THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Cardiac electrophysiological effects of local anesthetics

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1. Introduction

Local anesthetic agents have a great importance in anesthesia during diagnostic interventions and surgical procedures, in postoperative and obstetric analgesia, as well as in the treatment of chronic pain. These are essential drugs in dentistry, since dental treatments and minor oral surgical procedures are usually performed under local anesthesia. The choice of proper local anesthetic is of paramount importance, since pathological conditions of dental origin cause very intensive pain, wich is further enhanced by the fear of possibly painfull treatment. Systemic - central nervous system and cardiovascular toxicity are the most severe, potentially life-threatening complications of the currently used local anesthetic agents, which are generally accepted as safe. Therefore their use requires great caution.

1.1. Relation of physicochemical properties and anesthetic efficacy

Local anesthetics available for clinical applications contain tertiary amine attached to a substituted aromatic ring by an intermediate chain with either an ester or an amide linkage (aminoesters and aminoamides). The two groups have different pharmacokinetic and pharmacodynamic properties. The aromatic ring lends a lipophilic character to this part of the compound, which is necessary for the transmembrane penetration. Lipid-solubility can be enhanced by substituting the aromatic ring and by increasing the size of the alkyl substituents. This property is proportional to the intrinsic potency and toxicity of the compound. Protein binding is related to the strength of linkage to the receptor-site and the duration of action. Offset from the receptor becomes faster with decreasing molecular weigth. The time of onset is determined by the pKa of the local anesthetic solution, and the pH of the tissues. Local anesthetic agents, except lidocaine have an asymmetric tetrahedron carbon attached to four different substituents, therefore they exist as two enantiomers (levoisomer S and dextroisomer R), with common physicochemical properties, but due to their different three-dimensional structures, their affinities to their binding site may be significantly different.

1.2. Effect of local anesthetics on neural structures

Spread of afferent (sensory) and efferent (motor, sympathic) informations necessitates fast signal transmission over long distances, which is realized by transmembrane ion currents, generation and propagation of the „all or none" - type electrical response, action potential. Local anesthetics at the site of the action reversibly prevent the generation and conduction of electrical impulses by the membranes of the nerves. The temporal sequence of the loss of various functions is block of sensation of sharp pain, later that of temperature, touch, and finallly motor block develops. The sensitivity of different types of sensation to local anesthetics highly depends on the firing frequency of the nerve, the slowest conducting fibres are the most resistent. The different fibres may express ion channels of different sensitivity to local anesthetics, but penetration of the drug through several anatomical layers of different width can also significantly modify the clinically experienced blockade.

1.3. The voltage–gated sodium channel

Local anesthetics prevent the generation and conduction of an action potential by blocking the fast sodium current through the voltage-gated sodium channels. Sodium channels are transmembrane glycoprotein molecules consisting of an ion-conducting and voltage–sensing alpha subunit, also responsible for ion-selectivity, and two modulating beta subunits. Different tissues express different alpha subunit isoforms, characterized by a high degree of homology regarding their aminoacid sequencies. Sodium channels are dynamic molecules, wich drastically change their conformation according to the changes of the membrane potential. They can occupy an open, a closed, a fast inactivated and several slow inactivated states. The channel is closed at the resting membrane potential. During depolarization the voltage sensor moving in extracellular direction initiates conformational changes resulting in channel opening, and sodium ions flow into the cell. Within cca 1,5 ms the influx of sodium ions practically ends due to the closure of the fast inactivation gate in the cytoplasmic mouth of the channel pore, creating a non-conducting

inactivated conformational state, from wich the channel can open again only after repolarization of the cell membrane.

Channels go to different slow inactivated states on prolonged depolarization. Slow inactivation is characterized by conformational changes of the channel and is thought to play a role in the regulation of excitability.

1.4. Effects of local anesthetics on the voltage-gated sodium channel

Local anesthetic agents are present overwhelmingly as positively charged cations and a smaller portion as uncharged neutral free base under physiological circumstances. The lipid-solubile neutral molecules reach their binding site within the sodium channel through the lipid phase of the membrane moving along the faster hydrophobic path, while the charged cation form passes along the slower hydrophilic path, through the cytoplasmic mouth of the pore to the receptor site. Local anesthetics show two mechanisms of voltage-gated sodium channel inhibition: During activation of the channel the charged local anesthetic molecule entering the channel through the cytoplasmic mouth migrates to its binding site below the selectivity filter. In the open channel the charged alkylamino end of the molecule interacts with a highly conservative phenylalanine molecule in all alpha subunit isoforms by van der Waals forces, while the aromatic group interacts with a highly conservative tyrosine.

Located asymmetrically in the channel, the drug blocks sodium ion conduction partly by mechanically occluding the pore, partly by creating a positive electrostatic barrier. Interacting with the residues close to the selectivity filter, local anesthetics also alter the gating mechanisms of the channel, by stabilizing the slow inactivation conformation and the voltage sensor. These drugs usually bind with higher affinity to the open and inactivated conformations of the channel. During repetitive stimulations more channels bind the drug and blockade is increased, thus at higher stimulating frequencies a frequency-dependent, use-dependent or phasic block develops. The highly conservative phenylalanine molecule is hidden from the local anesthetic drug in the closed conformation of the channel, but hydrophobic interaction develops between the pore lining residues and the neutral tertiary amines. This form of block (so called resting phase or tonic block) develops at low stimulating frequencies, requires relative high concentration of the drug and is characterized by low affinity

binding to the closed channel. This form of block does not affect the operation of the voltage-sensor. Hydrophobic molecules are characterized by more expressed tonic, while charged molecules by more pronounced use-dependent block.

1.5. Pharmacokinetic properties of local anesthetics

The therapeutic effect of local anesthetics – prevention of generation and conduction of electrical impulses by the membrane of the nerve – depends on the local concentration of the drug. This effect is decreased by the systemic absorption, determined by the distance of the site of deposition from the nerves to be anesthetized, by the level of vascularization, local blood flow and the amount of fatty tissue at the site of application and cardiac output. After administration the peak concentration of local anesthetic agents in the plasma depends on the dose applied, on the rate of administration and systemic absorption, protein binding, distribution and elimination of the drug.

1.6. Local anesthetic systemic toxicity

Since voltage-gated sodium channels ubiquitously appear in excitable membranes, local anesthetic agents entering the systemic blood flow come into contact with all these structures and may alter their functional integrity. Free fraction of local anesthetics in the plasma resulting from administration of excessive doses or inadvertent intravascular injection leads to the development of central nervous system and cardiovascular toxicity. The magnitude of toxic effects depends on the concentration of the free drug in the plasma and its toxic potency.

Central nervous system toxicity occurs at lower plasma concentration, its excitatory, convulsive phase caused by suppression of cortical inhibitory neurons is followed by generalized central nervous system depression with hypoventillation, apnea and coma at rising plasma levels. Further increasing plasma concentration induces cardiotoxicity. Local anesthetic cardiotoxicity can be divided into a central nervous system mediated biphasic indirect and a direct myocardial component. Indirect cardiotoxicity demonstrates an initial stimulating effect in the form of hyperdynamic circulation, later, with further elevating plasma level, the general central nervous system depression manifests in bradycardia and hypotension.

Direct cardial effects of local anesthetics (negative inotropic, chronotropic and dromotropic effects) are related to the intrinsic potency of varying drugs and result from the effects on the cardiac action potential and the underlying ion currents at cellular level.

