Philosphy Doctor (Ph.D.) Thesis

Role of the $[Ca^{2+}]_i$-dependent currents in the cardiac arrhythmogenesis

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INTRODUCTION

The release of Ca^{2+} from internal stores is triggered by the action potential, which in turn is modulated by $[Ca^{2+}]_i$ in myocardial cells. Thus, the $[Ca^{2+}]_i$-dependent modulation of membrane voltage allows a feed-back regulation for the Ca^{2+} release. However, it may also function as substrate of arrhythmogenesis under pathological conditions. A major candidate in arrhythmogenesis is the spontaneous Ca^{2+} release from the sarcoplasmic reticulum, which can induce a depolarizing transient inward current (I_{TI}) at the resting membrane potential. The characterization of the various $[Ca^{2+}]_i$-dependent ionic fluxes of the surface cell membrane when Ca^{2+} release from the sarcoplasmic reticulum is altered, therefore, may contribute to our understanding of the regulation of myocardial contractility and of arrhythmias.

Ca^{2+} release from the sarcoplasmic reticulum may trigger or modulate several charge carrying mechanisms of the surface cell membrane. In certain cases the regulation of the gating process is governed exclusively by $[Ca^{2+}]_i$, thus the transmembrane voltage determines only the driving force of the ionic flux. Such gating properties were related to the $[Ca^{2+}]_i$-activated chloride current and to the $[Ca^{2+}]_i$-activated nonspecific cationic current. The estimation of the intensity of these currents during action potentials seems to be relatively straightforward provided $[Ca^{2+}]_i$ is known. In another group of membrane processes the situation is more complicated as $[Ca^{2+}]_i$, voltage and time have a compound effect on the conductance. Such regulation has been described for certain potassium channels, and for the L-type Ca^{2+}-channels. The conformational changes of the Na^+-Ca^{2+} exchanger molecules are not related to membrane voltage, however, their function still possesses a complicated voltage-dependence. This is due to the fact that during the action potential and the resultant $[Ca^{2+}]_i$ transient not only the voltage but the reversal potential of Ca^{2+} is markedly changed. This situation is further complicated by the limited available information about the subsarcolemmal Na^+ and Ca^{2+} concentrations. All of these factors make the reconstruction of $I_{Na/Ca}$ during the action potential speculative. Despite of these difficulties, however, the role of the Na^+-Ca^{2+} exchanger mechanism in myoplasmic Ca^{2+} removal as well as in Ca^{2+} entry have been firmly established. Moreover, the modulation of this mechanism by changes in external and internal Ca^{2+} concentrations as well as by changes in external Na^+ concentrations were described.

In situations of $[Ca^{2+}]_i$-overload, cardiac arrhythmias have been attributed to delayed afterdepolarizations, which are carried by a transient inward current I_{TI}. In the 1970s this current was extensively studied in cardiac Purkinje fibres. It was assumed that I_{TI} reflects at least two different membrane conductivities: (1) the Na^+/Ca^{2+}-exchanger and (2) a $[Ca^{2+}]_i$-dependent nonspecific cation channel. Membrane currents carried by the Na^+/Ca^{2+}-exchanger ($I_{Na/Ca}$) have been identified in a large variety of species and tissues, including human atrial myocytes. In the latter, $I_{Na/Ca}$ contributes to the duration of the action potential and to the generation of delayed afterdepolarizations.

The first description of a $[Ca^{2+}]_i$-dependent

nonselective cation conductance in cardiac cells was given by Kass et al. in 1978 for calf Purkinje fibres, which exhibited a depolarizing transient inward current under strophanthidin treatment. In 1981, Colquhoun et al. described a $[Ca^{2+}]_i$-dependent nonspecific cation channel, which could be identified on the single-channel level in cultured neonatal rat cardiomyocytes. In the following years similar channels were found in a large variety of species and tissues. Most of these channels exhibit a voltage-independent behaviour, ohmic conductance, poor selectivity among cations and a single channel conductance around 30 pS. Thus, it was assumed that $[Ca^{2+}]_i$-dependent nonspecific cation channels form a unique class of channels, called CAN-channels ($[Ca^{2+}]_i$-activated nonspecific), although different types seem to exist as well. In cardiac cells, this channel type has been identified in neonatal rat and adult canine and guinea-pig cardiomyocytes.

In human atrial myocytes, a $[Ca^{2+}]_i$-dependent and 4-aminopyridine (4-AP) resistant component of the transient outward current I_{to} has been identified and called brief outward current I_{bo}. This current exhibited properties, which suggest a dependence on sarcoplasmic reticulum Ca^{2+} release, since it could be inhibited by caffeine and the calcium channel blocking agent Co^{2+}. A similar component of I_{to} could be identified as a $[Ca^{2+}]_i$-dependent chloride current $I_{Cl(Ca)}$ in atrial, ventricular and Purkinje cells of rabbit and in canine ventricular cardiomyocytes. This current generated oscillatory membrane depolarizations in situations of $[Ca^{2+}]_i$-overload or isoproterenol treatment. The identification of $I_{Cl(Ca)}$ as a component of I_{to} in these species gave rise to the hypothesis, that I_{bo} in human atrium is believed to be a chloride-current as well. This hypothesis could not be confirmed in a later studies, where demonstrated that I_{to} in human atrial cells is completely suppressed when pipette K^+ is replaced by Cs^+ and that the remaining currents appear unaltered by $[Cl^-]_o$-substitution. Furthermore, the results of the latter study do not support a $[Ca^{2+}]_i$-dependence of I_{bo}.

$[Ca^{2+}]_i$-dependent currents in the heart may underlie arrhythmogenic mechanisms, such as delayed afterdepolarizations, in conditions of $[Ca^{2+}]_i$-overload. High levels of $[Ca^{2+}]_i$ may lead to spontaneous oscillatory release of Ca^{2+} from the sarcoplasmic reticulum (SR), which may then activate $[Ca^{2+}]_i$-dependent membrane currents. If such $[Ca^{2+}]_i$-oscillations are sufficiently synchronised during diastole these currents may initiate premature action potentials. Therefore, $[Ca^{2+}]_i$-dependent currents may reflect important arrhythmogenic mechanisms in a variety of pathophysiological conditions, which are associated with $[Ca^{2+}]_i$-overload, such as hypertrophy, ischemia and reperfusion, treatment with cardiac glycosides and catecholamines.

AIMS

Aims#1-3:

The main aims of this study was to identify $[Ca^{2+}]_i$-dependent membrane currents in the absence of Na^+/Ca^{2+}-exchange current and $[Ca^{2+}]_i$-dependent K^+-currents in human atrial *(Aim#1)* and ventricular *(Aim#2)* cardiomyocytes and in rabbit atrial, ventricular and Purkinje cardiac cells *(Aim#3)*.

Aims#4:

In this work we demonstrate a new method for the characterization of the various $[Ca^{2+}]_i$-dependent charge carrying processes contributing to the ventricular action potential. Current injections at constant rate resulted first in steady Ca^{2+} loading of the sarcoplasmic reticulum of Fura-2 dialysed isolated ventricular myocytes of the rabbit under whole cell current clamp. This was accompanied by the development of action potentials and $[Ca^{2+}]_i$ transients of constant shapes. Then a single rapid and brief application of caffeine enhanced and thereafter attenuated Ca^{2+} release from the sarcoplasmic reticulum. This protocol allowed the direct comparison of the steady state action potentials to those which were distorted by the enhanced or reduced activation of the various $[Ca^{2+}]_i$-dependent membrane processes in the same cells. Pharmacological interventions under these conditions and additional voltage clamp experiments were performed to discriminate among the $[Ca^{2+}]_i$-related mechanisms and to estimate their relative $[Ca^{2+}]_i$-sensitivity. The range of $[Ca^{2+}]_i$ change due to caffeine might be similar to certain pathological conditions (e.g. reperfusion, heart failure), therefore, our observations might also have pathophysiological significance. We demonstrate $I_{Ca,L}$ and I_{TI} as major mediators of the $[Ca^{2+}]_i$-induced changes in the action potential shape. Finally, our observations indicate relatively low $[Ca^{2+}]_i$-sensitivity of the Na^+-Ca^{2+} exchanger and, therefore, small alteration in $I_{Na/Ca}$ when Ca^{2+} release is modulated by caffeine.

