Anesthesiology 2003; 98:1139–46

Local Anesthetics Modulate Neuronal Calcium Signaling through Multiple Sites of Action

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Background: **Local anesthetics (LAs) are known to inhibit voltage-dependent Na^+ channels, as well as K^+ and Ca^{2+} channels, but with lower potency. Since cellular excitability and responsiveness are largely determined by intracellular Ca^{2+} availability, sites along the Ca^{2+} signaling pathways may be targets of LAs. This study was aimed to investigate the LA effects on depolarization and receptor-mediated intracellular Ca^{2+} changes and to examine the role of Na^+ and K^+ channels in such functional responses.**

Methods: **Effects of bupivacaine, ropivacaine, mepivacaine, and lidocaine (0.1–2.3 mM) on evoked $[Ca^{2+}]_i$ transients were investigated in neuronal SH-SY5Y cell suspensions using Fura-2 as the intracellular Ca^{2+} indicator. Potassium chloride (KCl, 100 mM) and carbachol (1 mM) were individually or sequentially applied to evoke increases in intracellular Ca^{2+}. Coapplication of LA and Na^+/K^+ channel blockers was used to evaluate the role of Na^+ and K^+ channels in the LA effect on the evoked $[Ca^{2+}]_i$ transients.**

Results: **All four LAs concentration-dependently inhibited both KCl- and carbachol-evoked $[Ca^{2+}]_i$ transients with the potency order bupivacaine > ropivacaine > lidocaine ≥ mepivacaine. The carbachol-evoked $[Ca^{2+}]_i$ transients were more sensitive to LAs without than with a KCl prestimulation, whereas the LA-effect on the KCl-evoked $[Ca^{2+}]_i$ transients was not uniformly affected by a carbachol prestimulation. Na^+ channel blockade did not alter the evoked $[Ca^{2+}]_i$ transients with or without a LA. In the absence of LA, K^+ channel blockade increased the KCl-, but decreased the carbachol-evoked $[Ca^{2+}]_i$ transients. A coapplication of LA and K^+ channel blocker resulted in larger inhibition of both KCl- and carbachol-evoked $[Ca^{2+}]_i$ transients than by LA alone.**

Conclusions: **Different and overlapping sites of action of LAs are involved in inhibiting the KCl- and carbachol-evoked $[Ca^{2+}]_i$ transients, including voltage-dependent Ca^{2+} channels, a site associated with the caffeine-sensitive Ca^{2+} store and a possible site associated with the IP_3-sensitive Ca^{2+} store, and a site in the muscarinic pathway. K^+ channels, but not Na^+ channels, seem to modulate the evoked $[Ca^{2+}]_i$ transients, as well as the LA-effects on such responses.**

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Department of Anesthesiology, New York University Medical Center, New York, New York. Submitted for publication August 19, 2002. Accepted for publication December 17, 2002. Dedicated to the memory of Eraldo Antonini, Professor of Biochemistry at the University of Rome, eminent biochemist, prematurely deceased twenty years ago, on March 19th, 1983. Supported by grant No. R01GM50686 from the National Institutes of Health, Bethesda, Maryland (to Dr. Blanck), and in part by the Anesthesia Research Fund of the Hospital for Special Surgery, New York, New York. Presented in part at the American Society of Anesthesiologists Annual Meeting, October 14-18, 2000, San Francisco, California, October 12-16, 2002, Orlando, Florida.

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THE majority of work concerning the mechanisms of local anesthetics (LAs) has focused on LA actions on the Na^+ and K^+ channels. LAs are known to reduce both Na^+ and K^+ currents,[1–3] which contribute to their anesthetic action. However, neuronal excitability is also determined by Ca^{2+} availability which is controlled by regulatory mechanisms of cytosolic Ca^{2+} ($[Ca^{2+}]_i$). Alteration of those regulatory mechanisms by LAs can lead to changes in presynaptic transmission and postsynaptic excitability. A few studies have shown that bupivacaine, ropivacaine, and lidocaine can affect the voltage-dependent Ca^{2+} currents in different systems.[4–6]

In the neuron-like human neuroblastoma cell line, SH-SY5Y, we have previously demonstrated that volatile anesthetics can either augment or attenuate intracellular Ca^{2+} responses to stimuli.[7] The net result is determined by the action of volatile anesthetics on the entire spectrum of Ca^{2+} regulatory mechanisms, including Ca^{2+}-entry, -mobilization, and -removal (-extrusion and -sequestration). Due to the essential role of intracellular Ca^{2+} in physiological functions and neuronal excitability, we wanted to determine whether LAs also affected intracellular Ca^{2+} signaling and the regulation of Ca^{2+} homeostasis in a similar manner as the volatile anesthetics.

