This project was designed to investigate the properties of the most important *calcium-modulated ion channels* in the canine (and occasionally in guinea pig or undiseased human) heart, which was extended also to epithelial cells. Accordingly, the individual topics are discussed separately in this final report.

(1) Properties of calcium-sensitive chloride current in canine and human ventricular myocardium

Calcium-sensitive chloride current ($I_{Cl\text{-}Ca}$), mediated by TMEM16A and/or Bestrophin-3, is believed to be involved in the generation of cardiac arrhythmias, however, the true profile of $I_{Cl\text{-}Ca}$ during an actual ventricular action potential (AP) is poorly understood. Therefore, this profile was studied systematically under physiological conditions, i.e under action potential voltage-clamp (APVC) conditions with normal Ca^{2+} cycling, and also under conditions designed to modify intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). 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 $I_{Cl\text{-}Ca}$, identified as a 9-anthracene carboxylic acid (9-AC) sensitive current under AP 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, corresponding to 1.1 μM, decreased $I_{Cl\text{-}Ca}$, while application of Bay K8644, isoproterenol, and faster stimulation rates increased the amplitude of $I_{Cl\text{-}Ca}$. Ca^{2+}-entry through L-type Ca^{2+} channels was essential for activation of $I_{Cl\text{-}Ca}$. 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. Activation of $I_{Cl\text{-}Ca}$ in canine ventricular cells requires Ca^{2+}-entry through the neighboring L-type Ca^{2+} channels but is only augmented by SR Ca^{2+}-release. In summary, substantial activation of $I_{Cl\text{-}Ca}$ requires high Ca^{2+} concentration in the submembrane fuzzy space.

Early afterdepolarization (EAD) formation has been reported to be modified by $I_{Cl\text{-}Ca}$ while others failed to corroborate this result. Therefore the role of $I_{Cl\text{-}Ca}$ in spatial and temporal heterogeneity of cardiac repolarization and EAD formation was also examined. These experiments were performed on the following cells of canine left ventricle: *subepicardial*, *midmyocardial* and *subendocardial* cells, as well as *apical* and *basal* cells of the midmyocardium. $I_{Cl\text{-}Ca}$ was blocked by 0.5 mM 9-AC and AP changes were tested with sharp microelectrodes. The whole-cell 9-AC-sensitive current was measured with either a conventional square pulse voltage-clamp or using APVC. Protein expression of TMEM16A and Bestrophin-3 was detected by Western blot. 9-AC reduced phase-1 repolarization in each cell type and increased AP duration in a reverse rate-dependent manner in all cell types except for subepicardial cells. Neither $I_{Cl\text{-}Ca}$ density recorded with square pulses, nor the normalized expression of TMEM16A and Bestrophin-3 proteins differed significantly among the studied groups of cells. The early outward component of $I_{Cl\text{-}Ca}$ was significantly larger in subepicardial than in subendocardial cells under APVC conditions. In both subepicardial and subendocardial cell populations, application of a typical subepicardial AP as a command pulse resulted in

a significantly larger early outward component compared to the experiments when a typical subendocardial AP was applied. Inhibition of $I_{Cl\text{-}Ca}$ generated EADs at low stimulation rates and their incidence was further increased by beta-adrenergic stimulation. 9-AC also increased the short-term variability of repolarization and the isoproterenol-induced reduction of the variability was smaller after the inhibition of $I_{Cl\text{-}Ca}$. Our results highlight the antiarrhythmic properties of $I_{Cl\text{-}Ca}$ which is reflected by a smaller spatial and temporal heterogeneity of cardiac repolarization and a reduced incidence of EADs.

(2) Properties of late sodium current in human, canine and guinea pig ventricular myocardium