METHODS

Cell isolation from rabbit heart

Single atrial, ventricular and Purkinje cells from rabbit heart were obtained by a method described briefly, New Zealand White rabbits of either sex, weighing 1·3-1·8 kg, were anaesthetized by I.V. administration of sodium pentobarbitone (40 mg (kg body weight)$^{-1}$). The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. Coronary thrombosis was prevented by simultaneous injection of heparin (1000 i.u. kg^{-1}). The heart was rapidly excised, placed in cold (3°C) normal Tyrode solution, mounted on a Langendorff apparatus and washed by retrograde perfusion with normal Tyrode solution at 37°C. The heart activity returned after 10-15 s and regularly 2 min of perfusion removed most of the blood from the coronary system. The heart was perfused with a nominally Ca^{2+}-free Tyrode solution for an additional 10 min and then with enzyme solution I (nominally Ca^{2+}-free modified Tyrode containing 0·2 mg ml^{-1} collagenase A) for 10

min and finally with enzyme solution II (nominally Ca^{2+}-free Tyrode containing 0·2 mg ml^{-1} collagenase A, plus 0·1 mg ml^{-1} protease Type XIV) for 10 min. All perfusion solutions were warmed to 37°C and saturated with 100 % O_2 prior to use. Thick strips (1-2 mm) were then dissected from both the left and the right auricles and from the papillary muscle of the left ventricle. The Purkinje fibres were then carefully dissected out. Muscle strips and Purkinje bundles were dissociated into single cells by incubating in enzyme solution II. Isolated cells were washed repeatedly with Tyrode solution containing 0·18 mM $CaCl_2$, and then resuspended in normal Tyrode solution and stored at room temperature (22-23°C).

Isolation of human atrial myocytes

All investigations on human tissues conform with the principles outlined in the Declaration of Helsinki and were approved by the ethics committee of the university of Cologne. Patients undergoing therapeutical operations gave informed consent prior to the operation.

Right atrial appendages were obtained from 46 patients undergoing open heart surgery. Samples were immersed immediately after excision in a cardioplegic solution, taken to the laboratory, and maintained at ≈ 4°C. Myocardial specimens were chopped with scissors into small chunks of ≤1 mm^3 and placed in flasks, containing 10 ml of a nominally Ca^{2+}-free modified Tyrode's solution. All steps were carried out at 37°C and continuous agitation during all steps of the isolation procedure was ensured by oxygen bubbling from the bottom of the flask. This washing procedure was performed for 5 min and repeated twice. Afterwards the tissue was incubated in 10 ml of a similar solution, containing 11 mg Worthington-collagenase type CLS II and 0.8 mg Protease Type XIV. After 30 min the tissue was filled into two separate flasks of 15 ml, each flask was filled with nominally Ca^{2+}-free modified Tyrode's solution, and centrifuged (500 *g* for 1 min). The supernatant was discarded and the remaining tissue was poured into a flask again. The flask was filled with nominally Ca^{2+}-free modified Tyrode's solution and the centrifugation procedure was repeated. After removal of the supernatant, the tissue was reincubated in nominally Ca^{2+}-free modified Tyrode's solution, containing 11 mg of Worthington-collagenase type CLS II. After 10–20 min (depending on cell yield and morphology), the cell suspension was washed and centrifuged in two steps as described before. Cells were stored in nominally Ca^{2+}-free modified Tyrode's solution at room temperature (≈20°C) and remained viable for hours. Cells were transferred from this medium directly to the examination chamber and superfused with 2 mM Ca^{2+} containing Tyrode's solution. Some cells were contracting spontaneously under these conditions and went over into irreversible contracture. However, only those cells which were not spontaneously contracting in the presence of $[Ca^{2+}]_o$ of 2 mM, which were typically shaped and without visible blebs on the surface under these conditions, were used for our experiments.

Isolation of human ventricular myocytes

Human left ventricular myocardium was

obtained from explanted hearts of 22 patients undergoing heart transplantation due to terminal heart failure (17 patients) or from donor hearts without heart failure (5 patients), which could not be transplanted for technical reasons. Explanted hearts were immersed in cardioplagic solution (≈4°C) and taken to the laboratory immediately after explantation. A part of the left ventricular wall (10–20 cm^2) was excised with its artery branch. The wall segment was perfused via this arterial branch for 30 min with a nominally Ca^{2+}-free modified Tyrode's solution to wash out blood and Ca^{2+}. All steps were performed at 37°C and solutions were oxygenated throughout the entire isolation procedure. After the washing phase the segment was perfused in a recirculating manner for 40 min with the same solution containing 25.5 mg/50 ml Worthington-collagenase type CLS II and 3 mg/50 ml protease type XIV. Afterwards, the enzyme was washed out for 15 min with modified Tyrode's solution containing 200 μM Ca^{2+}. Myocytes were taken from the midmyocardial region and suspended in modified Tyrode's solution containing 200 μM Ca^{2+}. After filtering this suspension through a nylon mesh, $[Ca^{2+}]$ was increased in 0.5 mM steps every 15 min up to a final concentration of 2 mM. The suspension was stored at room temperature (≈20°C) and cells remained viable for up to 5 h.

Current-clamp, voltage-clamp and $[Ca^{2+}]_i$ measurements

Cells were studied at room temperature with the whole-cell patch-clamp technique, using an Axopatch-1D amplifier. Pipettes were fabricated from thick-walled glass capillaries. Filled with the pipette solution tip resistances were typically between 1.2 and 3.4 MΩ. Stimulation and data acquisition were controlled by the pClamp 6.0.2. software. Currents were filtered at 2 kHz by a lowpass Bessel filter and were not leak corrected. Sampling frequency was 400 Hz, yielding an effective time resolution of 2.5 ms.

$[Ca^{2+}]_i$ was measured with a fluorescent indicator (100 mM K_5Fura-2). The experimental apparatus was built around an inverted microscope. Isolated cells were illuminated alternatively at two wavelengths (340 and 380 nm). The excitation wavelengths were selected by the dual monochromator system of the Deltascan-1 apparatus. The emitted fluorescence was collected from a restricted area of the visual field, set by a variable slit. This area typically covered the total two-dimensional image of the cell and thus provided an optical signal that reflected the average $[Ca^{2+}]_i$. The two-dimensional image of cells was continuously monitored using a video camera and a monochrome monitor. The intensity of the emitted fluorescence was measured by a photomultiplier and was not filtered. Background fluorescence was assessed in cell-attached mode prior to the establishment of the whole-cell configuration and was subtracted from the emitted fluorescence for both 340 nm and 380 nm excitations. Data acquisition of fluorescence was controlled by the OSCAR software. Sampling frequency was 200 Hz.

Electrophysiological measurements of human cells were performed in the whole-cell-mode of the patch-clamp technique using a patch-clamp amplifier (EPC 7) with a 100-MΩ feedback resistor. Microelectrodes were fabricated from borosilicate

glass capillaries and had resistances of 1.5–3.5 and 3.5–5.0 MΩ for ventricular and atrial cells, respectively. The experimental apparatus was constructed around a Zeiss Axiovert 35 inverted microscope with a photometer attachment. For fluorescence recordings, ultraviolet light emitted from a 75 W xenon arc lamp passed through 10 nm interference filters (340 or 380 nm wavelengths) and was reflected into the objective. Fluorescence emitted from the cell passed through a 510–540 nm bandpass filter to the photomultiplier tube. Fluorescence and current signals were digitised and stored on a personal computer for off-line analysis (sampling rate 100–200 Hz). Membrane capacitance was determined in each cell by integrating the capacitance current upon repolarizing to -80 mV from hyperpolarizing pulses.

Experimental protocols

Cells were continuously superfused by a perfusion system positioned close (100 μm) to the cells that offered an effective exchange time of 500 ms. In order to obtain an estimate about the maximal available $[Ca^{2+}]_i$-sensitive membrane conductance, large Ca^{2+} transients were achieved by brief (300 ms) applications of caffeine. Brief caffeine pulses elevate $[Ca^{2+}]_i$ to high levels for several hundreds of milliseconds in a spatially homogeneous manner providing steady $[Ca^{2+}]_i$-dependent channel activation. Caffeine-evoked Ca^{2+} transients regularly peaked above 1 μM free Ca^{2+} concentration and were accompanied by visible contractures. Instantaneous current-voltage relationships at such relatively constant levels of channel activation were assessed by short (200 ms) voltage ramps (0·7 V s^{-1}) in the range of -60 to +80 mV. Voltage ramps were applied before (R_1), during (R_2) and after (R_3) current transients in all cell types. Caffeine pulses (300 ms) were given between the first and the second voltage ramps. Membrane currents during the first and the third voltage ramps (R_1, R_3) were considered as controls characterizing background currents. In those instances, subtraction of background current (recorded during R_1 or R_3) from the current seen during R_2 provided the current-voltage relation for the pure $[Ca^{2+}]_i$-dependent current.

Junction potentials around the pipette tip were zeroed before formation of the membrane-pipette seal in normal Tyrode solution. After dialysing the intracellular space, the original solution interface at the pipette tip vanishes, and as a result of the original compensation a new tip potential develops. Junction potential measurements indicated that the error due to this effect was less than 2 mV.