1.7. Cardiac ventricular action potential and the underlying ion currents

Action potentials are generated by changes in conductivity of voltage- and time-dependent plasmalemmal ion channels. Ventricular cardiomyocytes have a resting potential of – 80 mV. Stimulation to potentials above treshold evokes phase 0 depolarization, the rapid rising phase of the action potential, which achieves an overshoot of about 30-40 mV and is characterized by a maximum rate of depolarization of about 200V/s. The upstroke is followed by a short early (phase 1) repolarization that is especially intensive in ventricular midmyocardial and subepicardial cells. Action potentials in the subepicardial ventricular cells display an early repolarization of about 20-30 mV, followed by a small secondary depolarization giving the action potential the characteristic spike and dome appearance. The plateau phase (phase 2) results from the fine balance of inward and outward currents, and the slightly dominating outward currents result in a small net outward current. The plateau phase is followed by the final (phase 3) repolarization, by the end of which the membrane returnes to resting potential (phase 4).

The upstroke of the cardiac action potential is developed by the fast and large inward sodium current through the voltage gated sodium channels. After rising to a peak, sodium channels undergo rapid inactivation and can reopen only after recovery from inactivation at negative membrane potentials. In a small range of membrane potentials where steady-state activation and inactivation curves of sodium current overlap, a sodium current of small amplitude, the so called sodium window current can be detected. Sodium current can be inhibited by tetrodotoxin, saxitoxin and local anesthetics.

The 4-aminopyridine sensitive and intracellular calcium concentration independent component of the transient outward potassium current responsible for the early repolarization is formed by at least two different isoforms of the potassium channels (predominantly Kv4.3 and Kv1.4 in humans and in dogs). I_{to} has a

significance in early repolarization, and developing the driving force for the proper calcium influx.

The plateau phase of the action potential is characterized by the fine balance of inward and outward currents. The inward L-type calcium current (I_{Ca}) through the L-type voltage gated calcium channels and repolarizing potassium currents (especially I_{Kr}) flowing through the fast delayed rectifier potassium channels are parallelly active at depolarized membrane potentials. There is a small I_{to} contribution to the outward currents during the early plateau phase, while the inward sodium window current also plays a role in the plateau phase, hence in the determination of action potential duration.

In cardiomyocytes L-type calcium current (I_{Ca}) is the main mechanism of calcium influx from the extracellular space. The resulting rise in $[Ca^{2+}]_i$ triggers calcium release from the stores of the sarcoplasmic reticulum through the ryanodin receptors. The increase in the intracellular Ca^{2+} concentration allows for binding of Ca^{2+} to troponine C, hence initiating the contraction. During diastole the cytosolic concentration of Ca^{2+} decreases as a significant amount of intracellular Ca^{2+} is transported back into the sarcoplasmic reticulum. Inhibitors of L-type calcium current are nifedipin derivates, verapamil and diltiazem.

A fast (I_{kr}) and a slow component (I_{Ks}) of delayed rectifier potassium current activating during repolarization can be distinguished. The pore-forming HERG subunit and the associated regulating subunit, the MIRP1 protein together form the ion channel responsible for I_{Kr} current, wich plays the main role in the physiological ventricular repolarization. This current activates at depolarizations positive to -40 mV. Due to its C-type inactivation the channel shows pronounced inward rectification. The channel is selectively blocked by dofetilide, d-sotalol and E4031.

Kv7.1 channel protein (encoded by the gene KCNQ1, previously named LQT1) together with the minK protein underlies cardiac I_{Ks}. In the human heart I_{Ks} activates very slowly during plateau phase, then it deactivates relatively rapidly within few hundred ms. Activation of I_{Ks} is supposed to play a role in the prevention of further extreme prolongation of action potential during prolonged action potentials. I_{Ks} is now accepted as part of the repolarization reserve, rather then as a current controlling repolarization. Selective inhibitor of this current is chromanol 293B.

The inward rectifier potassium current (I_{K1}) is responsible for the negative resting membrane potential between the action potentials. The inward rectification enables

the channels to be open at potentials around the resting membrane potential, but they close on depolarization. I_{K1} also plays an important role in the acceleration and completion of terminal repolarization. The dominant isoform carrying the current is Kir2.1 in humans and dogs. I_{K1} has no completely selective inhibitor, but a relatively selective block may be achieved with the application of 50 μM $BaCl_2$.

1.8. New local anesthetics in clinical practice

1.8.1. Ropivacaine ((S)-N-2,6-dimethylphenyl-1-propylpiperidine-2-carboxamide)

Ropivacaine is the first pure S stereisomer local anesthetic agent wich was developed after evidence of severe bupivacaine-related toxicity. It is characterized by lower lipid-solubility, protein binding and significantly shorter terminal half-time, than bupivacaine. In clinical practice the two drugs have similar anesthetic profiles in equipotent doses. It is generally accepted that in spite of their similar anesthetic properties, ropivacaine has lower cardiotoxic potency than bupivacaine. However, cases of cardiac arrest and severe arrhythmias have also been reported on ropivacaine anesthesia, as a result of applying large amounts of drug or following inadvertent intravascular administration. In animal experiments ropivacaine showed weaker negative inotropic and chronotropic effect and was less arrythmogenic than bupivacaine. Even though ropivacaine applied in equipotent doses seemed to be less cardiotoxic than bupivacaine, application of sublethal doses resulted in qualitatively and quantitatively similar hemodynamic changes. Contrary to this bupivacaine showed a more pronounced blocking effect on the ventricular conduction after intracoronary administration. In spite of its widespread clinical use, detailed experiments on volunteers and animal models little is known about ropivacaine's cellular cardiac effects. Besides its negative inotropic effect, ropivacaine decreased the maximum rate of depolarization in a use-dependent fashion. This effect was weaker than that of the bupivacaine, but was more pronounced than that of lidocaine. Ropivacaine decreased the maximum rate of depolarization at lower rates of stimulation when comparing to lidocaine. The time constant for recovery from block in case of ropivacaine was much longer than that of lidocaine, but shorter than that of bupivacaine.

Ropivacaine was reported to inhibite several cloned human ion channels e.g. Kv4.3/KChIP mediating I_{to} , Kv1.5 underlying $I_{Kur,}$ an important repolarizing current of the human atria at concentrations higher than the usual plasma levels. Ropivacaine blocked channels mediating I_{kr} with the same potential as bupivacaine in toxicologically relevant concentrations.

I_{Na} and I_{Ca} were decreased by ropivacaine in ventricular myocytes of guinea pigs, though I_K and I_{K1} were not influenced significantly. Inhibition of these ion channels may also contribute to the cardiotoxicity of ropivacaine.

1.8.2. Articaine ((*RS*)-methyl 4-methyl-3-(2-propylaminopropanoylamino)thiophene-2-carboxylate)

Articaine is one of the most widely applied local anaesthetic drugs in dentistry. Used as a racemate, articane is unique among the amide type local anesthetics, as it contains a tiophene ring, which improves lipid-solubility, and an ester group enabling the metabolism by the tissue and plasma esterases. Its intrinsic potency, local and systemic adverse effects seem similar to those of lidocaine. Plasma protein binding of articaine is 70 %, it diffuses better through soft tissues and bone than other local anesthetics. Though primarily used in dentistry, its usage for regional anesthesia in a day-case setting may be beneficial due to quicker onset and shorter elimination as well lower plasma concentrations compared to lidocaine.

Importantly, in contrast with other local anaesthetics, like bupivacaine, lidocaine or ropivacaine, no cardiac arrest has been reported with articaine up to now. During application in dentistry articaine has showed no significant cardiovascular inhibition. In spite of its widespread clinical use little information is available on the cardiac electrophysiological effects of the compound. In an early study 141 μM articaine was shown to decrease the overshoot, amplitude and velocity of depolarization of rabbit ventricular action potentials. However, no data on cardiac ion currents have been reported with articaine, except for HERG channels expressed in CHO cells, indicating an articaine-induced block at high concentrations. Little is known about the effects of articaine on other excitable structures. In rabbit striated muscle articaine decreased the activity of Ca^{2+}-dependent ATPase of sarcoplasmic reticulum, decreasing the binding of calcium ions to the high-affinity binding sites, this way

decreasing the rate of Ca^{2+} reuptake. All these effects may lead to prolonged muscle spasm once the drug diffuses into the muscle.