In the present study, we have chosen four LAs commonly used in our clinical practice. Bupivacaine, ropivacaine, mepivacaine, and lidocaine are all amide-linked LAs. Bupivacaine, ropivacaine, and mepivacaine differ only in the length of an alkyl side chain (R) on the piperidine ring contained in their amine domain (R = C_4H_9 for bupivacaine, R = C_3H_7 for ropivacaine, and R = CH_3 for mepivacaine), while lidocaine differs from the other three in the composition of its amine group (no piperidine ring) and the length of its intermediate chain. These four LAs have different hydrophobicities (the octanol/buffer partition coefficients) correlating with their very different anesthetic potency.[8,9]

To assess the LA-effects on evoked $[Ca^{2+}]_i$ transients, single (100 mM potassium chloride [KCl] or 1 mM carbachol) or sequential (100 mM KCl followed by 1 mM carbachol and *vice versa*) stimuli were used. In addition, we examined whether the LA-effects on the evoked $[Ca^{2+}]_i$ transients were related to their actions on Na^+ and/or K^+ channels, using coapplication of LAs and Na^+ or K^+ channel blockers. Our results showed distinct LA-effects on KCl- and carbachol-evoked $[Ca^{2+}]_i$ transients with no clear involvement of Na^+ channels, but involvement of K^+ channels. In addition, the filling sta-

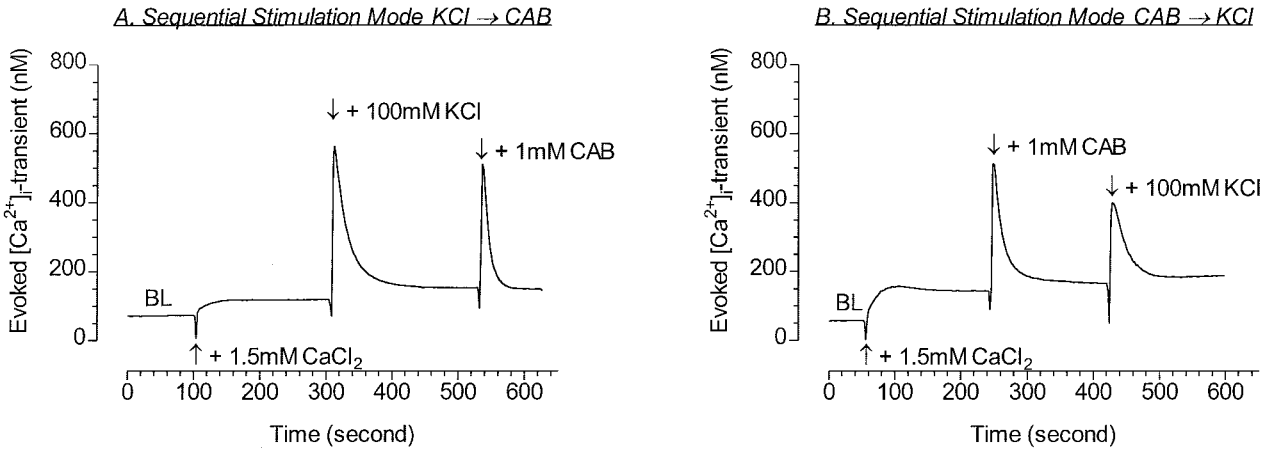

Fig. 1. Representative traces of $[Ca^{2+}]_i$ responses in sequential stimulation using 100 mM KCl followed by 1 mM carbachol (CAB) (*A*) or 1 mM CAB followed by 100 mM KCl (*B*).

tus of the Ca^{2+} stores appeared to modulate the LA effect on KCl- and carbachol-evoked $[Ca^{2+}]_i$ transients.

Materials and Methods

All chemicals used for this study were obtained from Sigma Chemicals (St. Louis, MO), unless otherwise indicated.

Cell Culture

Human SH-SY5Y neuroblastoma cells (originally provided by June Biedler, Ph.D., Sloan-Kettering Institute for Cancer Research, Rye, NY, at the time that the cells were provided; current position and affiliation, Distinguished Resident Scientist, Fordham University, Bronx, NY), passages 58–77, 90, 100–101, were maintained in RPMI 1640 medium containing 12% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and fungizone (1 μg/ml), passaged every 3 weeks. All cell culture components were GIBCO BRL products and purchased from Life Technologies (Rockville, MD).