In the action potential measurements fifteen consecutive action potentials (AP_1-AP_{15}) were evoked by brief current injections using the whole-cell current clamp at 0.2 Hz stimulation. Sarcoplasmic Ca^{2+}-loading and action potential configuration reached steady state during the first 3-5 action potentials. Cells were continuously superfused by a home made perfusion system positioned close (<100 μm) to the cells which offered an effective exchange time of 500 ms. In order to obtain an estimate about the maximal $[Ca^{2+}]_i$-dependent effect on the plateau of the steady state action potential, large $[Ca^{2+}]_i$ transients were evoked by brief (300 ms) applications of caffeine

during the early plateau phase of AP_9. This elevated $[Ca^{2+}]_i$ to high levels for several hundreds of milliseconds in a spatially homogenous manner. Confirmingly, caffeine-evoked $[Ca^{2+}]_i$ transients were accompanied by stronger visible contractures during AP_9 than those evoked by the action potentials before (e.g. AP_8) or after the caffeine application (AP_{10}). Moreover, contractions during AP_{10} were regularly missing, indicating intracellular Ca^{2+} depletion. This allowed the evaluation of the effect of increased, as well as decreased $[Ca^{2+}]_i$ transients on action potential configuration within a short period of time while the $[Ca^{2+}]_i$-independent membrane processes did not change significantly. Action potential durations during large (AP_9) and small $[Ca^{2+}]_i$ transients (AP_{10}) were normalized to those obtained under steady-state (AP_8) at various levels of repolarization to illustrate the effects of the $[Ca^{2+}]_i$-dependent conductances.

Data acquisition

Values are represented as mean ± S.E.. The number of different cells examined under identical conditions is indicated by "n". Significance of differences was tested by the paired t-test and when appropriate by the one way ANOVA test.

RESULTS

Activation of transient inward membrane currents by application of caffeine in rabbit atrial and ventricular cells

$[Ca^{2+}]_i$-activated transient currents have previously been explored in Purkinje cells. This work demonstrated two current peaks during caffeine-induced Ca^{2+} transients and both were attributed to $[Ca^{2+}]_i$-activated Cl^- currents. We illustrated a typical transient inward membrane currents following transient caffeine applications in isolated rabbit atrial myocytes in NMDG chloride extracellular solution. The membrane potential was held at -60 mV and caffeine (10 mM) was applied for 300 ms. Ventricular cells exhibited currents similar to those seen in atrial cells following caffeine application. These experiments confirmed the existence of I_{TI} in all three cell types. However, the amplitudes and kinetics of the membrane currents varied with cell type. Atrial cells gave peak current densities between -1·86 and -22·79 pA pF^{-1} with a mean value of -12·3 pA pF^{-1}. In ventricular cells, the peak current densities fell between -0·28 and -7·82 pA pF^{-1} with a mean of -4·9 pA pF^{-1}, whereas in Purkinje cells they ranged between -2·78 and -41·52 pA pF^{-1} with a mean value of -14·7 pA pF^{-1}. In contrast to the distinct, early peak (I_{fast}) in Purkinje cells, atrial and ventricular cells showed only one current peak at maximal $[Ca^{2+}]_i$. The duration of current transients varied as did the Ca^{2+} transients even within the same cell. Currents generally lasted longer when $[Ca^{2+}]_i$ remained high for longer periods. Current activation overlapped the rise in $[Ca^{2+}]_i$, but transient currents generally declined faster than the Ca^{2+} transients.

Current-voltage analysis of $[Ca^{2+}]_i$-activated current in rabbit cardiomyocytes

The experiments that followed investigated the charge carriers of the $[Ca^{2+}]_i$-dependent current. The I_{TI} persisted despite the removal of Na^+, I_K, and $I_{Ca,L}$. In our experimental conditions limited the potential charge carriers to Cl^- or Cs^+. It was necessary, therefore, to distinguish between permeation through $[Ca^{2+}]_i$-activated Cl^- channels, and the passage of monovalent cations through $[Ca^{2+}]_i$-activated non-specific channels. For a non-specific cation channel one would expect outward currents at all membrane potentials tested, due to the known Cs^+ permeability of such a channel, while $NMDG^+$ is known to be membrane impermeant.

The pure $[Ca^{2+}]_i$-dependent current had linear current-voltage characteristics and reversed at +12 mV when Cl^- was distributed symmetrically (E_{Cl} = -1 mV). This excluded any significant contribution of non-specific cation conductances to these currents. Then an asymmetrical Cl^- distribution (E_{Cl} = +42 mV) was used achieved by partially replacing Cl^- by $glutamate^-$. This manoeuvre shifted the reversal potential of the transient current to +27 mV. Our data indicates similar shifts in the reversal potentials (E_{rev}) in all cell types examined. The results of ramp experiments confirm a current-voltage analysis during caffeine-induced $[Ca^{2+}]_i$ transients for the Purkinje cell and additionally demonstrate that such currents are also related to the activation of a $[Ca^{2+}]_i$-sensitive pore which is highly permeable for Cl^- in atrial and ventricular cells of the rabbit heart.

Possible contribution of the Na^+- Ca^{2+} exchanger mechanism to the $[Ca^{2+}]_i$-activated current in rabbit cardiomyocytes

Subsequent studies were aimed at elucidating the possible role of the Na^+- Ca^{2+} exchanger as a charge carrier system in the generation of the transient current in isolated rabbit heart cells during Ca^{2+} transients. These studies examined the behaviour of the $[Ca^{2+}]_i$-activated membrane current in extracellular solutions containing Na^+ and compared the reversal potentials with those obtained in Na^+-free media.

The pure $[Ca^{2+}]_i$-dependent current reversed at +6·5 mV with a symmetrical Cl^- distribution, similar to results obtained when Na^+ was omitted from the extracellular space. When the extracellular Cl^- was partially replaced by $glutamate^-$ the current reversal was again shifted towards the new Cl^- equilibrium potential to +28 mV. Statistical comparison of reversal potentials obtained in Na^+-containing and Na^+-free solutions failed to show significant differences (P < 0·05) in any given cell type. These findings were corroborated by trials in the atrial cells which included the Na^+- Ca^{2+} exchange blocker Ni^{2+} (5 μM) in the extracellular solutions containing NMDG chloride or NMDG glutamate. This preserved both the time

course of the current and its reversal potential. These results exclude any major contributions of Na^+- Ca^{2+} exchange to the transient current that is produced by caffeine-induced Ca^{2+} transients in rabbit heart cells. The reversal potentials of the transient currents were not significantly different (P < 0·05) in atrial, ventricular and Purkinje cells, and hence we conclude that $[Ca^{2+}]_i$-activated currents seen in different types of cardiomyocytes are carried mainly by Cl^-. Nevertheless, Na^+- Ca^{2+} exchange did exist in these cells, because in the presence of extracellular Na^+ the slope of the declining phase of Ca^{2+} transients showed clear voltage dependence.

The experimental error introduced by liquid junction potentials was less than 5 mV. Thus, the present study may indicate a small permeability of the $I_{Cl(Ca)}$ to glutamate. This may also explain the difference between the calculated and measured reversal potentials. Assuming independence of ion movements the maximal hypothetical relative glutamate$^-$ permeability of the $[Ca^{2+}]_i$-dependent pore can be estimated using the Goldman-Hodgkin-Katz voltage equation. On average, E_{rev} was -16 mV in the atrial cells, -13 mV in ventricular cardiomyocytes and -14 mV in Purkinje cells. The calculated relative permeabilities ($P_{glutamate}/P_{Cl}$) were 0·423 in atrial cells, 0·505 in ventricular cells and 0·476 in Purkinje cells.

The effect of anthracene-9-carboxylic acid on the $[Ca^{2+}]_i$-activated current in rabbit cardiomyocytes

Anthracene-9-carboxylic acid (hereafter referred to as anthracene) exerted a major influence on $[Ca^{2+}]_i$-dependent membrane currents recorded in NMDG chloride solution. Anthracene markedly reduced the outward currents. The inward currents at -60 mV right after the voltage ramps were not significantly different in the presence and absence of anthracene. This indicates that the currents were similarly activated, and consequently the activating Ca^{2+} signals had to be similar, too. The reduction of outward currents, therefore, reflects the voltage-dependent blockade by anthracene of the transient current. Similar effects of anthracene were seen in four ventricular cells and in one Purkinje cell. The effect of anthracene was fully reversible in all cell types examined. Anthracene reduced the mean current amplitude by 90 % at +80 mV, did not affect the reversal potential and slightly decreased the inward currents. Stilbene derivatives are considered to be potent inhibitors of the $[Ca^{2+}]_i$-activated Cl^- current, although in some reports the blockade caused by DIDS (0·1 mM) was not complete. In our experiments, SITS (2 mM) and DIDS (0·1 mM) had no effect on current rectification and it was practically impossible to distinguish whether the occasional variations in current amplitudes were due to an incomplete blockade caused by the drug or were related to the concomitant changes in activating $[Ca^{2+}]_i$.

