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Differential Effects of Bupivacaine and Ropivacaine Enantiomers on Intracellular Ca^{2+} Regulation in Murine Skeletal Muscle Fibers

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Background: **Increased intracellular Ca^{2+} concentrations are considered to be a major pathomechanism in local anesthetic myotoxicity. Racemic bupivacaine and *S*-ropivacaine cause Ca^{2+} release from the sarcoplasmic reticulum of skeletal muscle fibers and simultaneously inhibit Ca^{2+} reuptake. Examining the optical isomers of both agents, the authors investigated stereoselective effects on muscular Ca^{2+} regulation to get a closer insight in subcellular mechanisms of local anesthetic myotoxicity.**

Methods: ***R*- and *S*-enantiomers as well as racemic mixtures of both agents were tested in concentrations of 1, 5, 10, and 15 mM. Saponin-skinned muscle fibers from the extensor digitorum longus muscle of BALB/c mice were examined according to a standardized procedure. For the assessment of effects on Ca^{2+} uptake and release from the sarcoplasmic reticulum, agents were added to the loading solution and the release solution, respectively, and force and Ca^{2+} transients were monitored.**

Results: **The effects of *S*-enantiomers on both Ca^{2+} release and reuptake were significantly more pronounced than those of racemic mixtures and *R*-enantiomers, respectively. In addition, the effects of racemates were markedly stronger than those of *R*-enantiomers. With regard to Ca^{2+} release, the effects of bupivacaine isomers were more pronounced than the isomers of ropivacaine.**

Conclusions: **These data show that stereoselectivity is involved in alterations of intracellular Ca^{2+} regulation by bupivacaine and ropivacaine. *S*-enantiomers seem to be more potent than *R*-enantiomers, with intermediate effects of racemic mixtures. In addition, lipophilicity also seems to determine the extent of Ca^{2+} release by local anesthetics.**

LOCAL anesthetic–induced myotoxicity seems to be a rather uncommon side effect of local and regional anesthesia.[1] However, recent studies revealed that certain anesthesia techniques—especially retrobulbar and peribulbar blocks—are related to a relatively high postoperative rate of significant muscular dysfunction directly caused by these agents.[2] Therefore, skeletal muscle damage has to be considered a potentially serious complication after local anesthetic application.

Intramuscular injections of local anesthetics regularly result in striated muscle damage and myonecrosis, with an agent-specific and dose-dependent rate of toxicity.[3–5] In this respect, hypercontracted myofibrils are the first histologic signs to become evident several minutes after injection, followed by lytic degeneration of striated muscle sarcoplasmic reticulum (SR), myocyte edema, and myonecrosis over the next hours. Intriguingly, myoblasts, vasculature, and basal laminae are not affected, and therefore, tissue regeneration may be ensured within 2–4 weeks.[6] Subcellular pathomechanisms of local anesthetic myotoxicity are still not completely revealed in detail. However, Benoit *et al.* suggested that excessively increased intracellular Ca^{2+} concentrations play a key role in myocyte injury, inducing numerous pathways of cell death.[7,8]

In previous studies, we showed that bupivacaine as well as ropivacaine destroy skeletal muscle fibers at the injection site, with ropivacaine being characterized by a significantly lower rate of myotoxicity in comparison with bupivacaine.[8] These findings can be explained by the specific effects of both agents on intracellular Ca^{2+} regulation: Bupivacaine and—to a smaller extent—ropivacaine both induce Ca^{2+} release from the SR and simultaneously inhibit Ca^{2+} reuptake into the SR, resulting in persistently increased Ca^{2+} concentrations.[9–11]

However, in these studies, myotoxic effects of the clinically applied preparations of both local anesthetics (*i.e.*, racemic bupivacaine and pure *S*(−)-ropivacaine) have been experimentally assessed, and the observed differences theoretically may have been the result of stereoselective effects or different lipophilicity. Therefore, the current study was conducted to assess the influence of stereoselectivity and lipophilicity on alterations of myocyte Ca^{2+} regulation using the well-established model of skinned murine skeletal muscle fibers.

