This project was designed to study four basic aspects of arrhythmia generation: (1) isoproterenol-induced currents, (2) consequences of changes in intracellular calcium concentration, (3) contribution of the individual ion currents and (4) beat-to-beat variability of action potential duration. Accordingly, these topics are discussed separately in this final report.

(1) Analysis of the isoproterenol-induced changes in action potential morphology and the underlying ion currents

Adrenergic activation of both L-type Ca^{2+} and various K^+ currents is a crucial mechanism of cardiac adaptation, however, it may carry substantial proarrhythmic risk as well. We have monitored the timing of activation of the most important isoproterenol-sensitive currents ($I_{Ca,}$ I_{Ks} and I_{Kr}) in isolated canine ventricular cells using conventional microelectrode, whole cell voltage clamp and action potential voltage clamp techniques in isolated canine ventricular myocytes. Isoproterenol (ISO, 10 nM) was found to elevate the plateau potential and shorten action potential duration (APD) in subepicardial and mid-myocardial cells, which effects were associated with multifold enhancement of I_{Ca} and I_{Ks} and moderate stimulation of I_{Kr}. The ISO-induced plateau-shift and I_{Ca}-increase developed faster than the shortening of APD and stimulation of I_{Ks} and I_{Kr}. Blockade of β_1-adrenoceptors (using 300 nM CGP-20712A) converted the ISO-induced shortening of APD to lengthening, decreased its latency and reduced the plateau-shift. In contrast, blockade of β_2-adrenoceptors (by 50 nM ICI 118,551) augmented the APD shortening effect and increased the latency of plateau-shift without altering its magnitude. All effects of ISO were prevented by simultaneous blockade of both receptor types. Inhibition of phosphodiesterases decreased the differences observed in the turn on of the ISO-induced plateau-shift and APD-shortening. In summary, the ISO-induced activation of I_{Ca} is turned on faster than the stimulation of I_{Ks} and I_{Kr} in canine ventricular cells due to the involvement of different adrenergic pathways and compartmentalization.

Using conventional microelectrode techniques in isolated canine ventricular cells we have found that in myocytes displaying a spike-and-dome action potential configuration (epicardial and midmyocardial cells) ISO caused reversible shortening of action potentials accompanied by elevation of the plateau. ISO-induced action potential shortening was absent in endocardial cells or in myocytes pretreated with 4-aminopyridine. Application of the rapid delayed rectifier K^+ current (I_{Kr}) blocker E-4031 failed to modify the ISO effect, while action potentials were lengthened by ISO in the presence of the slow delayed rectifier K^+ current (I_{Ks}) blocker HMR-1556. Both action potential shortening and elevation of the plateau were prevented by pretreatment with the L-type Ca^{2+} current (I_{Ca}) blocker nisoldipine. Action potential voltage clamp experiments revealed a prominent slowly inactivating I_{Ca} followed by a rise in I_{Ks}, both currents increased with increasing the cycle length. In conventional voltage clamp experiments ISO increased I_{Ks}, I_{Kr} and I_{Ca} to 420±4, 133±1 and 340±13 % of their baseline values, respectively. It was concluded that the effect of ISO in canine ventricular cells depends critically on action potential configuration, and the ISO-induced activation of I_{Ks} - but not I_{Kr} - may be responsible for the observed shortening of action potentials.

Since in many of our experiments ISO-induced I_{Ca} was suppressed by using Ca^{2+} channel blockers, the antagonistic interactions between the effects of various types of

Ca^{2+} channel blockers and ISO on the amplitude of I_{Ca} had to be examined as well. Whole cell version of the patch clamp technique was used to study the effect of ISO on I_{Ca} in the absence and presence of Ca^{2+} channel blocking agents, including nifedipine, nisoldipine, diltiazem, verapamil, $CoCl_2$, and $MnCl_2$. It was found that 5 µM nifedipine, 1 µM nisoldipine, 10 µM diltiazem, 5 µM verapamil, 3 mM $CoCl_2$, and 5 mM $MnCl_2$ evoked uniformly a 90-95% blockade of Ca^{2+} current in the absence of ISO. ISO (100 nM) alone increased the amplitude of Ca^{2+} current from 6.8±1.3 to 23.7±2.2 pA/pF in a reversible manner. ISO caused a marked enhancement of Ca^{2+} current even in the presence of nifedipine, nisoldipine, diltiazem, and verapamil, but not in the presence of $CoCl_2$ or $MnCl_2$. The results indicate that the action of isoproterenol is different in the presence of organic and inorganic Ca^{2+} channel blockers. $CoCl_2$ and $MnCl_2$ was able to fully prevent the effect of ISO on I_{Ca}, while the organic Ca^{2+} channel blockers failed to do so. This has to be born in mind when the effects of organic Ca^{2+} channel blockers are evaluated either experimentally or clinically under conditions of increased sympathetic tone.