Ca^{2+} Measurements

Cells in confluent culture were loaded with 5 μM Fura-2/AM (Molecular Probes, Eugene, OR) for 30 min at 37°C in the original culture medium. After removal of the dye-containing medium, the cells (still attached) were rinsed twice with Dulbecco phosphate-buffered saline without $CaCl_2$ and $MgCl_2$ (Gibco BRL, Rockville, MD) with 10 mM glucose-addition (pH 7.4), then gently resuspended in an incubation buffer (pH 7.4) containing 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose. Cells were incubated in incubation buffer containing 1.5 mM $CaCl_2$ for 1 h at room temperature to allow recovery. After a brief centrifugation (1 min at 300 rpm) prior to each measurement, the cell pellet was resuspended in the same incubation buffer and thermo-equilibrated at 37°C for 5 min. After a short baseline recording, 1.5 mM $CaCl_2$ was added to the suspension 3 min before cells were challenged by either 100 mM KCl or 1 mM carbachol, or one of the two sequential stimulation modes (see section on Sequential Stimulation and fig. 1). Fluorescence ratios were monitored at 510 nm after alternated excitation at 340 and 380 nm by an SLM 8000 Aminco fluorescence spectrofluorometer (Aminco, Urban, IL). Maximal and minimal fluorescence ratios were acquired by using 0.01% sodium dodecyl sulfate and 60 mM ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid, and $[Ca^{2+}]_i$ was then determined from fluorescence ratios based on the method of Grynkiewicz *et al.*[10] and a K_d of fura-2 for Ca^{2+} of 224 nM, as described previously.[7]

Sequential Stimulation

In order to investigate the contribution of different calcium regulatory mechanisms on evoked $[Ca^{2+}]_i$ transients and to monitor the dynamic interaction between different intracellular Ca^{2+} stores, sequential stimulation was applied as follows: the cells, in the presence and absence of LA ± tetrodotoxin or tetraethylammonium (TEA), were challenged by either 100 mM KCl followed by 1 mM carbachol (a muscarinic receptor agonist and Ca^{2+}-releaser from the IP_3-sensitive store), or 1 mM carbachol followed by 100 mM KCl (fig. 1).

Drug Treatment

In the drug-treated groups, cells were preincubated with LA ± tetrodotoxin/TEA for 5 min at 37°C prior to $CaCl_2$-addition as follows:

1. LA (-) tetrodotoxin/TEA: Preincubation with one of the following LAs: bupivacaine (0.75% Sensorcaine-MPF, Astra Pharmaceutical Products, Westborough, MA, USA), or ropivacaine (0.5% Naropin, Astra USA), or mepivacaine (1.5% Polocaine-MPF, Astra Pharmaceutical Products, USA), or lidocaine (2% Lidocaine HCl,

Abbott Laboratories, Chicago, IL, USA). The concentrations applied varied from 0.1 to 2.3 mM for each LA.
2. Tetrodotoxin/TEA (-) LA: Preincubation with either 1 μM tetrodotoxin, or 10 mM TEA;
3. Tetrodotoxin (+) LA: Preincubation with 1 μM tetrodotoxin plus one of the LAs at 2.3 mM in both KCl- and carbachol-stimulation.
4. TEA (+) LA: Preincubation with 10 mM TEA plus one of the LAs.

The LA concentrations applied in this series of measurements were as follows: in KCl-stimulation, 0.25 mM for bupivacaine, 0.5 mM for ropivacaine and lidocaine, 1 mM for mepivacaine, respectively; in carbachol-stimulation, 0.25 mM for all four LAs. The selection of these LA-concentrations was based on the dose–response curve for each LA (see Results) for their inhibition of KCl- or carbachol-evoked $[Ca^{2+}]_i$ transients. The concentrations chosen resulted in partial inhibition of the evoked $[Ca^{2+}]_I$ transients so that a further effect by TEA in either direction could be observed.

Data Analysis

Comparison between different groups was performed using one-way ANOVA (Student-Neuman-Keuls test or Dunnett test) conducted by Sigmastat software (Jandel Scientific, San Rafael, CA). Comparison between two curves using F test was conducted by GraphPad Prism (Graphpad Software, San Diego, CA). A significance was considered to be $P < 0.05$.