Materials and Methods

With approval of the Institutional Animal Care Committee of the University of Heidelberg (Heidelberg, Germany), we chose the well-established model of murine skinned fiber preparations, using an experimental protocol described before.[9,12–14] Altering the composition of respective experimental solutions, this model ensures the clear assessment of specific local anesthetic effects on both Ca^{2+} release from the SR and on gross Ca^{2+} reuptake.

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Table 1. Experimental Solutions

	LRS	HRS	HAS	LS
ATP, mM	8	8	8	8
Creatine phosphate, mM	10	10	10	10
Creatine kinase, U/l	150	150	150	150
HEPES, mM	30	30	30	30
EGTA, mM	0.3	30	30	30
HDTA, mM	29.7	—	—	—
Ca^{2+}, μM	—	—	24.9	0.4
Mg^{2+}, mM	0.4	0.4	0.4	0.4
Na^{+}, mM	36	36	36	36
K^{+}, mM	66	66	66	66

ATP = adenosine triphosphate; HAS = high activating solution; HDTA = 1,6-diaminohexane-N,N,N′,N′-tetraacetic acid; HRS = high relaxing solution; LRS = low relaxing solution; LS = loading solution.

Experimental Solutions

Table 1 summarizes the ionic concentrations of the different basic experimental solutions. All solutions were adjusted to a pH of 7.0 and contained 60 mM HEPES buffer, 8 mM adenosine triphosphate, and an adenosine triphosphate–regenerating system consisting of 10 mM creatine phosphate and 150 U/l creatine kinase. In addition, all solutions contained 66 mM K^+, 36 mM Na^+, and 0.4 mM Mg^{2+} (free ion concentrations).

The high activating solution (HAS; 24.9 μM Ca^{2+}), and the high relaxing solution (HRS; without Ca^{2+}) included 30 mM EGTA with a high affinity to Ca^{2+} for "clamping" the free Ca^{2+} concentration. In contrast, the low relaxing solution (LRS; without Ca^{2+}) included 0.3 mM EGTA but 29.7 mM HDTA with a very low affinity to Ca^{2+} ions. Free ionic concentrations and the ionic strength of all solutions (Γ/2 = 157 mM at a pH of 7.00 and 23.0°C) were calculated using the REACT program by Smith and Miller.[15]

The skinning solution was obtained by adding saponin (50 μg/ml) to the LRS, and the loading solution (LS; 0.41 μM Ca^{2+}) was obtained by mixing equal volumes of HAS and HRS. The release solutions (RSs) consisted of LRS with caffeine (10 mM) or with pure *R*- enantiomers, pure *S*-enantiomers, or racemic mixtures of bupivacaine and ropivacaine, respectively (AstraZeneca Laboratories, Södertalje, Sweden).

To assess the [Ca^{2+}]–force relation, HAS and HRS were appropriately mixed to obtain six different concentrations (0.41, 0.62, 0.96, 1.62, 3.39, and 24.9 μM) of free [Ca^{2+}].

Fiber Preparation and Experimental Protocol

BALB/c mice were exposed to carbon dioxide before being killed during deep anesthesia by cervical dislocation. Subsequently, the extensor digitorum longus muscle, which predominantly consists of fast-twitch fibers (> 95%), was carefully dissected and stored in paraffin oil at 4°C.[12–14] Subsequently, a fiber bundle consisting of one to three single muscle cells (diameter between 70 and 150 μm) was isolated and glued to a force transducer recording system (based on AE 801; SensoNor A.S., Horten, Norway) and to a micrometer-adjustable metal pin. Analyzing the diffraction pattern of a helium–neon laser beam the sarcomere length was adjusted to 2.6 μm.[9,12–14,16]

