocked $[Ca^{2+}]_i$ responses evoked by lowering pH in VMS astrocytes. ATP hydrolyzing enzyme apyrase completely prevented propagation of $[Ca^{2+}]_i$ excitation in VMS astrocytes evoked by lowering external pH. These data suggest that Ca^{2+} responses induced by lowering external pH are mediated by ATP release and subsequent activation of P2 receptors. Inhibitors of vesicular transport brefeldine A and bafilomycin A both effectively abolished $[Ca^{2+}]_i$ excitation of VMS astrocytes evoked by lowering external pH. Incubation of astrocytes with FM 1-46 dye (vesicular marker) leads to selective labelling of intracellular vesicles. Acidification of external medium induced decrease in the fluorescence intensity of