2. OBJECTIVES

Articaine and ropivacaine have been applied in the clinical practice for a relatively short time. In spite of their widespread use and generally accepted lower cardiotoxic potency little relevant information is available regarding their cellular cardiac effects. This study was undertaken to obtain data regarding the cellular cardiac electrophysiological effects of articaine and ropivacaine in isolated canine ventricular myocytes as electrophysiological properties of canine cardiomyocytes are believed to be the most similar to those of the human cardiomyocytes regarding distribution and kinetic of transmembrane ion currents.

We set out to answer the following relevant questions:

1. What are the concentration–dependent electrophysiological effects of articaine and ropivacaine on cardiomyocytes?
2. How do articaine and ropivacaine modify the configuration of action potential (amplitude of early repolarization and plateau phase) and its parameters (amplitude, rate of depolarization, action potential duration) at different pacing frequencies?
3. What changes in underlying ion currents are involved in the alterations of the action potential configuration?
4. What are the effects of articaine and ropivacaine on the calcium handling of the myocardiocytes (amplitude of intracellulare Ca^{2+} transients, Ca^{2+} release and reuptake) and on contractility?
5. What kind of proarrhythmic risk may be anticipated during usual clinical application and in the case of accidental intravascular administration?

3. MATERIAL AND METHODS

3.1. Isolation of single canine ventricular myocytes

Hearts of the intravenously anesthetized animals were quickly removed and placed in Tyrode solution. Left anterior descending coronary artery was cannulated. Trabeculae were excised for measurement of contractility, and the remaining left ventricular tissue was used for preparation of SR vesicles.

Single myocytes were obtained by enzymatic dispersion using the segment perfusion technique. The preparation was perfused nominally Ca^{2+}-free Joklik solution) for 5 min. This was followed by 30 min perfusion with Joklik solution supplemented with 1mg/ml collagenase and 0.2% bovine serum albumine containing 50 μM Ca^{2+}. Portions of the left ventricular wall were cut into small pieces and the cell suspension, obtained at the end of the procedure predominantly from the midmyocardial region of the left ventricle, was washed with Joklik solution. Finally the Ca^{2+} concentration was gradually restored to 2.5 mM. The cells were stored at 15 C°until use.

3.2. Recording of action potentials

All electrophysiological measurements were performed at 37 ^{o}C. Rod-shaped viable cells showing clear striation were sedimented in a plexiglass chamber allowing continuous superfusion with oxygenized Tyrode solution. Transmembrane potentials were recorded using 3 M KCl filled sharp glass microelectrodes having tip resistance between 20 and 40 MΩ. These electrodes were connected to the input of an Axoclamp-2B amplifier. The cells were paced through the recording electrode at steady cycle length of 1 s using 1 ms wide rectangular current pulses with 120% of threshold amplitude. Since the cytosol was not dialyzed, time dependent changes in action potential duration were negligible for at least 60 min under these experimental conditions.

Concentration-dependent effects of articaine and ropivacaine were determined in a cumulative manner by applying increasing concentrations of the drug between 1 and 300 μM. Each concentration was superfused for 3 min and the washout lasted for 10 min. These incubation and washout periods were sufficient to develop steady-state drug effects and practically full reversion. When

performing frequency-dependent measurements, the cycle length was set to 5 s, and following equilibration at least for 5 min the cycle length was continuously varied to the shorter values. Action potentials were digitized at 200 kHz using Digidata 1200 A/D card and stored for later analysis.

3.3. Recording ion currents using conventional voltage clamp

The cells were superfused with oxygenized Tyrode solution.at 37C°. Membrane currents were recorded with the Axopatch-2B amplifier using the whole cell configuration of the patch clamp technique. After establishing high (1-10 GΩ) resistance seal by gentle suction, the cell membrane beneath the tip of the electrode was disrupted by further suction or by applying 1.5 V electrical pulses for 1-5 ms. The series resistance was typically 4-8 MΩ before compensation (usually 50-80%). Experiments were discarded when the series resistance was high or substantially increasing during the measurement (in less than 10% of the experiments). Outputs from the clamp amplifier were digitized at 100 kHz under software control (pClamp 6.0, Axon Instruments). Ion currents were normalized to cell capacitance, determined in each cell using short hyperpolarizing pulses from -10 mV to -20 mV.

3.4. Recording ion currents using action potential voltage clamp

After formation of the gigaseal, action potentials were recorded in current clamp mode from myocytes superfused with Tyrode solution. The cells were continuously paced through the recording electrode at steady stimulation frequency of 1 Hz so as a 1-2 ms gap between the stimulus artifact and the upstroke of the action potential could occur. Ten subsequent action potentials were recorded from each cell, which were digitized and averaged. This averaged signal was delivered to the same cell at the identical frequency as the command voltage after switching the amplifier to voltage clamp mode. The current trace obtained under these conditions is a horizontal line positioned at the zero level except for the very short segment corresponding to the action potential upstroke. Articaine and ropivacaine were applied in a cumulative manner (from 10 to 1000 μM concentration). The profile of the ion currents blocked by articaine and

ropivacaine was determined by subtracting the pre-drug curve from the post-drug one. This procedure resulted in composite current profiles containing three distinct current peaks after reverting its polarity: an early outward for I_{to}, an inward for I_{Ca}, and a late outward for $I_{Kr}+I_{K1}$.

3.5. Measurement of contractility

Thin ventricular trabeculae, having diameters less than 1 mm, were mounted in a plexiglass chamber allowing continuous superfusion with Krebs solution at a temperature of 37^{o}C, and pH was adjusted to 7.4±0.05. Developed force was recorded under isometric conditions using a capacitive mechano-electronic transducer fixed to a micromanipulator. Each preparation was stretched to the length at which maximum developed force was evoked, and was allowed to equilibrate for 60 minutes while pacing at a constant cycle length of 2 s. Articaine and ropivacaine were applied at cumulatively increasing concentrations (each for 20 minutes) followed by a 60 minutes period of washout. Records were digitized at 1 kHz using Digidata 1200 A/D card and stored for later analysis.

3.6. Recording of cytosolic Ca^{2+} transients

Changes in intracellular free Ca^{2+} concentration were assessed by the ratiometric technique using the calcium-sensitive fluorescence dye fura-2. Isolated myocytes were loaded at room temperature in Tyrode solution containing 3 μM l^{-1} of the acetoxymethyl ester of Fura-2 in the presence of Pluronic F-127 for 10 minutes. After loading, a period of at least 30 minutes was allowed for de-esterification of the dye, then cells were stored at 15 ^{o}C until use. Loaded cells were placed in a superfusion chamber fixed to the stage of an inverted microscope. Measurements were performed at 37 ^{o}C. Cells were field stimulated at a constant cycle length of 1 s through a pair of platinum wires. Articaine and ropivacaine were applied at cumulatively increasing concentrations (each for 3 min) followed by a 10 min period of washout.

Excitation wavelengths of 340 and 380 nm were obtained from a xenon arc lamp by a dual-wavelength excitation monochromator and an on-line connected microcomputer. Fluorescence emission was monitored at 510 nm using a photomultiplier tube. Changes in intracellular free Ca^{2+} levels were approximated

by the ratio of the fluorescence intensity obtained at 340 and 380 nm excitation after correction for nonspecific background fluorescence and recorded. 10 consecutive $[Ca^{2+}]_i$ transients were averaged and analyzed off-line.