Results

Local Anesthetics Inhibited Both KCl- and Carbachol-evoked $[Ca^{2+}]_i$ Transients

In the absence of LA, the basal $[Ca^{2+}]_i$ measured was 61 ± 19 nM (mean ± SD, n = 536) in SH-SY5Y cells incubated in nominally Ca^{2+}-free buffer. Upon adding 1.5 mM $CaCl_2$, the $[Ca^{2+}]_i$ reached a higher steady state level of 93 ± 42 (mean ± SD, n = 157). None of the LAs showed significant effect on this Ca^{2+}-evoked $[Ca^{2+}]_i$ increase prior to stimulation (fig. 2).

Figures 1A and 1B are control traces representing the two sequential stimulation modes used in the present study. While the KCl-evoked $[Ca^{2+}]_i$ transient is dependent on the external Ca^{2+}, the carbachol-evoked $[Ca^{2+}]_i$ transient can be induced in the nominally Ca^{2+}-free medium in freshly prepared cells (data not shown). Both KCl- and carbachol-evoked $[Ca^{2+}]_i$ transients were significantly inhibited in a concentration-dependent fashion by bupivacaine, ropivacaine, mepivacaine, and lidocaine at concentrations 0.1–2.3 mM, which are lower than those applied to nerves during spinal or nerve blocks[11,12] but higher than those found in the plasma during epidural anesthesia.[13,3] Nonlinear fitting of the dose–response curves (figs. 3A and 3B) showed the order of the IC_{50} values as follows: bupivacaine < ropivacaine < lidocaine < mepivacaine and bupivacaine ≈ ropivacaine < lidocaine ≈ mepivacaine for KCl-evoked and carbachol-evoked $[Ca^{2+}]_i$ transient without a prestimulation, respectively (table 1). The difference of IC_{50} values between the most (bupivacaine) and the least (mepivacaine) potent LAs for inhibition of the KCl-evoked $[Ca^{2+}]_i$ transients was elevenfold (fig. 3A & table 1). Statistical analysis also showed that the differences between four LAs for their inhibitory effects on the KCl-evoked $[Ca^{2+}]_i$ transients at different LA concentrations were mostly significant ($P < 0.05$, table 2a). In contrast, the IC_{50} range (0.13–0.28 mM) for inhibiting the carbachol-evoked $[Ca^{2+}]_i$ transients was much narrower (fig. 3B, table 1). In fact, the potency of the four LAs was shown to be very similar ($P > 0.05$, table 2) in their inhibition of the carbachol-evoked $[Ca^{2+}]_i$ transients.

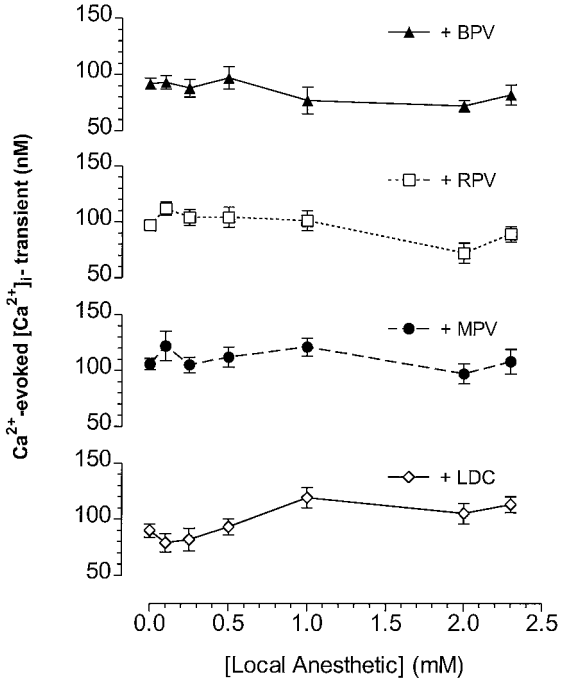

Fig. 2. Effects of local anesthetics (LAs) bupivacaine (BPV), ropivacaine (RPV), mepivacaine (MPV), and lidocaine (LDC) on the Ca^{2+}-evoked $[Ca^{2+}]_i$ increases (the baseline $[Ca^{2+}]_i$ increases). Cells were preincubated with one of the four LAs (0.1–2.3 mM) for 5 min before 1.5 mM $CaCl_2$ as added. Values presented are mean ± SEM, (n = 4–88). The difference between control- and LA-treated groups was tested using the Dunnett test. No significance was found between control and treated groups at any LA concentration.