Caffeine activates a transient membrane current in the absence of Na^+ and K^+ in human atrial cardiomyocytes

In all experiments in human cardiac cells, test

pulses were preceded by five depolarizing prepulses from -60 to +10 mV (1 Hz) in order to load the SR with Ca^{2+} to a similar extent.

To identify contaminating voltage dependent currents, experiments were performed in the absence of caffeine. Sustained outward currents could be seen during the test potentials, but no transient currents. When caffeine (10 mM) was applied for 250 ms, beginning 250 ms after clamping the cell to the test potential a transient currents were activated ~150 ms after onset of the caffeine application with a reversal potential near 0 mV. Digital subtraction of the control-registration from the pulse after addition of caffeine yielded the caffeine-induced currents. Similar currents could be observed in a total number of 46 from 81 cells, using either lithium chloride or cesium chloride solutions and caffeine concentrations of 1, 2.5, 5 and 10 mM. The mean reversal potential for 13 cells tested with either Li^+ or Cs^+ as major intra- and extracellular cations was -7.1 mV and -3.3 mV, respectively, when using test potentials from -60 to +60 mV and 10 mM caffeine. The mean current density at -60 mV (-1.13 pA/pF for Li^+ and -0.66 pA/pF for Cs^+) exhibited a remarkable variability. Currents of this type could never be observed without caffeine application.

Caffeine-induced current is $[Ca^{2+}]_i$-dependent in human atrial cardiomyocytes

Since it is known that caffeine activates a non-specific cation channel in smooth muscle cells of *Bufo marinus*, we investigated whether the caffeine-induced current is activated by caffeine directly or by SR-Ca^{2+} release.

The effect of rapid caffeine application in the presence of 10 mM EGTA in the pipette solution was examined shortly after a whole-cell-patch was established. Under these conditions, caffeine failed to induce a contraction of the cell (judged by visual control) and no transient currents after caffeine application could be observed. Similar results were obtained in all cells tested under these conditions. In these experiments, Ca^{2+}-currents were very prominent, compared with those in absence of EGTA.

Ionic nature of the $[Ca^{2+}]_i$-dependent non-specific current

In the absence of Na^+ and K^+, $[Ca^{2+}]_i$-dependent currents may represent $[Ca^{2+}]_i$-dependent chloride currents or $[Ca^{2+}]_i$-dependent non-specific cation currents. Since $[Ca^{2+}]_i$-dependent chloride currents have been described in atrial, ventricular and Purkinje cells of rabbits and in canine ventricular cardiomyocytes, it has been postulated that the 4-AP-resistant component of the transient outward current in human atrial myocytes, called I_{bo} by Escande et al., may reflect $I_{Cl(Ca)}$. In our study, we tested the contribution of chloride to the $[Ca^{2+}]_i$-dependent current by using different intra- and extracellular chloride concentrations. Membrane currents were tested using either CsCl pipette solution with (1) NMDG-Cl, (2) NMDG-glutamate extracellular solutions, or (3) Cs-glutamate pipette solution with NMDG-Cl extracellular solution. According to the

Nernst equation, the chloride-reversal-potentials E_{Cl}^- were calculated as: (1) -1 mV; (2) +41 mV; (3) -46 mV. With respect to the calculated reversal potentials, test potentials ranged from -60 to +60 mV. Our data illustrates three representative results, showing no clear reversal of the observed currents. A reversal of the current might be expected only negative of -60 mV, since small outward currents can be observed even at -60 mV. Therefore, it must be concluded that the $[Ca^{2+}]_i$-dependent current in human atrial myocytes is not carried by chloride. Since Cs^+ and Li^+ appeared to be permeable, as demonstrated us, a non-specific cation current must be assumed. Furthermore, $NMDG^+$ seems to be impermeable. We tested the permeability for $NMDG^+$ by switching the extracellular solution to a 110 mM $NMDG^+$ containing solution, after it had been demonstrated that inward currents were observed under experimental conditions described for our Li^+- and Cs^+-experiments. In 14 cells, no inward currents could be seen, after the extracellular solutions had been replaced by NMDG-glutamate solution. Caffeine-induced currents, carried by either Cs^+ or Li^+, were identical. Absence of transient currents in the presence of $NMDG^+$ could be demonstrated upon hyperpolarization to -100 mV, indicating a permeability ratio for $NMDG^+$ in comparison with Li^+ of <0.019, according to the Goldman–Hodgkin–Katz equation. These results suggest complete non-permeability for $NMDG^+$, which is in accordance with previous studies on CAN-channels. Since pipette solutions as well as extracellular solutions contained 20 mM TEA^+, inward currents carried by this cation could be expected at negative potentials, if any permeability is assumed.

In conclusion, the ionic permeability of the $[Ca^{2+}]_i$-dependent current indicates that this current is a non-specific cation current and will be referred to as I_{CAN} in the following sections.

Does I_{CAN} represent a non-selective conductivity through $[Ca^{2+}]_i$-dependent [SK]-K^+-channels in human atrial cardiomyocytes?

$[Ca^{2+}]_i$-dependent K^+ channels represent an ubiquitous channel type, abundant in a large variety of mammalian and non-mammalian tissues. In single channel experiments two types of $[Ca^{2+}]_i$-dependent K^+ channels could be identified: (1) channels with high conductivity of 200–300 pS, called [BK]-channels and (2) channels with low conductivity of 10–14 pS, called [SK]-channels. The former type can be blocked by TEA^+ from outside in concentrations of 0.1–1 mM and from inside in concentrations of 50–100 mM. The bee venom polypeptide apamin has no effect. The latter type is TEA^+-resistant, but can be blocked with high selectivity by apamin.

Since all intra- and extracellular solutions in the present study contained 20 mM TEA^+, it can be assumed that $[Ca^{2+}]_i$-dependent [BK]-K^+ channels, if present, were reliably blocked. However, a non-selective conductivity through TEA^+-resistant [SK]-channels could not be excluded, although it had not been described for this channel type. We tested this

hypothesis by using apamin as a tool for a selective blockade. Caffeine was used at a low concentration (1 mM) to allow for longer experiments. Apamin was dissolved in modified Tyrode's solution to a final concentration of 1 μM and was applied by the fast solution-exchange system. Caffeine induced I_{CAN} even after 15 min of exposition to apamin and no obvious decrease in current amplitude could be observed. In summary, four cells from three different preparations revealed similar results after 5 min (1 cell), 10 min (2 cells) and 15 min (1 cell) of incubation. These results indicate, that I_{CAN} is not carried by $[Ca^{2+}]_i$-dependent-[BK]-K^+ channels.

Human ventricular cardiomyocytes

For the following experiments, human ventricular cardiomyocytes from the midmyocardial region of the left ventricle were chosen. According to the permeability of Cs^+ and Li^+ to the $[Ca^{2+}]_i$-dependent non-specific cation channel in atrial cells, CsCl solution or LiCl solution was used as pipette and extracellular solutions. There was no transient currents could be observed after caffeine application. To test whether this feature may be due to the inability of caffeine to induce a sufficient $[Ca^{2+}]_i$-transient in these cells, we performed fluorescence measurements with fura-2 at 340 and 380 nm excitation wavelengths. Although caffeine induced a $[Ca^{2+}]_i$-transient, it failed to induce I_{CAN} at potentials where this current should be large with respect to the expected reversal potential of 0 mV.

The effect of $[Ca^{2+}]_i$ on the shape of the action potential in rabbit ventricular cells

The role of $[Ca^{2+}]_i$ as a modulator of the action potential shape has been investigated in isolated ventricular myocytes of the rabbit under whole-cell current clamped conditions. Ca^{2+} release from the sarcoplasmic reticulum was modulated by caffeine application which resulted in the enhancement of the $[Ca^{2+}]_i$ transient during action potential. The caffeine induced increase in $[Ca^{2+}]_i$ was previously used to characterize the transient inward current (I_{TI}) known to induce arrhtyhmogenic depolarization at the resting membrane potential. Such transient depolarizations, or even the development of extrasytoles could be noticed when caffeine was applied during the diastolic periods of electrically paced ventricular myocytes. These observations demonstrated the presence of the $[Ca^{2+}]_i$-dependent I_{TI} under our experimental conditions.

In this study we primarily focused on the $[Ca^{2+}]_i$-dependent modulation of the action potential plateau where $[Ca^{2+}]_i$-dependent alterations of voltage-independent (i.e. I_{TI}) as well as voltage-dependent mechanisms are expected to interact. Thus, next the caffeine application was paired to the electrical stimulation.