3.7. Preparation of heavy SR vesicles

Heavy sarcoplasmic reticulum vesicles, containing vesicles formed from membrane fragments of the terminal cisternae of the SR, were isolated from canine left ventricular muscle samples according to Lai and Meissner with slight modifications. Following homogenization and centrifugation at 4500 g the crude microsomes were collected by centrifugation at 40,000 g from the supernatant. Actomyosin contamination was removed by solubilization in 600 mM KCl, the microsome fraction was collected at 109,000 g. The pellet was resuspended and loaded onto a 20–45% linear sucrose gradient. Heavy sarcoplasmic reticulum (HSR) vesicles were extracted from the 36–38% region of the continuous sucrose gradient, collected by centrifugation, and resuspended in 0.4 M sucrose, 10 mM K-PIPES, at pH=7.0. Protein concentration was measured by the Biuret method.

3.8. Determination of ATPase activity

ATPase activity was determined at 37ºC by a coupled enzyme assay. Total hydrolytic activity was measured as the decrease of optical density at the NADH absorbance wavelength (340 nm). The total hydrolytic activity of the HSR vesicles ranged from 2.71 to 2.82 IU, and Ca^{2+}-dependent ATPase activity was identified as the portion of total hydrolytic activity inhibited by 5 mM thapsigargin. The drug was added to the preparation 5 min before starting the measurement of enzyme activity. The assay was performed in the presence of Ca^{2+} and EGTA mixtures, producing the estimated free Ca^{2+} concentration (2.4 μM) found to induce maximum SERCA activation.

3.9. Measurement of Ca^{2+} uptake and release in HSR vesicles

Ca^{2+} release from, and Ca^{2+} uptake into cardiac HSR vesicles was measured using the Ca^{2+}-sensitive dye antipyrylazo III. The absorbance was monitored at 710 nm by a spectrophotometer.

When measuring Ca^{2+} uptake, the vesicles were actively loaded with Ca^{2+} in the presence of articaine or ropivacaine at 37°C in the presence of 1 mM ATP/$MgCl_2$, and 0.25 mM antipyrylazo III at pH=7.0. Ca^{2+} loading was initiated by addition of 100 μM l^{-1} $CaCl_2$ to the medium, and the rate of uptake was monitored as a decrease of extravesicular Ca^{2+} concentration. The absorbance signal was calibrated by consecutive additions of $CaCl_2$.

When measuring Ca^{2+} release, the vesicles were passively loaded with Ca^{2+} during an overnight incubation in the presence of 200 μM/l $CaCl_2$. The sample was diluted so as to have a protein concentration of 10 mg/ml. Ca^{2+} release was induced by addition of 0.3 mM thymol 5 min after preincubation with the given concentration of articaine or ropivacaine. The initial rate of Ca^{2+} release was determined from the increase of extravesicular Ca^{2+} concentration.

3.10. Statistics

Results are expressed as mean ± SEM values. Statistical significance of differences was evaluated with using ANOVA followed by Student's t-test. Differences were considered significant when P was less than 0.05. Concentration-response curves were obtained by fitting data to the Hill equation using Microsoft Origin 6.0 software. Half-effective concentrations (EC_{50} values) and Hill coefficients were determined from these Hill fits. Action potential parameters were determined in off-line mode using Clampfit 9.0 software.

4.RESULTS

4.1. Effect of articaine on action potential configuration

Articaine treatment caused concentration-dependent changes in action potential morphology in canine ventricular myocytes, paced at a constant frequency of 1 Hz, including reduction of the amplitude and maximum velocity of depolarization (V_{max}) of the action potential, shortening of action potential duration, reduction of the amplitude of phase 1 repolarization, and depression of

the plateau. Reduction of action potential duration and V_{max} was statistically significant from concentrations of 10 and 30 μM, respectively. Fitting the V_{max} data to the Hill equation an EC_{50} of 162±30 μM and a Hill coefficient of 1.16±0.03 were obtained. All these effects were readily reversible (within 5 minutes) after superfusion with articaine-free Tyrode solution. Although articaine failed to induce statistically significant changes in the resting membrane potential of the cells, a tendency of depolarization was observed at higher concentrations (100 and 300 μM).

4.2.Frequency-dependent properties

Both the reduction of V_{max} and shortening of action potentials by articaine were frequency dependent. The former effect was prominent at fast driving rate, i.e. when the pacing cycle length was decreased from 5 to 0.5 s (normal frequency-dependence), while the latter was more pronounced at longer cycle lengths displaying features of reverse frequency-dependent action. Both are characteristics of ion channel blocking agents including several local anaesthetics.

Restitution kinetics of V_{max} and action potential duration were also determined. The myocytes were paced using a train of 20 basic stimuli delivered at a basic cycle length of 1 s. Each train was followed by a single extra stimulus applied with successively longer coupling intervals. The train of basic stimuli was reinitiated following the delivery of the extra stimulus. In this way, each 20th basic action potential was followed by a single extra action potential occurring at gradually increasing diastolic intervals. The diastolic interval was defined as the time from APD_{90} of the last basic action potential of the train to the upstroke of the extra action potential. Recovery curves were generated by plotting the V_{max} or APD of each extra action potential against the respective diastolic interval. In addition to the 16±2 % tonic block measured following the longest diastolic interval of 5 s, a marked rate-dependent block also became evident on shortening the diastolic interval. The offset kinetics of this rate-dependent block was estimated by fitting the articaine data to a single exponential yielding an offset time constant of 91±20 ms. This is shorter than the offset time constant obtained with any other local anaesthetic. The restitution curves constructed for action potential duration in the

presence of articaine were flat, indicating that action potential duration evoked after various diastolic intervals has lost its characteristic frequency-dependent nature seen in normal Tyrode solution.

4.3. Effect of articaine on cardiac ion currents measured by conventional voltage clamp

In these experiments, performed under conventional voltage clamp conditions, cumulative concentration-dependent drug-effects were studied between 10 and 1000 μM, increasing the concentration of articaine in steps of half decade.

L-type calcium current (I_{Ca}) was recorded at +5 mV using 200 ms long depolarizations arising from the holding potential of –40 mV. At this holding potential I_{Na} is inactivated which minimize the distortion of I_{Ca}. In these experiments Tyrode solution was supplemented with 3 mM 4-aminopyridine, 1 μM E 4031, and 30 μM chromanol 293B in order to block K^+ currents. Articaine blocked I_{Ca} in a concentration-dependent manner. An EC_{50} value of 471±75 μM and a Hill coefficient of 1.07±0.14 were obtained when fitting the results to the Hill equation. Articaine failed to influence the inactivation kinetics of I_{Ca}.

The transient outward current (I_{to}) was activated by depolarization to +50 mV from the holding potential of –80 mV and having duration of 200 ms. Before each test pulse a short (5 ms) depolarization to –40 mV was applied in order to inactivate the fast Na^+ current, while Ca^{2+} current was blocked by 1 μM nisoldipine. The blocking effect of articaine was characterized with an EC_{50} of 365±62 μM and a Hill coefficient of 1.02±0.04.

The inward rectifier K^+ current (I_{K1}) was studied by applying hyperpolarizations to –135 mV from the holding potential of -80 mV. The steady-state current was determined after 400 ms. I_{K1} was also blocked by articaine with an EC_{50} of 372±46 μM and a Hill coefficient of 1.63±0.09.

The rapid component of the delayed rectifier K^+ current (I_{Kr}) was activated by 1 s long depolarizing pulses to +40 mV arising from the holding potential of -80 mV. I_{Kr} was assessed as tail current amplitudes recorded following repolarization to -30 mV. I_{Ca} and I_{Ks} were suppressed by 1 μM nisoldipine and 30 μM chromanol 293B, respectively. The amplitudes of the I_{Kr} current tails were progressively decreased by increasing concentrations of articaine. The EC_{50} value and Hill coefficient were estimated 278±79 μM and 0.96±0.14, respectively.