Using sequential stimulation, *i.e.*, 100 mM KCl followed by 1 mM carbachol or *vice versa*, the LA effects on both KCl-evoked and carbachol-evoked $[Ca^{2+}]_i$ transients were further examined. All four LAs inhibited both carbachol-evoked and KCl-evoked $[Ca^{2+}]_i$ transients in a concentration-dependent manner when the intracellular Ca^{2+} stores were partially depleted by a KCl or a carbachol prestimulation, respectively. Concentration–response curves (figs. 3C and 3D) showed an order of the

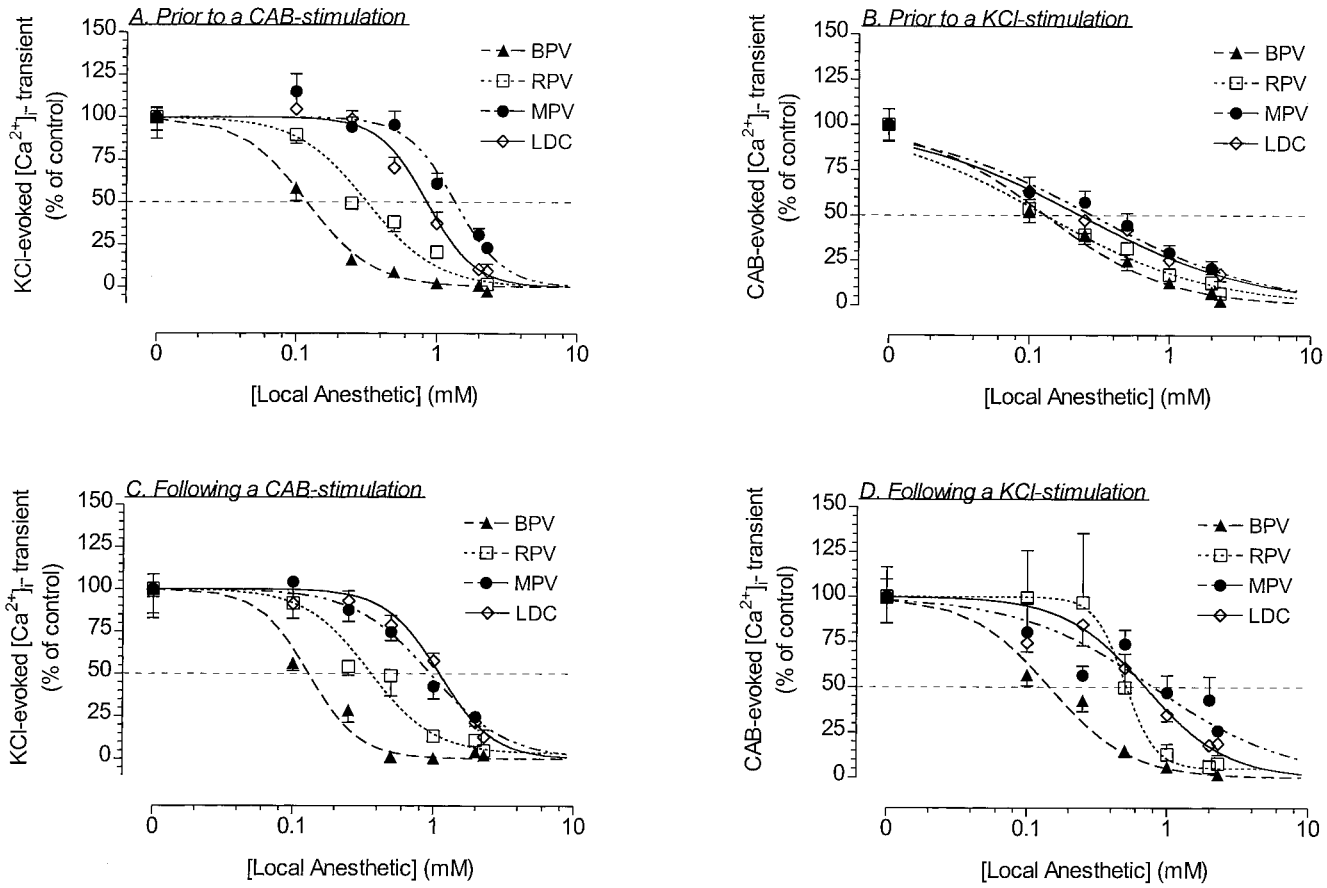

Fig. 3. Effects of local anesthetics (LAs) bupivacaine (BPV), ropivacaine (RPV), mepivacaine (MPV), and lidocaine (LDC) (0.1–2.3 mM for all) on sequentially evoked $[Ca^{2+}]_i$ transients. In the presence of 1.5 mM $CaCl_2$ in the reaction buffer, 100 mM KCl was applied (*A*) followed by 1 mM CAB (*D*), or *vice versa* (*B* and *C*). Values (mean ± SEM, n = 3–46 for the most groups and n = 2 for the following groups: all 2 mM BPV groups; 2 mM RPV group in *B*; 2.3 mM LDC group in *D*) for the LA-treated groups are presented in percentage of the corresponding control groups. Dose–response curves were generated by nonlinear fitting using the following equation: $f_u(x) = f_u(m)/(1 + 10^{[(\log IC_{50} - \log_x) \cdot -h}$, where $f_u(x)$ is the remaining $[Ca^{2+}]_i$ transients (in percent of the untreated control values) at an LA concentration x, and $f_u(m)$ is the maximal $[Ca^{2+}]_i$ transients (*i.e.*, 100%). IC_{50} is the LA concentration resulting in 50% inhibition of evoked $[Ca^{2+}]_i$ transients, and h is the Hill slope that may indicate the extent of drug–ligand interaction under specific conditions.[27]

IC_{50} values bupivacaine < ropivacaine < lidocaine ≈ mepivacaine, essentially indistinguishable between lidocaine and mepivacaine ($P > 0.05$). The potency of bupivacaine and ropivacaine for inhibiting the KCl-evoked $[Ca^{2+}]_i$ transients remained unchanged when the intracellular Ca^{2+} stores were partially depleted by a carbachol prestimulation, but mepivacaine was found to be less potent ($P < 0.05$) and lidocaine more potent ($P <$