Caffeine induced alterations of action potential configuration in rabbit ventricular cells

Using our standard pipette solution (containing 10 mM Na^+) and normal Tyrode extracellular solutions action potentials (AP_{1-15}) and $[Ca^{2+}]_i$ transients were routinely elicited 15 times by brief (3 ms) suprathreshold (1500 pA) depolarizing current injections at a constant pacing frequency (0.2 Hz). Under these conditions three to five consecutive

stimulations were required to achieve steady state Ca^{2+} loading of the sarcoplasmic reticulum and steady state action potential configuration. During the plateau of AP_9 rapid and brief application (300 ms) of extracellular caffeine (10 mM) induced a larger and longer lasting $[Ca^{2+}]_i$ transient than those evoked by the preceding action potential (i.e. AP_8). Therefore, distortions in the configuration of AP_9 reflected the potential modulatory effect of raised $[Ca^{2+}]_i$ on action potential configuration. After caffeine application Ca^{2+} stores became partially depleted, as it was indicated by the reduction of the amplitudes of $[Ca^{2+}]_i$ transients during AP. Thus, alterations during AP_{10} reflected the effect of reduced $[Ca^{2+}]_i$ on action potential shape. Steady state $[Ca^{2+}]_i$ transients and action potentials returned gradually after the perturbation induced by caffeine at the end of the action potential train. $[Ca^{2+}]_i$ transients enhanced by caffeine increased the rate of the repolarization and depressed the plateau. This was characterized by measuring the action potential durations (APD) at different levels of repolarization of AP_9. The reductions of AP_9 durations to $66\,\% \pm 6$, $70\,\% \pm 5$, and $76\,\% \pm 4$ at 30 %, 50 % and 90 % levels of repolarization, respectively using the steady state action potential (AP_8) as the reference (100 %). In contrast, $[Ca^{2+}]_i$ transients that were smaller than steady state ones were accompanied by elongated action potentials (AP_{10}-AP_{12}). To demonstrate this elongation, durations of AP_{10} were also determined at the same levels of repolarization. Results of this increases in APD to $160 \pm 10\,\%$, $142 \pm 9\,\%$ and $134 \pm 7\,\%$ at 30 %, 50 % and 90 % levels of repolarization, respectively. All of these action potential durations both during AP_9 and AP_{10} were significantly different from those during AP_8 ($p < 0.001$).

The faster repolarization during large $[Ca^{2+}]_i$ transient can be either the consequence of the increased outward currents or of the reduction of inward currents, whereas the prolongation of the plateau during attenuated $[Ca^{2+}]_i$ transients display the dominance of inward currents during the plateau of cardiac action potentials. In the next experiments we studied whether $[Ca^{2+}]_i$-dependent modulation of the L-type Ca^{2+} current can be correlated to the observed changes of action potential shape.

Caffeine induced alterations in L-type Ca^{2+} current in rabbit ventricular cells

In order to obtain an estimate about the $[Ca^{2+}]_i$-dependent modulation of the L-type Ca^{2+} current during the action potentials membrane currents of the same cells were also studied under whole-cell voltage clamp in the presence of identical ionic conditions. In these cases a train of 15 depolarizing voltage clamp steps from a -45 mV holding level to +10 mV with durations of 800 ms and at a frequency of 0.2 Hz were applied. This resulted in steady state Ca^{2+} loading of the sarcoplasmic reticulum and in reproducible inward membrane currents following three to five depolarizations. The amplitude of the inward current, which was defined as the difference between the early peak and the steady current level at the end of the depolarizing voltage step, reflected mostly $I_{Ca,L}$. $I_{Ca,L(8)}$ was considered as the steady state Ca^{2+} current, whereas during $I_{Ca,L(9)}$ brief caffeine application elevated $[Ca^{2+}]_i$ similarly to the previously performed experiments. $I_{Ca,L(9)}$ was greatly reduced by caffeine application, but $I_{Ca,L(10)}$ became larger than $I_{Ca,L(8)}$. The reduction in current amplitude during the 9th episode

accounted for a decrease to the 12 ± 2 % (n=8) of $I_{Ca,L(8)}$, whereas the increase during $I_{Ca,L(10)}$ reflected 122 ± 2 % (n=11) on the same relative scale. These changes in $I_{Ca,L}$ are consistent with both the shortening and the lengthening of the action potential during enhanced and depressed $[Ca^{2+}]_i$ transients. However, additional mechanisms cannot be excluded at this point. It is worth noting, for example, that an inward current was also induced by the caffeine application (immediately after the onset of caffeine application) at the -45 mV level during the 9th voltage clamp sweep. Moreover, the steady current level of $I_{Ca(9)}$ at +10 mV was more positive than that of $I_{Ca(8)}$ and $I_{Ca(10)}$. In the following experiments, therefore, the role of this caffeine induced transient current (most probably I_{TI}) was further investigated.

The effect of I_{TI} on the action potential plateau in the absence of $I_{Ca,L}$

To isolate the action potential modulatory role of I_{TI} from that of the Ca^{2+} current, $I_{Ca,L}$ was blocked by 500 µM Cd^{2+} in a different set of current-clamp experiments. First the complete blockade of $I_{Ca,L}$ by Cd^{2+} was verified using voltage-clamp depolarizations and thereafter the caffeine effects were further analysed under voltage and current clamp conditions. Five hundred µM Cd^{2+} abolished $I_{Ca,L}$ and prevented the enhancement of the inward membrane current upon depolarization that followed the caffeine application. This is illustrated by the similar membrane currents during the 8th and 10th voltage steps indicating that the late effects of caffeine on $I_{Ca,L}$ were not complicated by I_{TI}. Moreover, this finding also implied that the late effects of caffeine on the action potential configuration are probably mediated mainly by $I_{Ca,L}$. However, deflections of membrane currents to the outward (at +10 mV) as well as to the inward (at -45 mV) directions during caffeine application (episode No. 9) confirmed that the early caffeine effect indeed involved I_{TI}.

Elimination of $I_{Ca,L}$ by Cd^{2+} reduced the steady state action potential duration at the 90% repolarization level from 675 ± 64 ms to 389 ± 55 ms, while the resting membrane potential was -82 ± 2 mV in the absence and -80 ± 2 mV in the presence of 500 µM Cd^{2+}. While the change in APD90 was significantly different ($p = 0.022$), the resting membrane potential was not affected ($p = 0.381$). Although $I_{Ca,L}$ did not contribute to these action potentials, caffeine enhanced $[Ca^{2+}]_i$ transients could still induce shortening of the action potential. This shortening was not prominent at the 30 % level of the repolarization most probably because of the diminished plateau. However, at the 50 % and 90 % levels of repolarization the relative changes were about the same as under control conditions. The caffeine effect was restricted to $AP_{(9)}$, because no lengthening of the action potential occurred during $AP_{(10)}$ in the absence of $I_{Ca,L}$. This is also illustrated by the relative APD values of 97.5 ± 2, 101 ± 5 and 100 ± 5 at the 30 %, 50 % and 90 % levels of repolarizations, respectively. These experiments confirm the contribution of I_{TI} to the action potential shortening during large $[Ca^{2+}]_i$ transients, and underline the significance of $I_{Ca,L}$ in the determination of action potential configuration at relatively low $[Ca^{2+}]_i$.

The effects of altered Na^+ gradients on the caffeine induced changes of action potential configuration in

rabbit ventricular cells

The contribution of the currents generated by the electrogenic Na^+-Ca^{2+} exchanger ($I_{Na/Ca}$) to the action potential has been demonstrated under various experimental conditions. In further experiments, therefore, the Na^+-Ca^{2+} exchange function was modulated by changing the transmembrane Na^+ gradient. To test the significance of $I_{Na/Ca}$ in $[Ca^{2+}]_i$-dependent action potential modulation, predicted changes of $I_{Na/Ca}$ were compared to the measured caffeine induced distortions of action potential configuration.

When 67 mM extracellular Na^+ was substituted by equimolar Li^+, the substitution shifted the reversal potential of the Na^+-Ca^{2+} exchanger towards more negative potentials. This change, therefore, decreased the inward $I_{Na/Ca}$ and increased the probability of the outward current generating reverse function of the Na^+-Ca^{2+} exchanger. In accordance the duration of the steady state action potential decreased from 442 ± 40 ms to 247 ± 22 ms at the 90 % level of repolarization.

The caffeine induced reduction in the action potential duration of AP_9, however, could not be facilitated by the partial replacement of extracellular Na^+ with Li^+. On the contrary, relative APD values of AP_9 were significantly higher ($p < 0.045$) at all levels of repolarization in the presence of reduced Na^+ gradient than under control conditions. On the other hand, no difference ($p = 0.055$) was found between the relative APD(30) values and small, but significant ($p < 0.04$) differences were found at the APD(50) and APD(90) levels in the presence of high and low extracellular Na^+ concentrations during AP_{10}. Partial replacement of extracellular Na^+ by Li^+ decreased the resting membrane potential from -82 ± 1 mV to -74 ± 2 mV.