The slow component of the delayed rectifier K^+ current (I_{Ks}) was also evaluated as tail currents. The current was activated by 3 s long depolarization to +50 mV, and the amplitude of tail currents was determined at the holding potential of -40 mV after repolarization. I_{Ca} was inhibited by 1 μM nisoldipine and I_{Kr} was blocked by 1 μM E 4031. The EC_{50} was 326±65 μM and the Hill coefficient 0.87±0.07.

4.4. Effect of articaine on the ion currents under action potential clamp conditions

The profile of an ion current may be markedly different when compared under conventional voltage clamp and action potential clamp conditions. An advantage of the action potential clamp technique is that the effect of any drug on the net membrane current can be recorded allowing thus to monitor drug-effects simultaneously on more than one ion current. Furthermore, this technique enables us to record true current profiles flowing during an actual cardiac action potential. Of course, in the case of a drug acting on more than one ion current, such as articaine is, a series of peaks can be detected on the current trace, each of them corresponding to the fingerprint of an individual ion current. Accordingly, the early outward current peak arises when I_{to} is suppressed, while the inward deflection indicates a blockade of I_{Ca}. The late outward current peak, coincident with the terminal repolarization of the action potential, is composed of I_{K1} plus I_{Kr} in a ratio of 3:1. Articaine significantly blocked I_{to}, I_{Ca}, and the late current peak containing both I_{K1} and I_{Kr}. Inhibition of these currents increased with increasing the concentration of articaine up to 1000 μM and was readily reversible. In contrast to results of the conventional voltage clamp measurements, where the EC_{50} obtained for I_{Ca} was the highest, 100 μM articaine suppressed the inward current (I_{Ca}) more effectively than the late outward current peak.

4.5. Effect of ropivacaine on action potential configuration

Ropivacaine treatment caused concentration-dependent changes in action potential morphology in canine ventricular myocytes, paced at a constant frequency of 1 Hz, including reduction of V_{max}, shortening of action potential duration, reduction of the amplitude of phase 1 repolarization, and depression of

the plateau. From these effects reduction of phase 1 repolarization and V_{max} was statistically significant from 10 μM, and shortening of action potentials was significant from 30 μM. Fitting the V_{max} data to the Hill equation an EC_{50} of 81±7 μM and a Hill coefficient of 1.02±0.08 were obtained. Suppression of V_{max} and phase 1 repolarization was fully reversible within the 10 min period of washout. Interestingly, APD_{90} showed a marked rebound phenomenon: it increased above the normal level after superfusion with ropivacaine-free Tyrode solution. Action potential amplitude was decreased by 100 μM ropivacaine from 114.6±2.2 mV to 100.8±2.8 mV, which returned to 113.8±2.9 mV after 10 min of washout. Although ropivacaine failed to induce statistically significant changes in the resting membrane potential of the cells, a tendency of depolarization was observed at large concentrations (above 100 μM).

4.6. Frequency-dependent properties

Both reduction of V_{max} and shortening of action potentials by ropivacaine were frequency-dependent. Reduction of V_{max} was more prominent at faster pacing rates (normal frequency dependence) which is due to the use-dependent action of ropivacaine on Na channels. The APD shortening effect showed the properties of reverse rate-dependent action, as it became more pronounced with lengthening the pacing cycle length, and was absent at cycle length shorter than 1000 ms. This reverse rate-dependent action is a general feature of several ion channel blocking agents, including antiarrhythmic drugs and local anesthetics, and is attributable to the smaller net outward current flowing during the plateau of the longer action potential observed at longer cycle lengths. Thus the shortening effect of ropivacaine is likely due to inhibition of I_{Ca} and window I_{Na}, changes that may have major influence on APD at longer cycle lengths.

Restitution kinetics of V_{max} and action potential duration were also determined. In addition to the 37±1 % tonic block measured following the longest diastolic interval of 5000 ms, a marked rate-dependent block also became evident on shortening the diastolic interval. The offset kinetics of this rate-dependent block was estimated by fitting the data to a single exponential yielding an offset time constant of 340±40 ms. Curves describing the APD - diastolic interval relation in the presence of 100 μM ropivacaine were flat, and in contrast to the control

situation where APD increased with increasing diastolic interval, APD_{50} values actually decreased in the presence of ropivacaine when the diastolic interval was increased.

4.7. Effect of ropivacaine on cardiac ion currents measured by conventional voltage clamp

In these experiments cumulative concentration-dependent drug-effects were studied between 10 and 1000 µM, increasing the concentration of ropivacaine in steps of half decade. The voltage protocolls and ion channel inhibitors were the same as in the experiments with articaine. Under voltage clamp conditions a variety of ion currents were blocked by ropivacaine: L-type calcium current (I_{Ca}) (EC_{50} = 263±67 µM), transient outward current (I_{to}) (EC_{50} = 384±75 µM), inward rectifier potassium current (I_{K1}) (EC_{50} = 372±35 µM),the rapid component of the delayed rectifier potassium current (I_{Kr}) (EC_{50} = 303±47 µM), and the slow component of the delayed rectifier potassium current (I_{Ks}) (EC_{50} = 106±18 µM).

Reversibility of the ropivacaine-induced suppression of ion currents showed marked diversity. Blockade of I_{to} was fully reverted within the initial 3 min of washout. Inhibition of I_{Ca} was not fully reversible, however, the current was restored to 80% of the initial pre-drug level following 10 min washout. In contrast, I_{Kr}, I_{Ks}, and particularly I_{K1} displayed slow and only partial reversion within the 10 min of washout period.

4.8. Effect of ropivacaine on ion currents under action potential clamp Conditions

Under action potential voltage clamp, increasing the concentration of ropivacaine between 10 and 1000 µM, ropivacaine significantly blocked I_{to}, I_{Ca}, and the late current peak, containing both I_{K1} and I_{Kr}. Inhibition of these currents increased with increasing the concentration of ropivacaine up to 1000 µM. Ropivacaine blocked I_{to} with the highest sensitivity under action potential clamp conditions. Suppression of the inward and the late outward current peak increased symmetrically with increasing concentrations of ropivacaine.

4.9. Effects of articaine and ropivacaine on contractility

Both articaine and ropivacaine caused a concentration-dependent negative inotropic action on ventricular trabeculae paced at a constant frequency of 0.5 Hz. This effect was statistically significant ($p<0.05$) at the concentration of 10 μM, and suppression of the contractile force was practically complete at 1000 μM. These effects of articaine and ropivacaine were partially reversible (to 73±21 % and 85±10 % of the respective pre-drug control values) after 60 min superfusion with drug-free Tyrode solution. The morphology of the contraction curve was slightly modified by high concentrations of the compounds. There was a small, but statistically significant, lengthening in half-relaxation time induced by 300 μM articaine (from 74±4 to 85±6 ms) and ropivacaine (from 75±2 to 88±4 ms, $p<0.05$). No change in the time required to reach peak tension was observed (100±5 and 102±10 ms before and after 300 μM articaine, the respective values with the same concentration of ropivacaine were 91±6 and 95±4 ms). Suppressive effects of the drugs on contractile force were characterized using Hill plots, yielding EC_{50} values of 73.7±10.4 and 72.8±14.1 μM for articaine and ropivacaine respectively, with the corresponding Hill coefficients of 0.81±0.08 and 1.08±0.19. No statistically significant differences between the effects of the two drugs on contractile parameters were observed.