Table 1. IC_{50} Values and Hill Coefficients of Local Anesthetics

	IC_{50} (mM)				Hill Coefficient			
	BPV	RPV	MPV	LDC	BPV	RPV	MPV	LDC
KCl-evoked $[Ca^{2+}]_i$-transient	—	—	—	—	—	—	—	—
Without CAB prestimulation*	0.12	0.32	1.35	0.85	1.85	1.75	2.5	2.55
After CAB prestimulation†	0.13	0.35	0.95	1.10	2.4	1.9	1.6	2.25
CAB-evoked $[Ca^{2+}]_i$-transient	—	—	—	—	—	—	—	—
Without KCl prestimulation‡	0.13	0.13	0.28	0.23	1.0	0.75	0.71	0.70
After KCl prestimulation§	0.15	0.50	0.80	0.70	1.5	4.0	0.9	1.5

Local anesthetics tested for inhibiting sequentially evoked $[Ca^{2+}]_i$ transients. In the presence of 1.5 mM $CaCl_2$, * 100 mM KCl was applied, ‡ followed by 1 mM CAB in the reaction buffer, or †§ vice versa.

Values are obtained by non-linear fitting using the following equation: $f_u(x) = f_u(m)/(1 + 10^{[(\log IC50 - \log x) \cdot -h]}$. For the non-linear fitting purpose, only group means (shown in fig. 3 with their deviations), instead of the individual experimental data, were used. See the legend of the figure 3 for explanations of the data and the individual components of the equation.

BPV = bupivacaine; CAB = carbachol; MPV = mepivacaine; LDC = lidocaine; RPV = ropivacaine.

Table 2. Statistical Significance between Different LA Treatments in Inhibiting KCl-evoked and CAB-evoked $[Ca^{2+}]_i$ Transients at Each Single LA Concentration Applied

	RPV	MPV	LDC
100 mM KCl-evoked $[Ca^{2+}]_i$ transient*	—	—	—
BPV	a, b, c, d	a, b, c, d, e, f	a, b, c, d
RPV	—	a, b, c, d, e, f	b, c, d
LDC	—	c, d, e, f	—
1 mM CAB-evoked $[Ca^{2+}]_i$ transient†			
BPV	NS	d, f	NS
RPV	—	b, f	NS
LDC	—	f	—

Values compared between LA treatment inhibiting * KCl-evoked and † Carbachol-evoked $[Ca^{2+}]_i$ transients.