At the beginning of each experiment, the fibers were placed in the skinning solution for 5 min. After the skinning procedure, the fiber was dipped for 5 s into the RS to unload the SR, for 5 s into the HRS, and for 2 min into the LRS for equilibration. Then, the fibers' SRs were loaded with Ca^{2+} for 40 s in the LS, and subsequently, the preparations were dipped into the HRS for 5 s before transferring them again into the LRS for 2 min. In the following, the fibers were exposed to the respective RS, and the force transient induced by 10 mM caffeine was recorded. Finally, the maximal force transient was measured in the HAS at 24.9 μM Ca^{2+}. Because saponin skinning may reduce the Ca^{2+} loading capacity of the SR and to control the permeabilization procedure of the fibers, the initial caffeine release had to be at least 30% of maximal force; otherwise, the preparation was discarded.

To investigate the ability of all tested agents to induce Ca^{2+} release from the SR, the Ca^{2+} loaded fibers were exposed to a "modified" RS containing respective isomers or racemates, respectively, but no caffeine. Subsequently, all local anesthetic isomers and racemates were added to the LS, and force transients were measured after transferring the preparations into RSs containing caffeine alone. Finally, control measurements in the absence of all tested agents were performed to demonstrate the return to baseline.

At the end of the experimental protocol, individual [Ca^{2+}]–force relations were assessed for each fiber at different Ca^{2+} concentrations.[17] These measurements were performed without any addition to the solutions, with caffeine, and with bupivacaine and ropivacaine isomers and racemates, respectively (table 2).

Throughout this protocol, all measurements were performed at four different concentrations of the agents (1, 5, 10, and 15 mM). Two to four fiber bundles per animal were dissected, and six fiber bundle preparations per agent were examined at each concentration level.

Data Analyses and Statistics

All force transients were normalized to the corresponding maximum force at 24.9 μM Ca^{2+}. [Ca^{2+}]–force relations representing the individual Ca^{2+} sensitivity of the contractile apparatus were quantified by fitting a Hill curve to the measured data points.[17] Each curve is characterized by the Hill coefficient (n) as an indicator of maximal steepness of the sigmoid graph and the pCa_{50} value (negative decadal logarithm of [Ca^{2+}] at which isometric force is half maximal) as an indicator of Ca^{2+} sensitivity of the contractile apparatus. For each fiber, the characteristic n and pCa_{50} values were used to transform force transients into free myoplasmic Ca^{2+} transients according to an algorithm described by Uttenweiler *et al.*[18]

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Table 2. Experimental Protocol

	Ca^{2+} Loading	Release
1. Control	LS	RS
2.	LS	LRS + local anesthetics (1/5/10/15 mM)
3.	LS + local anesthetics (1/5/10/15 mM)	RS
4. Control	LS	RS
5. [Ca^{2+}]/force relation	0.03–24.9 μM Ca^{2+}	
6. [Ca^{2+}]/force relation	0.03–24.9 μM Ca^{2+} + caffeine (10 mM)	
7. [Ca^{2+}]/force relation	0.03–24.9 μM Ca^{2+} + local anesthetics (1/5/10/15 mM)	

Local anesthetics: *R*-ropivacaine, racemic ropivacaine, *S*-ropivacaine, *R*-bupivacaine, racemic bupivacaine, and *S*-bupivacaine.
LRS = low relaxing solution; LS = loading solution; RS = release solution.

For statistical analysis, the Kolmogorov-Smirnov test to confirm normal distribution for each group, one-way analysis of variance, and the Student *t* test with Bonferroni correction. All *P* values were two tailed and were considered significant at 0.05 or less. All data are presented as mean ± SD.

Results

Ca^{2+} Release from the Sarcoplasmic Reticulum

The force transients induced by the tested isomers and racemates are shown in figure 1A. Using fiber-specific n and pCa_{50} values, these transients have subsequently been transformed into corresponding Ca^{2+} transients (fig. 1B): *R*-ropivacaine, *S*-ropivacaine, and racemic ropivacaine dose-dependently induced Ca^{2+} transients, whereas the effects of racemic ropivacaine were markedly stronger than those of the *R*-enantiomer but less pronounced than those of the *S*-isomer.