(2) Effects of cytosolic calcium changes on the incidence of early afterdepolarizations

We have studied the influence of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) on APD and on the incidence of early afterdepolarizations (EADs) in canine ventricular cardiomyocytes. Action potentials of isolated cells were recorded using conventional sharp microelectrodes and concomitant $[Ca^{2+}]_i$ was monitored by the fluorescent dye, Fura-2. EADs were evoked at 0.2 Hz pacing rate by inhibiting the rapid delayed rectifier K^+ current with dofetilide, by activating the late sodium current with veratridine, or by activating the L-type calcium current with BAY K8644. These interventions progressively prolonged the action potential and resulted in initiation of EADs. Reducing $[Ca^{2+}]_i$ by application of the cell-permeant Ca^{2+} chelator BAPTA-AM lengthened the AP at 1 Hz if it was applied alone or in the presence of veratridine and BAY K8644. BAPTA-AM, however, shortened the AP after pretreating the cells with dofetilide. The incidence of the evoked EADs was decreased by BAPTA-AM strongly in dofetilide and moderately in veratridine, while it was increased by BAPTA-AM in the presence of BAY K8644. Based on these experimental data changes in $[Ca^{2+}]_i$ have marked effects on APD as well as on EAD incidence, however, the underlying mechanisms may be different depending on the mechanism of EAD generation. As a consequence, reduction of $[Ca^{2+}]_i$ may eliminate EADs under some – but not all – experimental conditions.

(3) Properties of the individual ion currents involved in arrhythmia generation

Beyond the ISO-induced changes discussed earlier, three ion currents, each strictly related to generation of afterdepolarizations, were investigated in details. These are the L-type Ca^{2+} current, the late Na^+ current, and the Ca^{2+}-sensitive Cl^- current.

In voltage clamp experiments tetrodotoxin (TTX) is used to block Na^+ current in all excitable tissues including mammalian ventricular cardiomyocytes, however, the TTX-sensitivity of the latter is lower than other excitable tissues by three orders of

magnitude. Studying the TTX-sensitivity of I_{Ca} in isolated canine ventricular cells using conventional voltage clamp and action potential voltage clamp techniques TTX was found to block I_{Ca} in a reversible manner without altering inactivation kinetics of I_{Ca}. Fitting results to the Hill equation an IC_{50} value of 55±2 μM was obtained with a Hill coefficient of unity (1.0±0.04). The current was fully abolished by 1 μM nisoldipine indicating that it was really I_{Ca}. Under action potential voltage clamp conditions the TTX-sensitive current displayed the typical fingerprint of I_{Ca}, which was absent in the presence of nisoldipine. Stick-and-ball models for Cav1.2 and Nav1.5 channel proteins were constructed to explain the differences observed between action of TTX on cardiac I_{Ca} and I_{Na}. To reveal the details, the TTX-sensitivity of I_{Ca} was studied in isolated canine ventricular myocytes as a function of channel phosphorylation, extracellular pH and the redox potential of the bathing medium using the whole cell voltage clamp technique. 55 μM TTX caused 60% ± 2% inhibition of I_{Ca} in acidic (pH = 6.4), while only a 26% ± 2% block in alkaline (pH = 8.4) milieu. Similarly, the same concentration of TTX induced 62% ± 6% suppression of I_{Ca} in a reductant milieu (containing glutathione + ascorbic acid + dithiothreitol, 1 mM each), in contrast to the 31% ± 3% blockade obtained in the presence of a strong oxidant (100 μM H_2O_2). Phosphorylation of the channel protein (induced by 3 μM forskolin) failed to modify the inhibiting potency of TTX; an IC_{50} value of 50 ± 4 μM was found in forskolin. The results are in a good accordance with the predictions of our model, indicating that TTX binds, in fact, to the selectivity filter of cardiac L-type Ca channels. In light of this, quite surprisingly, TTX failed to inhibit $Ca_v1.2$ current expressed in HEK cells, up to the concentration of 100 μM - in spite of the fact that the kinetic properties of the $I_{Ca,L}$ and $Ca_v1.2$ currents were macroscopically similar. This finding may question the suitability of a single pore-forming channel subunit, expressed in a transfection system, for electrophysiological or pharmacological studies.