In an additional set of experiments Na^+ concentration was reduced to zero in the pipette solution while extracellular Na^+ concentration was kept at 137 mM. Reducing intracellular Na^+ concentration promoted inward $I_{Na/Ca}$ and inhibited the reverse mode of the Na^+-Ca^{2+} exchanger. Accordingly, this increased the APD(90) values of the steady-state action potentials to 1013 ± 129 ms while the resting membrane potential was -79 ± 2 mV. However, omission of Na^+ from the pipette solution did not prevent the caffeine induced early action potential shortening as that was demonstrated by the relative (APD(30) and APD(50)) values of AP_9. Moreover, the relative changes at these levels of repolarization were about the same when Na^+ was present or missing from the pipette solution. The effect of caffeine at more polarized membrane potentials was variable. In some cells the action potential shortening was still prevalent at the level of 90 % repolarization, whereas in some others lengthening was observed. In a third group of cells repeating the protocol resulted in either a decrease or an increase of APD(90). This explains the relatively high scatter of the APD(90) values of AP_9 in the absence of intracellular Na^+. Despite of this scatter the mean value of this relative APD(90) was significantly higher ($p < 0.036$) than in the presence of intracellular Na^+. Similarly to the experiments when external Na^+ was partially substituted, lengthening of AP_{10} in the absence of internal Na^+ was only slightly altered.

In conclusion, changes in the steady state action potential duration were consistent with the predicted alterations of $I_{Na/Ca}$. Based on the comparison between

relative APD changes during altered Na^+ gradients and relative APD changes under control conditions, however, we could not relate significant role to $I_{Na/Ca}$ in the $[Ca^{2+}]_i$-dependent distortions of the plateau phase of the action potential in rabbit ventricular myocytes. Experiments with increased Na^+ gradient, however, did not exclude that the late repolarization processes were influenced by $I_{Na/Ca}$ during large $[Ca^{2+}]_i$ transients.

DISCUSSION

Transient inward current in rabbit cardiomyocytes

Our study demonstrates that Cl^- is the dominant charge carrier of the transient inward currents in isolated rabbit atrial, ventricular and Purkinje cells. $[Ca^{2+}]_i$-dependent currents in these cell types seen during brief applications of external caffeine or spontaneous activity had relatively long durations and variable, although mostly large, amplitudes. The experiments thus extend earlier studies which demonstrated transient currents and their charge carriers in Purkinje fibres and ventricular myocytes. The behaviour of current reversal in different experimental solutions makes it likely that the most significant charge carrier of I_{TI} is Cl^-. This finding has been also supported by the blocking effect of the known Cl^- channel blocker, anthracene-9-carboxylic acid. The unchanged reversal potentials in the presence or absence of Na^+ indicate that a significant contribution by the Na^+- Ca^{2+} exchanger or by the non-specific cation channel can be excluded.

Transient membrane currents of atrial, ventricular and Purkinje cells were provoked by caffeine to mobilize the Ca^{2+} available for release from their intracellular stores at different membrane potentials. Ca^{2+} transients were accompanied by transient inward currents at negative holding potentials in all cell types examined and the characteristics of the current were also very similar in the three cell types. Experimental conditions in general were designed to exclude voltage-dependent currents and K^+ as charge carriers. Our method of applying short voltage ramps had the advantage of testing current-voltage relations when the $[Ca^{2+}]_i$-dependent conductance of the surface membrane did not change significantly, since $[Ca^{2+}]_i$ fell steadily and slowly after reaching a peak. It is also likely that $[Ca^{2+}]_i$ was distributed homogeneously in the myoplasmic space after reaching its maximum. The application of bracketing voltage ramps before and after our test ramps characterized background currents, and pointed to the stationary state of our preparations. Such a method is appropriate for the characterization of instantaneous current-voltage relations of ligand-gated channels which show no significant time dependence in their gating.

The reversal potential of the transient current moved in the direction of the change of the equilibrium potential for Cl^-. However, the reversal potential of the transient current was somewhat different from that predicted from the Nernst equation. Such a difference might arise from an experimental error originating from a junction potential between solution interfaces, surface charge effects, or incomplete cell dialysis, or might be related to

incomplete channel selectivity. With our experimental protocol the error introduced by junction potentials was small. On the other hand, large relative permeabilities for Cl^- substitutes have been reported for several anion channels, and therefore the possibility of incomplete channel selectivity cannot be excluded. In a recent study, poor Cl^- selectivity of the $[Ca^{2+}]_i$-activated transient outward current has also been observed in rabbit ventricular myocytes.

Non-selective cation currents have often been implicated in arrhythmogenesis of Ca^{2+}-overloaded myocardial cells, as has the Na^+- Ca^{2+} exchange mechanism. Non-selective cation currents have been described mainly in single channel experiments but they have also been demonstrated in multicellular preparations, where direct control of the composition of intra- and extracellular spaces was difficult. In the present study, where such technical limitations are less likely to be encountered, we were unable to demonstrate the activation of a non-specific cationic membrane conductance.

Based on voltage- and Na^+-dependent properties, previous experimental studies have demonstrated the involvement of the Na^+- Ca^{2+} exchange in I_{TI} generation in the hearts of chicks, guinea-pigs and rats. The kinetics of this current seemed to follow $[Ca^{2+}]_i$ kinetics in an almost linear fashion, and current amplitudes were found to be variable, although they were regularly less than 300 pA at negative holding potentials. The Na^+- Ca^{2+} exchanger has been shown to be significant in the handling of myoplasmic Ca^{2+} in guinea-pigs and rabbits. In rabbits, however, the arrhythmogenic role of this mechanism has not been clearly demonstrated. In addition, the distinct nature of I_{TI} and the Na^+- Ca^{2+} exchange mechanism in rabbit ventricular cells has been suggested by Giles & Shimoni. In contrast, in the same preparation, Laflamme & Becker demonstrated inward currents during spontaneous $[Ca^{2+}]_i$ oscillations when Cl^- was absent from both the external and the internal solutions. This current had no apparent reversal, had an amplitude of less than 120 pA at all membrane potentials and was related to the Na^+- Ca^{2+} exchanger. It is worth noting, however, that these authors used Ag-AgCl electrodes for both pipette and bath coupling, and therefore their solutions might have contained some Cl^-. The variable appearance of $I_{Cl(Ca)}$ and the absence of its blocking agent made it impossible to make a quantitative separation of $I_{Cl(Ca)}$ and $I_{Na/Ca}$ during caffeine-induced Ca^{2+} transients. Therefore, we had to rely on indirect evidence. In our study, the reversal potentials of I_{TI} followed the changes in Cl^- equilibrium. Removal of extracellular Na^+ did not abolish I_{TI}, and did not modify the reversal potentials either. In contrast to what has been described for the Na^+- Ca^{2+} exchanger in other species, in rabbit myocardial cells transient current amplitudes over 1 nA were frequently observed and the time courses of these currents were different from the Ca^{2+} transients. Based on these findings, one can exclude the Na^+- Ca^{2+} exchange mechanism as the major charge carrier system in rabbit heart during caffeine-induced Ca^{2+} release, but a relatively minor

contribution cannot be ruled out.

In our work we report first that extracellular anthracene (0·5 mM) blocks the $[Ca^{2+}]_i$-activated Cl^- current in heart. This blockade is predominantly of the outward current, although it is noticeable for the inward Cl^- current as well. Anthracene had no effect on the reversal potential, underlining Cl^- as the dominant charge carrier of the transient current elicited by the rise in $[Ca^{2+}]_i$.

Our results demonstrate that Ca^{2+} release from the sarcoplasmic reticulum may significantly alter the Cl^- conductivity of the cell membrane of rabbit atrial, ventricular and Purkinje cells. Differences in interspecies characteristics might be responsible for the different charge carrying mechanisms described previously for I_{TI} generation. $[Ca^{2+}]_i$-activated Cl^- currents dominate the membrane conductance changes induced by Ca^{2+} transients, and therefore they are likely to be involved in arrhythmogenesis, at least in rabbit heart.

Transient inward current in human atrial cardiomyocytes

The results of our study provide strong evidence for the expression of a $[Ca^{2+}]_i$-dependent ion channel in human atrial myocytes, which can be activated in the absence of Na^+ and K^+. We used caffeine as a tool to induce $[Ca^{2+}]_i$-transients independently of contaminating L-type-Ca^{2+} currents. In our study, we were unable to induce a transient membrane current under control conditions, i.e. in absence of caffeine. In these experiments, only a sustained current could be observed, very similar to the depolarization-induced non-specific cation channel in human atrial myocytes. A direct activation of the transient membrane current by caffeine, as described for a non-specific cation channel in smooth muscle cells of Bufo marinus is unlikely, since in our experiments with 10 mM EGTA in the pipette solution, caffeine failed to induce this current. Therefore it has to be concluded that the caffeine-induced current is activated by $[Ca^{2+}]_i$ and that very high $[Ca^{2+}]_i$-concentrations are required. In this study, $[Ca^{2+}]_i$ was determined only in a semiquantitative way, but it could be demonstrated that fluorescence at 340 nm was 3–4 times higher during the peak of the caffeine-induced $Ca^{2+}{}_i$-transients compared with the peak of depolarization-(i.e. calcium channel-) induced transients.