Analogously, the dose-dependent effects of *R*-bupivacaine on Ca^{2+} release from the SR were smaller than those of the racemate, and again, the *S*-isomers showed the strongest effects on Ca^{2+} release.

At the concentrations of 5, 10, and 15 mM, *S*-bupivacaine was more potent than *S*-ropivacaine, whereas racemic bupivacaine was more potent than racemic ropivacaine at some concentrations but not at others. Similarly, *R*-bupivacaine was more potent than *R*-ropivacaine at some concentrations but not at others.

Ca^{2+} Reuptake into the Sarcoplasmic Reticulum

Figure 2A shows caffeine-induced force transients after Ca^{2+} loading in the presence of all tested agents. Again, these transients have been transformed into corresponding Ca^{2+} transients with the help of fiber-specific n and pCa_{50} values (fig. 2B): The presence of *R*- ropivacaine, *S*-ropivacaine and racemic ropivacaine (5, 10, and 15 mM) during the loading procedure dose-dependently decreased caffeine-induced Ca^{2+} transients.

At the concentrations of 5, 10, and 15 mM, *S*-enantiomers of both ropivacaine and bupivacaine were more potent than the corresponding *R*-enantiomers. In addition, racemic ropivacaine was more potent than *R*-ropivacaine at some concentrations, but in contrast, the effects of racemic bupivacaine were not different from those of *R*-bupivacaine.

When compared with the corresponding enantiomers, *R*-bupivacaine was more potent than *R*-ropivacaine at 5 and 15 mM but not at 10 mM. However, at all tested concentrations, there was no significant difference between racemic bupivacaine and racemic ropivacaine. In addition, *S*-bupivacaine was more potent than *S*-ropivacaine at 5 mM but not at 10 or 15 mM.

According to our experimental protocol, another control measurement was performed after these experiments, to exclude long-term effects of after washout. There was no significant difference between these force transients and the transients assessed at the beginning of our protocol (data not shown graphically).

Ca^{2+} Sensitivity and Hill Coefficient Values

Changes in pCa_{50} values are displayed in figure 3A, representing alterations in Ca^{2+} sensitivity of the contractile apparatus in the presence of the tested agents. In comparison with control measurements, there was a dose-dependent increase in pCa_{50} after exposure to all examined local anesthetics. Changes in Hill coefficients are shown in figure 3B. These values decreased in a dose-dependent way in the presence of all local anesthetics, with effects being most pronounced at concentrations greater than 5 mM. However, with regard to changes in both parameters, no obvious differences between the experimental groups were found.

Discussion

An excessive increase in intracellular Ca^{2+} concentrations has been revealed to be an important mechanism in local anesthetic myotoxicity.[7] In this respect, highly myotoxic (racemic) bupivacaine has recently been identified to induce Ca^{2+} release from the SR and to inhibit Ca^{2+} reuptake simultaneously.[9,19] To a minor extent, however, the clinically used pure optical *S*-isomer of ropivacaine, which is characterized by a more moderate rate of myotoxicity *in vivo*, also induces Ca^{2+} release from the SR and inhibits reuptake.[8,10,11] In theory, these