Late Na^+ current ($I_{Na,L}$) has long been known to be involved in arrhythmogeneseis, but its contribution to the cardiac action potential has not been clarified in details. In spite of the rapidly growing interest toward this current, there is no publication available on experimental recording of the dynamic $I_{Na,L}$ current as it flows during an action potential with Ca^{2+} cycling. Also unknown is how the current profile changes when the Ca^{2+}-calmodulin dependent protein kinase II (CaMKII) signaling is altered, and how the current contributes to the development of arrhythmias. Therefore we have used an innovative AP-clamp sequential dissection technique (referred also as onion peeling) to directly record the $I_{Na,L}$ current flowing during the action potential with normal Ca^{2+} cycling in guinea pig ventricular myocytes. The magnitude of $I_{Na,L}$ measured under action potential clamp conditions was substantially larger than earlier studies indicated. CaMKII inhibition using KN-93 significantly reduced the current. In addition, we recorded $I_{Na,L}$ together with I_{Ks}, I_{Kr}, and I_{K1} from the *same* cell to understand how these currents counterbalance to shape the action potential morphology. It was found that the amplitude and the total charge carried by $I_{Na,L}$ exceed that of I_{Ks}. Finaloly, facilitation of $I_{Na,L}$ by Anemone toxin II prolonged APD and induced Ca^{2+} oscillations that led to early and delayed afterdepolarizations and triggered action potentials; these arrhythmogenic activities were eliminated by buffering Ca^{2+} with BAPTA. In conclusion, $I_{Na,L}$ contributes a significantly large inward current that prolongs APD and unbalances the Ca^{2+} homeostasis to cause arrhythmogenic APs.

Understanding the role of ionic currents in shaping the cardiac action potential has great importance as channel malfunctions can lead to sudden cardiac death by inducing arrhythmias. Therefore, inhibitors are frequently used with the hope of selectively blocking an ion channel. For the calcium-activated chloride current ($I_{Cl(Ca)}$) 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and 9-anthracene carboxylic acid (9-AC) are generally used for this purpose. In voltage clamped canine ventricular myocytes DIDS- (0.2 mM) and 9-AC- (0.5 mM) sensitive currents were identical regardless of intracellular Ca^{2+} buffering. DIDS-sensitive current amplitude was larger with the increase of stimulation rate and correlated well with the rate-induced increase of calcium transients. Both drugs increased APD to the same extent but the elevation of the plateau potential was more pronounced with 9-AC at fast stimulation rates. On the contrary, 9-AC did not influence either the action potential amplitude or the maximal rate of depolarization but DIDS caused marked reduction of V_{max}. Both inhibitors reduced the magnitude of phase-1 but at slow stimulation rates this effect of DIDS was larger. All of these effects were readily reversible upon washout. Increasing concentrations of 9-AC between 0.1-0.5 mM in a cumulative manner gradually reduced phase-1 amplitude and increased APD. The EC_{50} value for 9-AC was 160 μM with a Hill coefficient of 2. The amplitudes of $I_{Ca,L,}$ $I_{Ks,}$ I_{Kr} and I_{K1} were not changed by 0.5 mM 9-AC. It was concluded that - although DIDS may also be used for dissecting $I_{Cl(Ca)}$ under voltage-clamp conditions - 9-AC is superior in action potential measurements for studying the physiological role of $I_{Cl(Ca)}$ due to the lack of sodium channel inhibition. In summary, 9-AC appears to be a selective inhibitor of $I_{Cl(Ca)}$, as it had no effect on other ion currents including $I_{Ca,L}$, I_{Kr}, I_{Ks}, and I_{K1}.