In cardiac cells, $[Ca^{2+}]_i$-dependent membrane currents, which are not attributed to the Na^+/Ca^{2+}-exchange system, have been described as a component of the transient outward current I_{to}. Escande et al. identified a brief outward current I_{bo} as a 4-AP-resistant component of I_{to} in human atrial myocytes. A similar, component of I_{to} has been identified as a chloride-current in atrial, ventricular and Purkinje cells of the rabbit and in canine ventricular cardiomyocytes, called $I_{Cl(Ca)}$. Therefore it was assumed that I_{bo} may reflect $I_{Cl(Ca)}$ in human atrium as well. Li et al. were unable to demonstrate a contribution of chloride to I_{bo},

since I_{to} was completely suppressed by substitution of pipette K^+ and the remaining current was unaltered, when $[Cl^-]_o$ was replaced. In the present study, we could not demonstrate a contribution of Cl^- to the caffeine-induced current. Variation of $[Cl^-]_i$ and $[Cl^-]_o$ did not shift the reversal potential in a wide range of test potentials. With respect to these results we conclude that $[Ca^{2+}]_i$-dependent chloride channels are not expressed in human atrial cells. To determine the ionic nature of the caffeine-induced current, we tested the permeability for Cs^+ and Li^+, two cations which are generally permeable for CAN channels. The results of the present study demonstrate an almost equal permeability for these cations. In addition, $NMDG^+$ as a cation, for which impermeability to CAN channels is generally accepted, failed to carry inward currents down to -100 mV, when present as the major extracellular cation. Since TEA^+ was also present in pipette- and extracellular solutions (20 mM), small inward currents at -100 mV would be expected, if permeability for this cation is assumed. Permeability of CAN-channels to the large cations TEA^+ and Tris has been described in some reports. However, this property was not extensively examined in the present study. A non-selective conductivity through $[Ca^{2+}]_i$-dependent [SK]-K^+-channels could be ruled out, since apamin in high concentrations (1 µM) was unable to suppress the caffeine-induced current even at low concentrations of caffeine (1 mM).

In conclusion, the results of the present study are in accordance with criteria, which have been formulated for CAN-channels, i.e. $[Ca^{2+}]_i$-dependent activation, non-specific cation-permeability and non-permeability among anions, giving rise to the hypothesis that this current represents a $[Ca^{2+}]_i$-dependent non-specific cation channel in human atrium.

The physiological role of I_{CAN} remains unclear, but it can be assumed that it provides an arrhythmogenic mechanism in situations of $[Ca^{2+}]_i$-overload and may be involved in the generation of the transient inward current I_{TI} and delayed afterdepolarizations. The clinical relevance of such afterdepolarizations could be demonstrated in vivo recently, giving rise to the hypothesis that they are one possible mechanism for the initiation of sustained atrial non-reentry tachycardia in humans. The electrophysiological mechanisms of atrial fibrillation and flutter are very complex and reentry seems to play a major role. Nevertheless, triggered activity caused by delayed afterdepolarizations may be an appropriate substrate for initiation of atrial fibrillation/flutter since it may favour reentry-mechanisms by increasing dispersion of refractoriness in the tissue.

The present concept of the contribution of $[Ca^{2+}]_i$-dependent non-specific cation channels to cardiac arrhythmias was established due to their capability to activate during oscillatory SR-Ca^{2+} release, giving rise to depolarizing membrane currents, such as the transient inward current I_{TI} . However, this feature has not been described for human cardiomyocytes yet. Bènardeau et al. observed delayed afterdepolarizations in human atrial myocytes, which

could be suppressed by substitution of external Na^+ by Li^+. It was concluded that delayed afterdepolarizations in human atrial myocytes are mainly carried by the Na^+/Ca^{2+} exchanger. Although in our study was not designed to examine the contribution of I_{CAN} to delayed afterdepolarizations, it must be emphasised that part of the cells developed spontaneous membrane currents in the absence of Na^+ and K^+, i.e. in the absence of currents generated by the Na^+/Ca^{2+} exchanger or $[Ca^{2+}]_i$-dependent K^+-channels (not shown), suggesting a contribution of this current at least under pathophysiological conditions.

Transient inward current in human ventricular cardiomyocytes

We were unable to identify $[Ca^{2+}]_i$-dependent membrane currents in the absence of Na^+ and K^+ in human ventricular myocytes. An insufficient load of the SR with Ca^{2+} can be excluded, since all cells showed a strong contraction after caffeine application and currents were not present even in those cells in which SR-Ca^{2+} release could be demonstrated by fura-2-fluorescence. Furthermore, caffeine in concentrations of 10 mM are believed to be sufficient to induce a complete SR-Ca^{2+} release. Therefore we conclude that neither a $[Ca^{2+}]_i$-dependent chloride-, nor a non-specific cation channel is expressed in these cells. Cells were isolated from the midmyocardial region (M-region), according to the suspected region for the generation of delayed afterdepolarizations. However, that the $[Ca^{2+}]_i$-dependent non-specific cation channel may be present in cells from other regions, such as the subepicardial or subendocardial layer, cannot be excluded.

The results of the present study suggest that neither a $[Ca^{2+}]_i$-dependent chloride-, nor a non-specific cation-channel is expressed in human ventricular myocardium. Therefore, we conclude that arrhythmogenic currents in the presence of $[Ca^{2+}]_i$-overload, which are associated with the transient inward current I_{TI} and delayed afterdepolarizations, might be carried by the Na^+/Ca^{2+}-exchanger alone. However, this hypothesis is based on indirect evidence, i.e. the absence of $[Ca^{2+}]_i$-dependent currents in the absence of Na^+ and K^+ and should be confirmed by further studies.

The contribution of the $[Ca^{2+}]_i$-dependent modulation of the plateau phase of the ventricular action potential in rabbit

Steady state $[Ca^{2+}]_i$ transients and action potentials were modulated by a single, brief application of extracellular caffeine during the plateau phase of the action potential in electrically stimulated ventricular myocytes of the rabbit. The effect of caffeine was biphasic: first a large $[Ca^{2+}]_i$ transient occurred with a short action potential then attenuated $[Ca^{2+}]_i$ transients with elongated action potentials were registered. Among the various possible charge carrying mechanisms that may theoretically contribute to these changes in the shape of the action potential the roles of $I_{Ca,L}$, I_{TI} and $I_{Na/Ca}$, were examined. We have found that the enhanced repolarization during large

$[Ca^{2+}]_i$ transients is mainly the consequence of the $[Ca^{2+}]_i$-dependent reduction of $I_{Ca,L}$ and of the activation of a transient current, that is probably related to I_{TI}. The elongation of the action potentials during small $[Ca^{2+}]_i$ transients, on the other hand, was linked to the attenuation of the $[Ca^{2+}]_i$-dependent reduction of $I_{Ca,L}$. Major contribution of $I_{Na/Ca}$ to the $[Ca^{2+}]_i$-dependent action potential distortions could not be demonstrated at plateau voltages.

In this study, pronounced $[Ca^{2+}]_i$-dependent regulation of L-type Ca^{2+} channels was correlated to the caffeine-induced alterations in action potential configuration. The intimate relation between $[Ca^{2+}]_i$ and L-type Ca^{2+} current was confirmed during repetitive voltage-clamp depolarizations which also allowed the reproduction most of the experimental conditions applied during current clamp. Caffeine induced an immediate reduction in $I_{Ca,L}$ amplitude which was followed by a transient enhancement of the same parameter. The immediate effect of caffeine was complicated by the simultaneous appearance of I_{TI}. However, in the presence of 500 μM Cd^{2+} which effectively blocked $I_{Ca,L}$, I_{TI} could be separated. Moreover, no changes in the membrane currents were observed during the second voltage clamp depolarization after caffeine application under these conditions. Accordingly, Cd^{2+} could not eliminate the shortening of the action potential upon caffeine application, although, it prevented the caffeine-dependent action potential prolongation. This suggests that the reduction in action potential duration during large $[Ca^{2+}]_i$ transients was mediated by the $[Ca^{2+}]_i$-dependent reduction of $I_{Ca,L}$ and by an additional outward current, seemingly the reversed I_{TI}. The elongation of the action potential when Ca^{2+} release is attenuated, on the other hand, was governed chiefly by the reduced $[Ca^{2+}]_i$-dependent inactivation of $I_{Ca,L}$ and possibly by the increased driving force for Ca^{2+}. The feed-back regulation of $I_{Ca,L}$ by $[Ca^{2+}]_i$ which resulted in bi-directional changes of action potential duration suggests that myoplasmic $[Ca^{2+}]_i$ transients may preserve some constancy despite possible differences in sarcoplasmic Ca^{2+} content. Stronger Ca^{2+} release from the internal stores can be limited by the reduction of $I_{Ca,L}$, while reduced release capacities might be supported by the stronger trigger signal provided by larger $I_{Ca,L}$. This effect can theoretically substitute the specific Ca^{2+}-release activated Ca^{2+} entry mechanism known to exist in several preparations other than heart.