Although late sodium current ($I_{Na\text{-}late}$) has long been known to contribute to plateau formation of mammalian cardiac action potentials, lately it was considered as possible target for antiarrhythmic drugs. However, many aspects of this current are still poorly understood. Therefore the true profiles of $I_{Na\text{-}late}$ was studied in canine and guinea pig ventricular cells and compared them to $I_{Na\text{-}late}$ recorded in undiseased human hearts. $I_{Na\text{-}late}$ was defined as a tetrodotoxin-sensitive current, recorded under action potential voltage clamp conditions using either canonic- or self-action potentials as command signals. Under action potential voltage clamp conditions the amplitude of canine and human $I_{Na\text{-}late}$ monotonically decreased during the plateau (decrescendo-profile), in contrast to guinea pig, where its amplitude increased during the plateau (crescendo profile). The decrescendo-profile of canine $I_{Na\text{-}late}$ could not be converted to a crescendo-morphology by application of ramp-like command voltages or command action potentials recorded from guinea pig cells. Conventional voltage clamp experiments revealed that the crescendo $I_{Na\text{-}late}$ profile in guinea pig was due to the slower decay of $I_{Na\text{-}late}$ in this species. When action potentials were recorded from multicellular ventricular preparations with sharp microelectrode, action potentials were shortened by tetrodotoxin, which effect was the largest in human, while smaller in canine, and the smallest in guinea pig preparations. It is concluded that important interspecies differences exist in the behavior of $I_{Na\text{-}late}$. At present canine myocytes seem to represent the best model of human ventricular cells regarding the properties of $I_{Na\text{-}late}$. These results should be taken into account when pharmacological studies with $I_{Na\text{-}late}$ are interpreted and extrapolated to human. Accordingly, canine ventricular tissues or myocytes are suggested for pharmacological studies with $I_{Na\text{-}late}$ inhibitors or modifiers. Incorporation of present data to human action potential models may yield a better understanding of the role of $I_{Na\text{-}late}$ in action potential morphology, arrhythmogenesis, and intracellular calcium dynamics.

(3) Effect of cytosolic calcium concentration on action potential duration and its beat-to-beat variability in canine myocytes

Intracellular calcium concentration ($[Ca^{2+}]_i$) is often buffered by using the cell-permeant acetoxy-methylester form of the Ca^{2+} chelator BAPTA (BAPTA-AM) under experimental conditions. The time-dependent action of BAPTA-AM on action potential duration (APD) was studied in canine ventricular mtocytes. Action potentials were recorded with conventional sharp microelectrodes. The effect of BAPTA-AM on the rapid delayed rectifier K^+ current (I_{Kr}) was studied using the conventional voltage clamp

and action potential voltage clamp techniques. APD was lengthened by exposure to 5 μM BATA-AM, while shortened by the Ca^{2+} ionophore A23187 in a time-dependent manner. The APD-lengthening effect of BAPTA-AM was strongly suppressed in the presence of nisoldipine, and enhanced in the presence of BAY K8644, suggesting that a shift in the $[Ca^{2+}]_i$-dependent inactivation of L-type Ca^{2+} current may be an important underlying mechanism. However, in the presence of the I_{Kr}-blocker dofetilide or E-4031 APD was shortened rather than lengthened by BAPTA-AM. Similarly, the APD-lengthening effect of 100 nM dofetilide was halved by the pretreatment with BAPTA-AM. In line with these results, I_{Kr} was significantly reduced by BAPTA-AM under both conventional voltage clamp and action potential voltage clamp conditions. This effect was not related to the Ca^{2+} chelator effect BAPTA-AM. It is concluded that the APD-modifying action of BAPTA-AM is composed of two distinct effects: (1) a direct inhibitory effect of BAPTA-AM on I_{Kr} current and (2) an indirect effect on calcium-dependent ion channels related to chelation of $[Ca^{2+}]_i$. Consequently, BAPTA-AM has to be applied carefully to control $[Ca^{2+}]_i$ in whole cell systems because of its direct inhibitory action on I_{Kr}.

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. The magnitude of SV is basically determined by the intensity of cell-to-cell coupling in multicellular preparations and by the duration of the action potential. To compensate for the APD-dependent nature of SV, the concept of relative SV (RSV) has been introduced by normalizing the changes of SV to the concomitant changes in action potential duration. RSV is reduced by I_{Ca}, I_{Kr} and I_{Ks} while increased by I_{Na}, suggesting that ion currents involved in the negative feed-back regulation of action potential duration tend to keep RSV at a low level. Since the majority of these ion currents are strongly Ca^{2+}-dependent, RSV is also intimately influenced by changes in intracellular calcium concentration.