4.10. Effects of articaine and ropivacaine on $[Ca^{2+}]_i$ transients

In isolated canine ventricular cells articaine and ropivacaine displayed a concentration-dependent reduction of systolic $[Ca^{2+}]_i$, with a concomitant decrease in the amplitude of $[Ca^{2+}]_i$ transients. Diastolic $[Ca^{2+}]_i$ was not altered by the drugs. Suppression of $[Ca^{2+}]_i$ transients was statistically significant at concentrations of 10 μM or higher, and was almost fully reversible upon washout. Fitting $[Ca^{2+}]_i$ amplitude data to the Hill equation EC_{50} values of 87.4±12 and 99.3±17μM, and Hill coefficients of 0.87±0.09 and 0.96±0.15 were estimated for articaine and ropivacaine, respectively. Both drugs resulted in a moderate prolongation of relaxation of the $[Ca^{2+}]_i$ transient at high concentrations. While 300 μM articaine and ropivacaine increased the monoexponential decay time constant

(from 200±22 to 284±40 ms, and from 199±20 to 326±39 ms, respectively, $P<0.05$ in both cases), this effect was not significant at 100 μM concentration (the respective decay time constants were 222±31 and 228±20 ms). Again, differences between the effects of the two drugs were not statistically significant.

4.11. Effects of articaine and ropivacaine in heavy SR vesicles

Articaine and ropivacaine caused a moderate, but statistically significant, reduction in both Ca^{2+} release and Ca^{2+} uptake of the HSR vesicles at concentrations of 300 μM and above No significant differences were seen between these effects of ropivacaine and articaine. The decreased Ca^{2+} uptake was accompanied by proportional inhibition of the SR Ca^{2+} ATPase (significant from 200 μM). Although these effects were relatively small in amplitude, increasing the articaine concentration above 200 μM failed to further suppress Ca^{2+} uptake or ATPase activity. In contrast, the effect of ropivacaine on ATPase activity was not saturated at the highest applied concentration of 1 mM.

5. DISCUSSION

The primary pharmacological target of local anesthetics is the voltage-gated sodium channel. These agents reversibly inhibit the excitation and conduction of action potentials in the nervous system by blocking of the voltage-gated sodium channels. The major disadvantage of these drugs is a lack of selectivity. Suppression of different voltage-dependent K^+ currents ($I_{to,}$ $I_{Kr,}$ I_{Ks}) may enhance the anesthetic effect by increasing the inactivated sodium channel population during longer action potentials. Inhibition of I_{K1} current, wich plays an important role in controlling the resting membrane potential, has a mild depolarizing effect, which also promotes accumulation of sodium channels in inactivated states. Moreover, voltage-

gated sodium channels are also widely expressed in other excitable cells. The most severe complications of local anesthetics entering the systemic bloodstream due to an excessive dose or accidental intravascular injection are central nervous system and cardiac toxicity. Multiple sites of action are general properties of all local anesthetics - similarly to most antiarrythmic agents. Not suprisingly, local anesthetic agents may have antiarrhythmic effects of different mechanisms, but at the same time, their possible proarrhythmic activity, an essential element of their cardiotoxicity, is also caused by blockade of many ion channels.

5.1. Effects of articaine on action potential morphology are in line with the voltage clamp data

The cellular electrophysiological effects of articaine were first analyzed in this study. Articaine suppressed several ion currents in a concentration-dependent manner with the concomitant alterations of action potential morphology. These changes observed in the configuration of the action potential can be deduced from suppression of the various ion currents. Reduction of V_{max} and action potential amplitude are clearly consequences of inhibition of the fast Na^+ current (I_{Na}). Since V_{max} is an indicator of I_{Na} density, and is believed to be linearly related to I_{Na}, I_{Na} is the current which was most effectively blocked by articaine, considering the 162 μM of EC_{50} value obtained for the V_{max} block. In addition, the articaine-induced rate-dependent V_{max} block showed extraordinarily fast kinetics having offset time constant of 91 ms. Up to our best knowledge, this is the fastest time constant reported for I_{Na} blockade in cardiac tissues, suggesting that articaine may have class 1.B antiarrhythmic properties according to the Vaughan Williams classification. Suppression of I_{Na} is likely involved also in the articaine-induced shortening of action potentials, significant from the relatively low concentration of 10 μM, where articaine failed to significantly diminish V_{max}. It is not exceptional that a drug blocks the window Na^+ current at sufficiently low concentrations where the fast Na^+ current, and consequently V_{max}, remains unaffected . The other factor likely involved in the articaine-induced shortening of action potentials is the inhibition of I_{Ca}. Although articaine blocked I_{Ca} with the relatively high EC_{50} value of 471 μM under conventional voltage clamp conditions, action potential clamp experiments revealed that articaine evoked a much larger suppression of the inward than the late outward current peak within the

concentration rage of 30-100 μM congruently with its shortening effect. Depression of the action potential plateau may also be ascribed to the blockade of I_{Ca}. Finally, reduction of the amplitude of early (phase 1) repolarization may be explained by the inhibition of I_{to}, as it was demonstrated under both conventional and action potential voltage clamp conditions. Regarding the possible mechanism of the articaine-induced ion channel blockade it can be concluded that the drug likely associates with a single binding site on each channel protein (except for I_{K1}) as indicated by the Hill coefficients of close to unity.

5.2. Comparison with other local anaesthetics

As it was demonstrated in the voltage clamp experiments the EC_{50} values obtained with articaine ranged between 200 and 500 μM for the various ion currents. From this point of view articaine is markedly different from the other two most frequently used local anaesthetics, lidocaine and bupivacaine. For instance, bupivacaine was reported to suppress V_{max}, I_{to} and I_{Ca} more effectively than articaine. 1 μM bupivacaine reduced V_{max} by 26% I_{to} was blocked with an EC_{50} of 22 μM , and a 22% reduction of I_{Ca} was induced by 10 μM bupivacaine . Similar to articaine, I_{Na} was blocked by lidocaine with EC_{50} values of 95 and 226 μM , whereas the EC_{50} of lidocaine for blocking I_{Ca} was only 27 μM . This value is lower than our EC_{50} obtained with articaine more than one order of magnitude. Unlike articaine, neither bupivacaine, nor lidocaine inhibited I_{K1} up to concentrations of 1000 μM. However, relatively little differences were found in peak plasma concentrations measured in patients anaesthetized with bupivacaine, articaine, and lidocaine: typically values of 3-6 μM, 6-7 μM, and 5-10 μM were obtained, respectively.

Lidocaine is a potent class 1.B antiarrhythmic agent having cardiac side effects including negative inotropy, while bupivacaine was found to be proarrhythmic at higher concentrations. In contrast to bupicaine or lidocaine, no fatal cardiovascular complication has been reported with articaine up to now. Since the lowest concentration of articaine causing statistically significant changes in our study was higher than the usual peak plasma concentration measured with articaine in patients, it is likely that articaine fails to alter cardiac electrogenesis during normal anaesthesia. Although articaine was shown to

interfere with several cardiac ion currents at higher concentrations, its shortening effect on action potential duration was relatively moderate, and more importantly it seemed to saturate between 100 and 300 μM. Congruently with this, the clearest increase in the articaine-induced reduction of the late outward current peak was evident over this concentration range. Therefore one may conclude that the more and more pronounced aricaine-induced blockade of outward currents, appearing with increasing concentrations of articaine, can prevent the further shortening of action potential duration, which might be arrhythmogenic due to shortening of the refractory period. This property of articaine predicts less proarrhythmic potency comparing with other local anaesthetics in case of accidental overdose.