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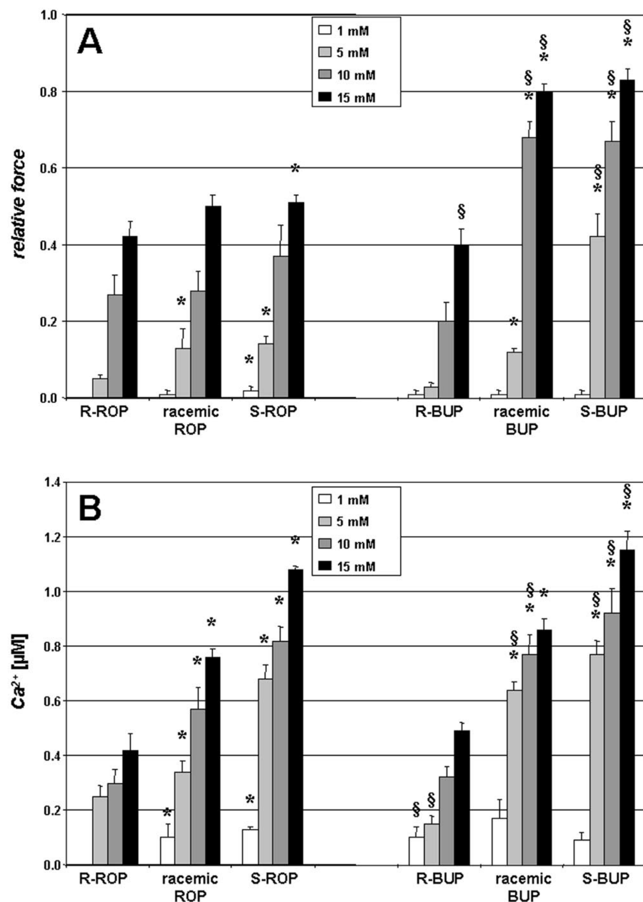

Fig. 1. (*A*) Mean force transients induced by the optical isomers and racemates of ropivacaine and bupivacaine, respectively, in concentrations of 1, 5, 10, and 15 mM. All force transients are normalized to maximum force transient at 24.9 µM Ca^{2+}. * *P* < 0.05 *versus R*-isomer in identical concentrations; § *P* < 0.05 *versus* corresponding ropivacaine isomers/racemate in identical concentrations. n = 6 fiber bundle preparations per agents at each concentration. (*B*) Transformation of force transients induced by tested local anesthetics (fig. 1A) into corresponding Ca^{2+} transients, based on the individual [Ca^{2+}]–force relation of each fiber. * *P* < 0.05 *versus R*-isomer in identical concentrations; § *P* < 0.05 *versus* corresponding ropivacaine isomers/racemate in identical concentrations. n = 6 fiber bundle preparations per agents at each concentration. BUP = bupivacaine; ROP = ropivacaine.