(4) Selectivity of the transient receptor melastatin 4 channel inhibitor 9-phenanthrol in canine ventricular myocytes

9-phenanthrol has been used for suppression of transient receptor melastatin 4 **(**TRPM4) channels in various cardiac preparations, however, the selectivity of the compound to these channels is uncertain. Therefore, the concentration-dependent effects of 9-phenanthrol on the major cardiac ion currents were investigated in enzymatically dispersed canine ventricular cells using whole cell configuration of the patch clamp technique. The pipette solution contained 10 mM BAPTA in order to prevent the activation of TRPM4 channels by 9-phenanthrol. 9-phenanthrol (10 and 30 μM) significantly suppressed the transient outward K^+ current, the rapid delayed rectifier K^+ current and the inward rectifier K^+ current with the blocking potency for $I_{K1}<I_{Kr}<I_{to1}$. These effects of 9-phenanthrol were partially reversible. L-type Ca^{2+} current was not affected by 9-phenanthrol up to the concentration of 30 μM. In addition to these effects, 9-phenanthrol induced a steady outward current at potentials positive to -40 mV. The amplitude of this current was larger at more positive voltages and increased with the concentration of 9-phenanthrol. Action potentials were recorded using sharp microelectrodes. The maximal rate of depolarization, phase-1 repolarization and terminal repolarization were significantly decreased and the plateau potential was depressed by 9-phenanthrol (3-30 μM) without alteration in the

resting membrane potential. These changes in action potential morphology are congruent with the observed alterations of ion currents. In conclusion, 9-phenanthrol inhibits cardiac Na^+ and K^+, but not Ca^{2+}, channels therefore the compound is not selective to TRPM4 in canine ventricular myocardium. As a consequence, its application as a TRPM4 blocker can be appropriate only in expression systems but not in native cardiac cells.

(5) Interspecies differences in the effect of isoproterenol are partially related to differences in action potential morphology

Since the effects of isoproterenol (ISO) is known to be markedly calcium-dependent, the repolarization response to ß-adrenergic stimulation was studied in guinea pig and canine ventricular myocytes. The ISO-iduced response differs between guinea pig and canine myocytes and, within the latter, between myocardial layers. Correlative analysis suggests that this may be due to differences in action potential contour. To test the hypothesis that AP contour may set the response of current and of repolarization to ß-AR stimulation (10 nM ISO). The responses of membrane potential and current to ISO were measured under current-clamp and action potential voltage clamp conditions in guinea pig (GP), dog epicardial (DOG-EPI) and dog subendocardial (DOG-ENDO) myocytes. Dynamic-clamp experiments were used to evaluate the impact of action potential parameters on the response to ISO. ISO prolonged action potential duration in GP myocytes, did not affect it in DOG-ENDO and shortened it in DOG-EPI ones. The current induced by ISO (I_{ISO}) sharply differed between GP and canine myocytes and, to a lesser extent, between DOG-ENDO and DOG-EPI cells. Differences in I_{ISO} profile are likely important in setting the parameters (duration, time-to-peak, time-to-reversal) of action potential. These differences were minimized when canine myocytes where clamped with GP action potential waveforms and vice versa. Introduction of a "notch" in GP action potential under dynamic clamp conditions was alone insufficient to affect the response to ISO, nevertheless, when incorporated in a GP action potential waveform, the main features (development of notch and low plateau potential) of canine action potentials caused I_{ISO} of GP myocytes to acquire canine features. Early repolarization contour and level of plateau potential may largely account for species-specificity of I_{ISO} profile, while action potential contour is crucial in setting the duration of action potentials in response to ß-adrenergic stimulation.

(6) Mechanism of the dantrolene-induced inhibition of ryanodine receptors

Ryanodine receptors are important determinants of calcium release from the sarcoplasmic reticulum, so they can effectively control cytosolic calcium concentration ($[Ca^{2+}]_i$), and thus the activity of all calcium-sensitive ion channels. Dantrolene is a ryanodine receptor (RyR) inhibitor, which is used to relax muscular structures when necessary. Although, dantrolene binds to the RyR protein, its mechanism of action is unknown, mainly because of the controversial data showing that dantrolene inhibited Ca^{2+}-release from intact cells and sarcoplasmic reticulum (SR) vesicles, but failed to inhibit single RyR channel currents in bilayers. Accordingly, it was concluded that an important factor for dantrolene`s action was lost during the purification procedure of RyR. Recently, Mg^{2+} was demonstrated to be the essential factor for dantrolene to inhibit Ca^{2+}-release. In Ca^{2+} release- and bilayer-experiments, using SR vesicles and solubilized channels, respectively, we have supported these results. Our Ca^{2+}-release experiments

demonstrated that the effect of dantrolene and Mg^{2+} was cooperative, and that ATP enhanced the inhibiting effect of the drug. Furthermore, RyR channel currents were found to be inhibited by 10 μM dantrolene in the presence of Mg^{2+} and ATP. The open probability of the channels decreased by 36.6±7%. Altogether, our data provide important complementary information that support the Mg^{2+}-dependent mechanism of dantrolene's action and suggest that dantrolene also requires ATP to inhibit RyR. We propose that RyR binds dantrolene only in a specific allosterically modified state.