5.3. Cardiac effects of ropivacaine

This is the first study to analyze the effects of ropivacaine on ion currents in canine ventricular cardiomyocytes. It is essential, as the he canine cardiomyocytes are believed to be the best model of the human myocardium regarding the action potential and the density and distributation of the underlying ion channels. The results revealed that ropivacaine suppressed several ion currents in a concentration-dependent manner with the concomitant alterations of action potential morphology. These changes observed in the configuration of the action potential can be deduced from suppression of the various ion currents. I_{Na} is the current which was most effectively blocked by ropivacaine, considering the 81 μM of EC_{50} value obtained for V_{max} block at the frequency of 1 Hz. This is in a good agreement with reported voltage clamp data in guinea pig ventricular myocytes. However, when comparing this EC_{50} to the 41 μM obtained with bupivacaine, the effect of ropivacaine on I_{Na} seems to be weaker than that of bupivacaine. Similar conclusion was drawn from V_{max} measurements in multicellular guinea pig ventricular preparations. The offset kinetics of this rate-dependent ropivacaine block was estimated by fitting the ropivacaine data to a single exponential yielding an offset time constant of 340 ms. This correlates with the offset time constant of an average class 1.B. antiarrythmic agent. Besides its well-known sodium channel blocking effect, we demonstrated that ropivacaine also suppresses other inward and outward currents, as I_{Ca}, th I_{to} I_{Kr}, $I_{Ks,}$ and $I_{K1.}$

The clinical relevance of the present electrophysiological data can be evaluated only when comparing the concentrations used in our experiments to the ropivacaine plasma levels measured in patients during anesthesia. The peak plasma level in ropivacaine anesthesia may reach 2.6-2.9 mg/l corresponding to the concentration of 10-12 μM. Although the lowest ropivacaine concentration that caused statistical significant effects on V_{max} and phase-1 repolarization was 10 μM in our study, ropivacaine is not likely to markedly alter cardiac electrogenesis at plasma levels typically obtained during neuraxial or regional anesthesia. But what can be anticipated in cases of ropivacaine overdose or intoxication caused by accidental intravenous injection of the drug? Under such extraordinary conditions much higher plasma levels may occur: 40 μM when calculating with 14 l of total extracellular fluid volume, or 180 μM if calculated with the 3 l of plasma volume (after intravenous injection of an ampoule containing 20 ml of 7.5 mg/ml ropivacaine). Although the EC_{50} values obtained with ropivacaine for I_{Ca}, I_{Kr}, I_{K1}, and I_{to} were found within a relatively narrow range of 250 - 400 μM, suppression of inward currents during the action potential plateau seems to be dominant as indicated by the APD shortening observed at cycle lengths of 1000 ms or longer. This may account for the flat APD - frequency and APD - diastolic interval relationships observed with ropivacaine.

Surprisingly, washing out of ropivacaine resulted in a prolongation of action potential duration beyond its control value. This rebound effect can be explained by the differences in kinetics of washout from the various ion channels, as it was demonstrated with ropivacaine. The ropivacaine-induced inhibition of V_{max} (indicator of I_{Na}) was rapidly and fully reversible, and 80% of I_{Ca} was also restored within 10 min. In contrast, currents responsible for repolarization (I_{Kr}, I_{Ks}, and more importantly I_{K1}) showed only slow and incomplete recovery during the 10 min period of washout. The reason for this variance in reversibility is unknown, it is probably due to differences in intramolecular localization of the binding sites. It is noteworthy, however, that the molecular structure of Kir channels (responsible for I_{K1}) is basically different from those of other ion channels. As a consequence of the sustained block of outward currents seen upon washout, action potential duration is likely increased throughout the period of drug elimination following anesthesia. This carries an extra proarrhythmic risk for patients having inherited or acquired form of long QT syndrome. All these patients have reduced

repolarization reserve due to relative deficiency of outward compared with inward currents, and therefore they are more susceptible to further drug-induced APD lengthening which may result in development of early afterdepolarizations threatening with torsades de pointes-type ventricular arrhytmias. Although I_{Ks} has little influence on repolarization at normal heart rates, it is an important component of the repolarization reserve. Due to the relatively high I_{Ks} blocking potency of ropivacaine (EC_{50} = 106 μM), patients having normal APD at rest with already decreased repolarization reserve may also have an increased risk for arrhythmias. Finally, since the contribution of an ion current to the action potential is not uniform within the ventricular wall, the transient asymmetrical blockade of ion channels may further increase the incidence of arrhythmias due the increased dispersion of repolarization. Indeed, ventricular fibrillation and cardiac arrest were reported in anesthesia induced by ropivacaine similarly to cases of bupivacaine-induced anesthesia. In conclusion, the present results suggest that ropivacaine is not expected to induce cardiac complications when applied regularly in the normal population, however, under extraordinary conditions (such as accidental intravenous injection, case of overdose, or patients susceptible to arrhythmias) special care has to be taken not only during the anesthesia but in the postoperative recovery period as well.

5.4. **Mechanism of the negative inotropic action of articaine and ropivacaine**

Articaine and ropivacaine reduced contractility and $[Ca^{2+}]_i$ transients in concentration-dependent way in canine ventricular cells. Regarding articaine this is the first report, while ropivacaine, similarly to bupivacaine, has been previously shown to decrease $[Ca^{2+}]_i$ transients in ferret papillary muscles in concentrations comparable to the present results obtained in canine myocytes. No significant differences were obtained between the suppressive effects of articaine and ropivacaine on contractility, $[Ca^{2+}]_i$ transients, and I_{Ca}, suggesting that cardiodepressant side effects of the two drugs may also be similar. Comparing with bupivacaine, less cardiodepressant effects are anticipated with articaine

since bupivacaine was reported to cause stronger suppression of I_{Ca} than articaine or ropivacaine in canine ventricular myocytes. In line with these results, the negative inotropic action of ropivacaine was found to be significantly weaker than that of bupivacaine in canine, rabbit, and guinea pig cardiac preparations. Suppression of contractility and $[Ca^{2+}]_i$ transients were statistically significant at 10 μM concentration, and displayed very similar concentration dependences: the respective EC_{50} values were 74 and 87 μM for articaine, while 73 and 99 μM for ropivacaine. It can be concluded, therefore, that the decreased contractility is due to the reduction in the $[Ca^{2+}]_i$ transient. Suppression of I_{Ca} required somewhat higher concentrations: it was significant from 30 μM, and half maximal block occurred close to 300 μM. The magnitude of I_{Ca} inhibition, induced by articaine and ropivacaine in the present study, is in good agreement with previous results obtained in canine and guinea pig ventricular cells.

Suppression of $[Ca^{2+}]_i$ transients can be caused by altered kinetics of Ca^{2+} release and/or Ca^{2+} reuptake in the SR, or alternatively, due to changes in transsarcolemmal Ca^{2+} fluxes. Since at concentrations lower than 200 μM both drugs failed to modify Ca^{2+} release and Ca^{2+} uptake in HSR vesicles, and also left SERCA ATPase activity unaltered, changes in transmembrane Ca^{2+} movements seem to be the underlying mechanism. This is congruent with the reduction of I_{Ca} observed in the presence of articaine and ropivacaine. However, at the concentration of 10 μM no significant reduction in I_{Ca} was observed in contrast to the significant suppression of $[Ca^{2+}]_i$ transients and contractility. This discrepancy can probably be ascribed to inhibition of Na^+ current by these local anesthetics, that leads to reduction in $[Na^+]_i$ resulting in increased Ca^{2+} efflux and decreased Ca^{2+} influx through the Na^+/Ca^{2+} exchanger. In summary, the decreased Ca^{2+} content of the SR due to reduction of net transsarcolemmal Ca^{2+} influx may be the reason for the reduced amplitude of $[Ca^{2+}]_i$ transient, and the concomitantly diminished contractility.