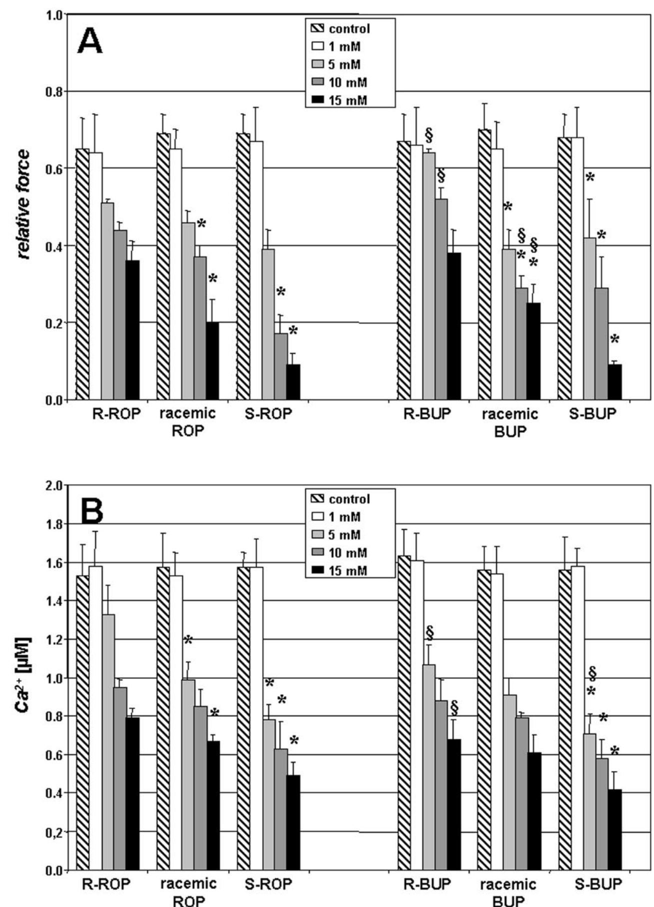

Fig. 2. (*A*) Caffeine-induced force transients after loading in the presence of the racemates and isomers of ropivacaine and bupivacaine, respectively, in concentrations of 1, 5, 10, and 15 mM. In comparison with control transients, all tested agents in concentrations of 5 mM or greater significantly decreased force transients (except *R*-bupivacaine at 5 mM; bars not marked for statistical significance). * *P* < 0.05 *versus R*-isomer in identical concentrations; § *P* < 0.05 *versus* corresponding ropivacaine isomers/racemate in identical concentrations. n = 6 fiber bundle preparations per agents at each concentration. (*B*) Transformation of force transients (fig. 2A) into corresponding [Ca^{2+}] transients. Again, all tested isomers and racemates, respectively, in concentrations of 5 mM or greater significantly decreased caffeine-induced Ca^{2+} transients in comparison with control values (bars not marked for statistical significance). * *P* < 0.05 *versus R*-isomer in identical concentrations; § *P* < 0.05 *versus* corresponding ropivacaine isomers/racemate in identical concentrations. n = 6 fiber bundle preparations per agents at each concentration. BUP = bupivacaine; ROP = ropivacaine.

obvious differences between toxic potencies might be the result of stereoselective effects on intracellular Ca^{2+} homeostasis or of the different lipophilicity of both agents. Therefore, the current study was conducted to assess the impact of stereoselective effects and lipophilicity of local anesthetics on Ca^{2+} regulation by the SR in murine striated myocytes. Testing the pure optical isomers as well as the racemates of ropivacaine and bupivacaine, respectively, we found out that all agents induce Ca^{2+} release from the SR and inhibit Ca^{2+} reuptake. However, *S*-enantiomers in general seemed significantly more effective than *R*-enantiomers, with intermediate effects of racemic mixtures.

Optical isomers, with the exception of rotation of the plane of light, share all physicochemical properties but have an asymmetric carbon atom. Because *S*-isomers of long-acting pipecoloxylidide derivatives have been recognized to be less cardiotoxic, *S*-ropivacaine and levobupivacaine (*S*-bupivacaine) have recently been introduced into clinical practice.[20] Although both levobupivacaine and ropivacaine are pure optical *S*-isomers, the butyl group of bupivacaine is replaced by a propyl group for ropivacaine. This change of molecular structure affects major physicochemical characteristics, such as molecular weight and lipophilicity, while not significantly affecting the pKa value.[20] In addition, both

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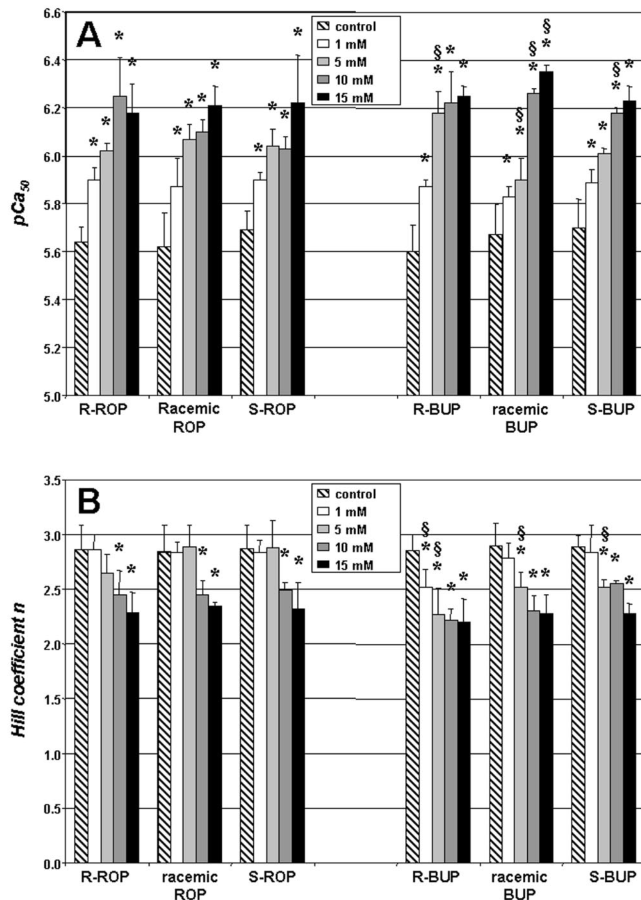