I_{TI} has been previously associated to $[Ca^{2+}]_i$-dependent depolarizations at the resting membrane potential. The existence of this mechanism was also confirmed under our experimental conditions. The role of I_{TI} during the plateau phase of the action potential has not been extensively examined yet. The major problem with this current is that despite significant experimental efforts, the nature of the current is still not fully understood. Although it is generally accepted that the $[Ca^{2+}]_i$-dependent Cl- conductance is the most significant contributor to this current and that non-specific conductances are practically missing in rabbit heart, a relatively small contribution by $I_{Na/Ca}$ cannot be ruled out. The development of I_{TI} required high $[Ca^{2+}]_i$ transients as it was only seen shortly after the caffeine applications during voltage clamp. This suggests that this current has high threshold for Ca^{2+}. The duration of caffeine induced I_{TI} was variable similarly to the caffeine induced $I_{Cl(Ca)}$, and I_{TI} reversed its direction above -45 mV when the calculated equilibrium potential of Cl- was -39 mV at 20 °C. The $[Ca^{2+}]_i$-

dependent Cl- conductance being also part of the transient outward current (I_{TO}) has been associated with the early repolarization processes of the ventricular action potential. Therefore, it would not be surprising if $I_{Cl(Ca)}$ as the putative dominant charge carrier of I_{TI}, was partially responsible for the reduction of action potential duration during caffeine applications, as well. Significant contribution of $I_{Na/Ca}$ to this repolarizing current is unlikely, as caffeine induced action potential shortening was maintained when outward currents by the Na^+-Ca^{2+} exchanger were prevented. Moreover, in the presence of 500 μM Cd^{2+} which is also known to induce a 60% reduction in $I_{Na/Ca}$ caffeine evoked outward currents were not attenuated during voltage-clamp depolarizations.

$[Ca^{2+}]_i$-dependent alterations of $I_{Na/Ca}$ have been previously related to the electrical restitution of ferret ventricular cells and to the modulation of the action potential in the rat. However, experiments in rabbits and guinea pigs failed to determine the time course or even the direction of the current carried by $I_{Na/Ca}$ during the action potential plateau. Under our experimental conditions the caffeine induced early action potential shortening and the subsequent action potential elongation could neither be prevented nor facilitated by the modulation of the transmembrane Na^+ gradient. Based on these findings it seems unlikely that the Na^+-Ca^{2+} exchanger plays a major role either in the $[Ca^{2+}]_i$-dependent shortening or in the $[Ca^{2+}]_i$-dependent lengthening of the plateau phase of the action potential in rabbit ventricular myocardial cells. Nevertheless, these experiments do not exclude the electrogenic Na^+-Ca^{2+} exchanger as a mechanism contributing to the action potential configuration during steady state. Moreover, the marked elongation in the steady state action potential duration when the Na^+ gradient was high and the decrease in steady state action potential duration when the Na^+ gradient was low support the hypothesis that inward $I_{Na/Ca}$ is a significant determinant of the steady-state action potential duration. All of these observations are in good agreement with the thermodynamic assumptions stating that $I_{Na/Ca}$ is highly sensitive for the transmembrane Na^+ gradient but significantly less sensitive for the changes in $[Ca^{2+}]_i$. However, we should also add that omission of intracellular Na^+ prevented the caffeine induced reduction in APD(90) during AP_9. Therefore, we believe that the inward $I_{Na/Ca}$ could be still facilitated by increased $[Ca^{2+}]_i$ at membrane potentials below the plateau level when the transmembrane Na^+ gradient is high. It is worth noting that an inward current peak was suggested for the $I_{Na/Ca}$ during the late repolarization phase of the action potential, therefore, it would not be surprising to assume that the Na^+-Ca^{2+} exchanger has the highest $[Ca^{2+}]_i$-sensitivity in this voltage range.

Maintaining intracellular $[Na^+]$ at 10 mM level increased the probability of the development of outward $I_{Na/Ca}$ not only during the overshoot but also during the plateau of the cardiac action potential. However, we have found no evidence of the influence of outward $I_{Na/Ca}$ on the action potential configuration under our experimental conditions.

Other $[Ca^{2+}]_i$-dependent charge carrying mechanisms were not examined in this study since the caffeine induced changes in action potential shape could be well explained on the basis of $I_{Ca,L}$, I_{TI} and $I_{Na/Ca}$. However, $[Ca^{2+}]_i$-dependent modulation of other membrane channels might be expected in different species as well. The overall influence of the various

$[Ca^{2+}]_i$-dependent processes on membrane potential will depend not only on the expression level of the different $[Ca^{2+}]_i$-dependent conductances but also on the actual $[Ca^{2+}]_i$, membrane potential, heart rate and on several intracellular messengers. Further studies are required to elucidate the interaction between these variables and to clarify their contribution to physiological excitation contraction coupling as well as to certain pathophysiological conditions.

SUMMARY

Gyula P. Szigeti, M.D.: *Role of the $[Ca^{2+}]_i$-dependent currents in the cardiac arrhythmogenesis*

Tutors: Prof. László Kovács, M.D. and Zoltán Papp, M.D.

Single atrial, ventricular and Purkinje cells, isolated enzymatically from either rabbit or human heart, have been studied under whole-cell voltage-clamp and were internally perfused with the fluorescent Ca^{2+} indicator, fura-2. Ca^{2+} release from the sarcoplasmic reticulum was induced by external application of caffeine. Membrane currents of cardiomyocytes from rabbit heart accompanying Ca^{2+} transients showed linear current-voltage characteristics. When Cl^- concentrations were kept symmetrical in the absence of the Na^+-Ca^{2+} exchange mechanism, transient currents had a reversal potential close to 0 mV. Reduction of external Cl^- concentration shifted this reversal potential towards the new Cl^- equilibrium potential. This current was blocked by the application of the Cl^- channel blocker, anthracene-9-carboxylic acid (0.5 mM). It is concluded, that $[Ca^{2+}]_i$-activated transient membrane currents in atrial, ventricular and Purkinje cells of rabbit heart are mainly due to the activation of a $[Ca^{2+}]_i$-dependent Cl^- current. In human atrial myocytes, caffeine also induced a transient membrane current in the absence of Na^+ and K^+. Cl^- did not contribute to this current. Experiments with different cations suggested non-selectivity for Cs^+ and Li^+. Voltage ramps indicated a linear current-voltage relation. In human ventricular cardiomyocytes, caffeine failed to induce transient currents. In human atrial cardiomyocytes, a $[Ca^{2+}]_i$-dependent nonspecific cation channel is expressed and may contribute to triggered arrhythmias in situations of $[Ca^{2+}]_i$-overload. In ventricular cells, neither a $[Ca^{2+}]_i$-dependent nonspecific cation channel nor a $[Ca^{2+}]_i$-dependent chloride channel seems to be expressed.

Effects of single, brief application of caffeine (10 mM) on action potential train were demonstrated on rabbit ventricular cells. Caffeine (during AP9) first enhanced and thereafter attenuated the $[Ca^{2+}]_i$ transients accompanying AP9 and AP10-AP12, respectively. This approach provided direct comparison between time courses of action potentials: during the initial steady state (e.g. AP8) and when Ca^{2+} release from the sarcoplasmic reticulum was increased by caffeine (AP9) or decreased by depletion (AP10). The increase in $[Ca^{2+}]_i$ facilitated repolarization and decreased action potential duration. However, action potentials at reduced Ca^{2+} release (AP10) had longer duration than during steady state. The caffeine-induced changes in L-type Ca^{2+} current ($I_{Ca,L}$), during voltage-clamp conditions partially explained the effects of caffeine on action potentials. When $I_{Ca,L}$ was blocked by 500 mM Cd^{2+}, enhanced $[Ca^{2+}]_i$ transients revealed an extra current component

which was outward at +10 mV and inward at the resting membrane potential (most probably the transient inward current). In the presence of Cd^{2+}, however, AP8 and AP10 had identical time courses, suggesting that $I_{Ca,L}$ alone was responsible for the lengthening of AP10. Alterations in the transmembrane Na^+ gradient resulted in changes of the steady state action potential durations (AP8) consistently with the expected modulation of the Na^+-Ca^{2+} exchange current. However, the contribution of this current to the $[Ca^{2+}]_i$-dependent behaviour of action potential plateau could not be demonstrated.

In extenso **publications used for PhD thesis:**

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