5.5. Effects of local anesthetics on SR Ca^{2+} handling

Although neither articaine, nor ropivacaine influenced Ca^{2+} release and Ca^{2+} uptake at concentrations lower than 300 μM in our canine HSR vesicles, at higher concentrations both drugs significantly suppressed Ca^{2+} release and uptake with

the concomitant reduction of SERCA ATPase activity. From this point of view actions of the two drugs were somewhat different. The articaine-induced inhibition saturated around 0.5 mM and its maximal magnitude was approximately 20%, while the inhibitory effect of ropivacaine increased progressively with increasing concentrations of the drug, similarly to the action of lidocaine on canine SR ATPase. As could be expected, inhibition of ATPase activity showed good correlation with reduction of Ca^{2+} uptake. There is no explanation, however, for the apparently similar actions on the Ca^{2+} release and Ca^{2+} uptake observed with high concentrations of articaine or ropivacaine. In absence of relevant data in the literature on the effects of articaine and ropivacaine on RyR2, our results can be compared only with similar actions of other local anesthetics. Conductance of the RyR2 channel of the sheep was reduced by procaine and QX222, and ryanodine binding was decreased by bupivacaine and tetracaine in porcine SR vesicles. Except for tetracaine, which had an EC_{50} value of 100 μM, all these effects were evident only in millimolar concentrations, suggesting unspecific interactions between RyR2 and the local anesthetic compound.

5.6. Therapeutic implications

The clinical relevance of the present data can be evaluated only when comparing the concentrations used in our experiments to the plasma levels of articaine and ropivacaine measured in patients during anesthesia. The typically found peak plasma levels were 6-7 μM for both articaine and ropivacaine, although peak concentrations of 10-12 μM were also measured after administration of high doses of ropivacaine. Since the lowest concentrations of articaine and ropivacaine that caused statistically significant reduction in the amplitude of $[Ca^{2+}]_i$ transients and force of contraction was 10 μM in our study, neither articaine nor ropivacaine is likely to alter cardiac Ca^{2+} handling and contractility markedly at plasma levels typically obtained during neuraxial or regional anesthesia. However, in case of overdose or intoxication caused by accidental intravenous injection plasma levels may increase above 100 μM for a short period of time. These concentrations may strongly compromise the mechanical performance of the heart.

6. SUMMARY

Articaine and ropivacaine are local anesthetics used in clinical practice for a relatively short time. In spite of their widespread application, little is known about their cellular cardiac effects. The purpose of this work was to analyse the cellular cardiac electrophysiological effects of articaine and ropivacaine in isolated canine cardiomyocytes, a preparation wich is generally believed to be the best model of the human heart.

Articaine and ropivacaine caused concentration-dependent changes in the action potential configuration: decrease in the amplitude and the maximum velocity of depolarization, shortening of the action potential, suppression of phase-1 repolarization, and depression of plateau. Characterization of the block of sodium channels by the decrease in maximal rate of depolarization at 1 Hz pacing frequency yielded an EC_{50} of 162±30 μM, and 81±7 μM for articaine and ropivacaine respectively. Besides this tonic block, a frequency-dependent V_{max} blockade of fast offset kinetic was detected with both drugs, which could be characterized by an offset time constant of 91±20 ms, and 340±40 ms for articaine and ropivacaine respectively.

Under voltage clamp conditions a variety of ion currents were blocked by articaine and ropivacaine: L-type calcium current with an EC_{50} of 471±75 μM and 263±67 μM, transient outward current with an EC_{50} of 365±62 and 384±75 μM, inward rectifier potassium current with an EC_{50} of 372±46 μM and 372±35 μM, rapid delayed rectifier potassium current with an $EC_{50\ o}$ of 278±79 μM and 303±47 μM, and slow delayed rectifier potassium current with an EC_{50} of 326±65 μM and 106±18 μM for articaine and ropivacaine respectively. Drugs likely associate with a single binding site on each channel protein as indicated by the Hill coefficients of close to unity. Inhibition of different ion channels was also demonstrated under action potential voltage clamp.

Articaine and ropivacaine caused a concentration-dependent reduction in amplitude of $[Ca^{2+}]_i$ transients (EC_{50} = 87,4±12 és 99,3±17 μM), wich was congruent with the suppression of contractility (EC_{50} = 73,7±10 és 72,8±14 μM for articaine and ropivacaine respectively). Neither articaine nor ropivacaine influenced directly the Ca^{2+} release and Ca^{2+} uptake at concentrations lower than300 μM in

our canine HSR vesicles. No significant qualitative differences could be found between the negative inotropic effects of articaine and ropivacaine.

Since neither articaine nor ropivacaine caused significant changes in the electrophysiological and mechanical parameters of the cells in clinically relevant micromolar concentrations, we can conclude that both drugs can be used safely. Articaine and ropivacaine alter the cardiac action potential and the underlying ion currents at concentrations higher than the therapeutic range, caused by accidental venous injection or overdose. Under these circumstances fewer side-effects can be expected in the case of articaine than during ropivacaine overdose. In addition in the case of ropivacaine an increased proarrhythmic risk may be anticipated even in the postoperative recovery period.

7. List of publications

Szabó A , Szentandrássy N, Birinyi P, Horváth B, Szabó G, Bányász T, Márton I, Nánási PP, Magyar J. Effects of articaine on action potential characteristics and the underlying ion currents in canine ventricular myocytes. Br J Anaesth. 2007; 99:726-33 **[IF=2,948]**

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Szentandrássy N, **Szabó A** , Almássy J, Jóna I, Horváth B, Szabó G, Bányász T, Márton I, Nánási PP, Magyar J. Effects of articaine and ropivacaine on calcium handling and contractility in canine ventricular myocardium. Eur J Anaesthesiol. 2010; 27:153-61 **[IF=1,55]**

New results

The cellular electrophysiological effects of articaine were first analyzed in this study. Articaine decreases amplitude and duration of ation potential, maximum velocity of depolarization (V_{max}), suppresses phase 1 repolarization and causes plateau depression in a concentration-dependent manner. Besides tonic block a fast-kinetic use-dependent block of V_{max} is also developed. Articaine suppresses several ion currents (I_{Ca}, I_{to}, I_{K1}, I_{Kr}, I_{Ks}) in concentrations higher than blood levels during usual clinical applications, showing relatively balanced effect on inward and outward currents. Due to the latter, only moderate alteration in action potential configuration can be anticipated during an incidental overdose.

The effects of ropivacaine on ion currents in canine ventricular cardiomyocytes were first analysed in our study. Canine ventricular preparations are believed to be the best human model for action potential, density and distribution of underlying ion currents. Besides its well-known block of sodium current, ropivacaine also supresses other inward and outward currents (I_{Ca}, I_{to}, I_{Kr}, I_{Ks}, I_{K1}) underlying the action potential. At plasma levels higher than obtained during ropivacaine anesthesia -e.g.in case of incidental overdose- arrhythmias may be expected during and especially following the anesthesia, since elimination of ropivacaine's effect on inward and outward currents is imbalanced.

This is the first study to analyze the mechanism of negative inotropic effect of articaine and ropivacaine. Both articaine and ropivacaine caused a reversible and concentration-dependent decrease in amplitude of $[Ca^{2+}]_i$ transients, wich is congruent with the reduction in the contractility force. Ca^{2+} release, Ca^{2+} uptake and SERCA ATPase activity in the sarcoplasmic reticulum were not modified significantly at concentrations of articaine and ropivacaine below 200 μM. Since articaine and ropivacaine block L-type Ca^{2+} current with similar affinity (EC_{50} = 327 and 263 μM), their similar negative inotropic effect can be primarily attributed to the reduction of transmembrane Ca^{2+} influx, though inhibition of transmembrane Na^{+} influx presumably also contributes to the decrease of $[Ca^{2+}]_i$.