Fig. 3. (*A*) pCa_{50} values (representing Ca^{2+} sensitivity of the contractile apparatus), assessed in the presence of isomers and racemates of bupivacaine and ropivacaine, respectively. No significant differences were found between experimental groups. * $P < 0.05$ in comparison with control values, assessed in the absence of local anesthetics; § $P < 0.05$ *versus* corresponding ropivacaine isomers/racemate in identical concentrations. n = 6 fiber bundle preparations per agents at each concentration. (*B*) Hill coefficients n, assessed in the presence of isomers and racemates of bupivacaine and ropivacaine, respectively. Again, no obvious differences were found between experimental groups. * $P < 0.05$ in comparison with control values, assessed in the absence of local anesthetics; § $P < 0.05$ *versus* corresponding ropivacaine isomers/racemate in identical concentrations. n = 6 fiber bundle preparations per agents at each concentration. BUP = bupivacaine; ROP = ropivacaine.

local anesthetics are characterized by similar pharmacokinetic profiles on protein binding and metabolism. Differences between the isomers of the anesthetics are caused by stereoselective binding of the isomers to the target site, which itself must be optically active.[20]

To systematically assess effects on intracellular Ca^{2+} regulation of striated myocytes, the pure optical isomers as well as the racemates of bupivacaine and less lipophilic ropivacaine, respectively, were examined in concentrations of 1, 5, 10, and 15 mM. With special regard to local anesthetic myotoxicity, these obviously high concentrations were necessary to simulate direct exposure of the myocytes to these agents in clinically relevant concentrations (15 mM = 0.5% bupivacaine solution).[1,9] As an experimental approach, we have chosen the well-established model of saponin-skinned murine muscle fibers to obtain direct information about the effects of ropivacaine and bupivacaine isomers on intracellular Ca^{2+} regulation by the SR and on Ca^{2+} sensitivity of the contractile proteins. In this experimental setup, isometric force transients are directly measured and mathematically transformed into corresponding intracellular Ca^{2+} transients.[12–14,17,18] This procedure is possible because several studies on intracellular Ca^{2+} distribution using fluorescence microscopy and digital image analysis have shown that because of the fast Ca^{2+} binding kinetics of troponin C, the time course of caffeine-induced force transients occurs under quasi–steady state conditions for intracellular Ca^{2+} concentrations and force.[14,21,22] Nevertheless, methodologic limitations of this model must be mentioned because intracellular $[Ca^{2+}]$ is not directly determined but only inferred from mechanical transients. It remains undetermined whether our results can be entirely transferred to slow-twitch fibers because fast-twitch fibers are predominant in murine extensor digitorum longus muscles.[12–14] However, marked differences between local anesthetic effects on both fiber types are not expected to be probable.[12–14,17,21]