$I_{Cl(Ca)}$ mediated by TMEM16A and/or Bestrophin-3 is believed to contribute to cardiac arrhythmias. The true profile of $I_{Cl(Ca)}$ during an actual ventricular action potential, however, has poorly been understood. Therefore the true profile of $I_{Cl(Ca)}$ was studied under physiological conditions (normal Ca^{2+} cycling and action potential voltage-clamp) as well as under conditions designed to change $[Ca^{2+}]_i$. The expression of TMEM16A and/or Bestrophin-3 in canine left ventricular myocytes and human ventricular myocardium was examined. The possible spatial distribution of these proteins and their co-localization with $Ca_v1.2$ was also studied. The profile of $I_{Cl(Ca),}$ identified as a 9-anthracene carboxylic acid-sensitive current under action potential voltage-clamp conditions, contained an early fast outward and a late inward component, overlapping early and terminal repolarizations, respectively. Both components were moderately reduced by ryanodine, while fully abolished by BAPTA, but not EGTA. $[Ca^{2+}]_i$ was monitored using FURA-2-AM. Setting $[Ca^{2+}]_i$ to the systolic level measured in the bulk cytoplasm (1.1 μM) decreased $I_{Cl(Ca),}$ while application of Bay K8644, isoproterenol, and faster stimulation rates increased the amplitude of $I_{Cl(Ca)}$. Ca^{2+}-entry through L-type Ca^{2+} channels was essential for activation of $I_{Cl(Ca)}$. TMEM16A and Bestrophin-3 showed strong co-localization with one another and also with $Ca_v1.2$ channels, when assessed using immunolabeling and confocal microscopy in both canine myocytes and human ventricular myocardium. In conclusion, activation of $I_{Cl(Ca)}$ in canine ventricular cells requires Ca^{2+}-entry through the neighboring Ca^{2+} channels and is only augmented by SR Ca^{2+}-release. Substantial activation of $I_{Cl(Ca)}$ requires high Ca^{2+} in the dyadic clefts which can be effectively buffered by BAPTA, but not EGTA.

(4) Beat-to-beat variability of action potential duration

Beat-to-beat variability of cardiac action potential duration (short term variability, SV) is a common feature of various cardiac preparations, including the human heart. Although it is believed to be one of the best arrhythmia predictors, the underlying mechanisms are not fully understood at present. Therefore, we have studied the role of the major cardiac ion currents, APD, and stimulation frequency on the magnitude of SV in canine ventricular myocytes. SV was an exponential function of APD, when APD was modified by current injections. To compensate for the APD-dependent nature of SV, the concept of *relative SV* has been introduced by normalizing the changes of SV to the concomitant changes in APD. Relative SV was increased by dofetilide, HMR 1556, nisoldipine and veratridine, while it was reduced by BAY K8644, tetrodotoxin, lidocaine, and isoproterenol. Relative SV was also increased by increasing the stimulation frequency. In summary, relative SV was decreased by ion currents involved in the negative feedback regulation of APD (I_{Ca}, I_{Ks} and I_{Kr}), while it was increased by I_{Na} and I_{to}. We conclude that drug-induced effects on SV should be evaluated exclusively in relation with the concomitant changes in APD. Since relative SV was decreased by ion currents playing critical role in the negative feedback regulation of APD, blockade of these currents, or the beta-adrenergic pathway, may carry also some additional proarrhythmic risk in addition to their well-known antiarrhythmic action.

SV was also intimately influenced by changes in intracellular calcium concentration ($[Ca^{2+}]_i$). Exposure of myocytes to the Ca^{2+} chelator BAPTA-AM (5 μM) decreased, while Ca^{2+} ionophore A23187 (1 μM) increased relative SV. Contribution of transient changes of $[Ca^{2+}]_i$ due to Ca^{2+} released from the sarcoplasmic reticulum was studied using 10 μM ryanodine and 1 μM cyclopiazonic acid: relative SV was reduced by both agents. Inhibition of the Na^+-Ca^{2+} exchanger by 1 μM SEA0400 increased relative SV. It is concluded that elevation of $[Ca^{2+}]_i$ increases relative SV significantly. More importantly, Ca^{2+} released from the sarcoplasmic reticulum is an important component of this effect.

Profound changes of tissue redox potential occur in the heart under conditions of oxidative stress associated frequently with cardiac arrhythmias. The redox potential in isolated canine ventricular cells was shifted toward a reduced state using a reductive cocktail (containing dithiothreitol, glutathione and ascorbic acid) while oxidative changes were initiated by superfusion with H_2O_2. Exposure of myocytes to the reductive cocktail decreased SV significantly without any detectable effect on APD. Application of H_2O_2 increased both SV and APD, but the enhancement of SV was the greater, so relative SV increased. Longer exposure to H_2O_2 resulted in development of early afterdepolarizations accompanied by tremendously increased SV. Pretreatment with the reductive cocktail prevented both elevation of relative SV and the development of afterdepolarizations. The results suggest that the increased beat-to-beat variability during an oxidative stress contributes to generation of cardiac arrhythmias.