(7) Calcium-dependent K^+ and Cl^- channels in epithelial cells

Primary fluid secretion in secretory epithelia relies on the unidirectional transport of ions and water across a single cell layer. This mechanism requires the asymmetric apico-basal distribution of ion transporters and intracellular Ca^{2+} signaling. The primary aim of the present study was to verify the localization and the identity of Ca^{2+}-dependent ion channels in acinar cells of the mouse lacrimal gland. Whole-cell patch-clamp-electrophysiology, spatially localized flash-photolysis of Ca^{2+} and temporally resolved digital Ca^{2+}-imaging was combined. Immunostaining of enzymatically isolated mouse lacrimal acinar cells was performed. We show that the Ca^{2+}-dependent K^+-conductance is paxilline-sensitive, abundant in the luminal, but negligible in the basal membrane; and co-localizes with Cl^--conductance. These data suggest that both Cl^- and K^+ are secreted into the lumen and thus they account for the high luminal [Cl^-] (~141 mM), but not for the relatively low [K^+] (<17 mM) of the primary fluid. Accordingly, these results also imply that K^+ must be reabsorbed from the primary tear fluid by the acinar cells. We hypothesized that apically-localized Na^+-K^+ pumps are responsible for K^+-reabsorption. To test this possibility, immunostaining of lacrimal acinar cells was performed using anti-Na^+-K^+ ATP-ase antibody. We found positive fluorescence signal not only in the basal, but in the apical membrane of acinar cells too. Based on these results we propose a new primary fluid-secretion model in the lacrimal gland, in which the paracellular pathway of Na^+ secretion is supplemented by a transcellular pathway driven by apical Na^+-K^+ pumps.

The plasma membrane of parotid acinar cells is functionally divided into apical and basolateral regions. According to the current model, fluid secretion is driven by transepithelial ion gradient, which facilitates water movement by osmosis into the acinar lumen from the interstitium. The osmotic gradient is created by the apical Cl^- efflux and the subsequent paracellular Na^+ transport. In this model, the Na^+-K^+ pump is located exclusively in the basolateral membrane and has essential role in salivary secretion, since the driving force for Cl^- transport via basolateral Na^+–K^+–$2Cl^-$ cotransport is generated by the Na^+-K^+ pump. In addition, the continuous electrochemical gradient for Cl^- flow during acinar cell stimulation is maintained by the basolateral K^+ efflux. However, using a combination of single-cell electrophysiology and Ca^{2+}-imaging, we demonstrated that photolysis of Ca^{2+} close to the apical membrane of parotid acinar cells triggered significant K^+ current, indicating that a substantial amount of K^+ is secreted into the lumen during stimulation. Nevertheless, the K^+ content of the primary saliva is relatively low, suggesting that K^+ might be reabsorbed through the apical membrane. Therefore, we investigated the localization of Na^+-K^+ pumps in acinar cells. We showed that the pumps appear evenly distributed throughout the whole plasma membrane, including the apical pole of the cell. Based on these results, a new mathematical model of salivary fluid

secretion is presented, where the pump reabsorbs K^+ from and secretes Na^+ to the lumen, which can partially supplement the paracellular Na^+ pathway.

(8) Inactivation kinetics of L-type calcium current as an indicator of submembrane calcium activity ?

Inactivation of L-type Ca^{2+} current ($I_{Ca\text{-}L}$) is known to be accelerated in response to elevation of intracellular Ca^{2+}, which can be – at least theoretically – used to determine submembrane Ca^{2+} concentration in the fuzzy space, which cannot be measured otherwise. This hypothesis was tested using L-type cardiac Ca^{2+} channels expressed in CHO cells, where the Ca^{2+}-dependence of Ca^{2+} channel inactivation was studied. In these experiments full assembly of Ca^{2+} channels, containing both the pore forming alpha-1 as well as the other auxiliary (beta and gamma) subunits were used. The monoexponential inactivation time constant was 62±15 ms at 10 µM intracellular Ca^{2+} (corresponding to the highest value estimated during systole) while it was 177±59 ms when studied in Ca^{2+}-free internal solution. This range of the decay time constant of L-type Ca^{2+} current, however, seems too small to be used as a measure of submembrane Ca^{2+} concentration.