In the current study, we found out that all tested local anesthetics dose-dependently induce Ca^{2+} release from the SR, inhibit Ca^{2+} reuptake (indicated by significantly decreased caffeine induced transients after loading in the presence of these agents), and sensitize the contractile apparatus to free Ca^{2+}. However, despite qualitative similarities in these effects on intracellular Ca^{2+} regulation, there were marked quantitative differences: At least in concentrations of 5 mM or greater, the effects of *S*-isomers of ropivacaine and bupivacaine on Ca^{2+} release and reuptake were significantly more pronounced than those of corresponding *R*-enantiomers, with intermediate effects of racemates in equimolar concentrations. These data prove that stereoselectivity is involved in local anesthetic effects on intracellular Ca^{2+} homeostasis in murine striated myocytes. According to the results of previous examinations, these alterations in myocyte Ca^{2+} regulation could be explained by direct interactions of local anesthetic isomers with the Ca^{2+} release channel ryanodine receptor (RyR) as well as with Ca^{2+}–adenosine triphosphatases of the SR[9,19,23]: Three forms of RyR have been identified, whereas RyR1 is expressed predominantly in skeletal muscle fibers.[24] Numerous agents have been shown to activate RyR1 and thus to induce Ca^{2+} release from the SR by increasing the opening probability of this receptor complex, but to our knowledge, no stereoselective effects on RyR1 have been described.[24] In contrast to RyR1 activation, stereoselectivity has been shown to be involved in the inhibition of SERCA adenosine triphosphatases by thapsigargin derivatives.[25] However, despite the fact that our current

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data clearly indicate stereoselective interactions of ropivacaine and bupivacaine isomers with both SR channels, basic molecular mechanisms of these observations remain unrevealed because our experimental setup is not suitable to clear this issue.

Another result of this experimental study was that the effects of highly lipophilic bupivacaine isomers on Ca^{2+} release were significantly more distinct than those of corresponding ropivacaine isomers in identical concentrations. In contrast, no significant differences were found between bupivacaine and ropivacaine enantiomers with regard to the inhibition of Ca^{2+} reuptake and to changes in Ca^{2+} sensitivity of the contractile apparatus, respectively. Therefore, next to stereoselectivity, we suggest that physicochemical properties of local anesthetics, particularly the specific lipophilicity, may also determine the extent of Ca^{2+} release from the SR in skeletal muscle fibers. Again, molecular mechanisms of these observations remain unrevealed.

In conclusion, we have shown that stereoselectivity is involved in alterations of striated myocyte Ca^{2+} homeostasis by pipecoloxylidide derivatives, with specific effects of *S*-isomers seeming most pronounced in inducing Ca^{2+} release from the SR and in inhibiting reuptake. In addition, at least with regard to Ca^{2+} release from the SR, specific lipophilicity of local anesthetic isomers seems to determine the extent of effects. Therefore, both stereoselectivity and specific lipophilicity are strongly supposed to play an important role in the pathogenesis of local anesthetic myotoxicity.

However, it remains doubtful whether these *in vitro* findings are appropriate to explain and determine differences between myotoxic potencies of local anesthetic agents *in vivo*. After continuous peripheral nerve blocks, racemic bupivacaine and *S*-ropivacaine in equipotent concentrations both induce skeletal muscle fiber necrosis, but racemic bupivacaine causes tissue damage of a significantly larger extent.[8] Intriguingly, with regard to our current findings and postulating a relative anesthetic potency of 1.5:1,[26] *S*-ropivacaine in equipotent concentrations seem more potent in increasing intracellular Ca^{2+} concentrations because specific effects of *S*-ropivacaine (15 mM) on both Ca^{2+} release and reuptake exceeded those of racemic bupivacaine (10 mM). This obvious contradiction might be resolved by the fact that because of its pronounced lipophilicity, racemic bupivacaine rapidly perturbs sarcolemmal structures, massively accumulates in the myoplasm, and thus may induce an immediate increase in intracellular Ca^{2+} concentrations *in vivo*. Consequently, the effective intracellular concentration of racemic bupivacaine is strongly supposed to be higher than that of less lipophilic *S*-ropivacaine as the latter—even when administered in higher, equipotent concentrations—perturbs the sarcolemmal barrier and accumulates intracellularly markedly slower.[1,8] Summarizing all these results and findings, we postulate that although tissue damage is likely to be a function of intracellular [Ca^{2+}], it may not be proportional to specific effects on Ca^{2+} regulation assessed in our *in vitro* study. Therefore, further studies are warranted to assess the various degrees of skeletal muscle toxicity for all tested compounds and to confirm this hypothesis.

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