One-way ANOVA (Student-Neuman-Keuls test) was used. A significant difference ($P < 0.05$) between any two LA-treated groups was labeled in the table as follows: treatments at a LA-concentration of 0.1 (a), 0.25 (b), 0.5 (c), 1, 2 (d), and 2.3 (e) mM, respectively.

BPV = bupivacaine; CAB = carbachol; LA = local anesthetic; LDC = lidocaine; NS = no significant difference was found at any LA concentration between that particular group pair; RPV = ropivacaine.

0.05) following carbachol prestimulation (fig. 3A *vs.* fig. 3C, table 1, row a *vs.* row b). In contrast, the carbachol-evoked $[Ca^{2+}]_i$ transients became less sensitive to the LAs and displayed a wider IC_{50} range (table 1, row c *vs.* row d) when the Ca^{2+} stores were partially emptied by a KCl prestimulation (fig. 3B *vs.* fig. 3D).

Effects of Tetrodotoxin and TEA on KCl-evoked and Carbachol-evoked $[Ca^{2+}]_i$ Transients and Effects of their Coapplication with Different LAs

The SH-SY5Y cells possess tetrodotoxin-sensitive Na^+ currents.[14,15] Tetrodotoxin at 1 μM (a concentration shown to completely block the voltage-dependent Na^+ currents in the SH-SY5Y cells)[15,16,17] had no significant effect on either KCl-evoked or carbachol-evoked $[Ca^{2+}]_i$ transients (fig. 4). In the presence of tetrodotoxin, LA inhibition of evoked $[Ca^{2+}]_i$ transients was identical to inhibition in the absence of tetrodotoxin, indicating that LA block of Na^+ channels does not contribute to LA inhibition of evoked $[Ca^{2+}]_i$ transients.

TEA (10 mM) alone produced a small yet significant increase in the KCl-evoked $[Ca^{2+}]_i$ transients. In the presence of any of the LAs, no significant difference was found between groups (±) TEA (fig. 5A). Since TEA augmented the KCl-evoked $[Ca^{2+}]_i$ transients, the results obtained in the presence of LA would suggest a greater degree of inhibition and an involvement of K^+ channels in KCl-evoked $[Ca^{2+}]_i$ transients, which was blocked by LAs.

In contrast, the carbachol-evoked $[Ca^{2+}]_i$ transients were significantly attenuated by TEA alone, and coapplication of TEA and any LA showed further inhibition (fig. 5B).

Discussion

This study demonstrates that LAs decreased KCl-evoked and carbachol-evoked $[Ca^{2+}]_i$ transients. Their potency appeared to be modulated by the filling level of intracellular Ca^{2+} stores (except for bupivacaine) and

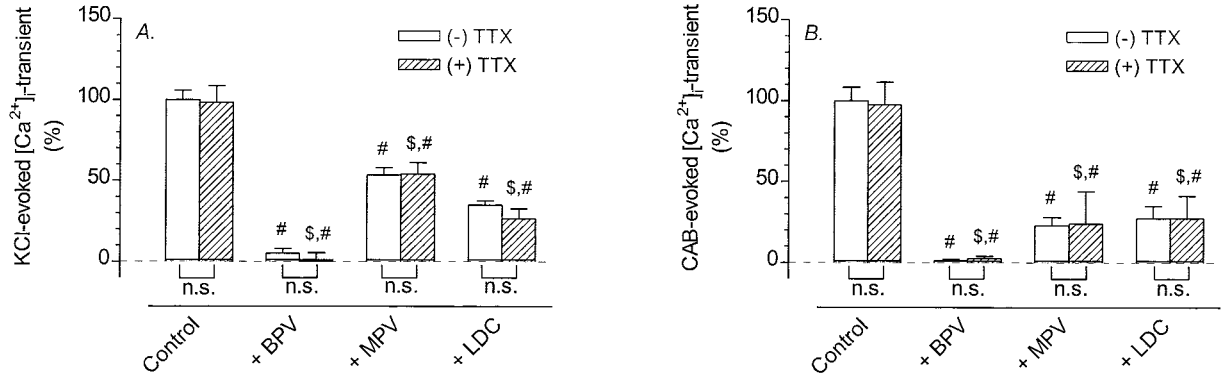

Fig. 4. Effects of Na^+ channel blocker tetrodotoxin (TTX, 1 μM) and local anesthetics (LAs) bupivacaine (BPV), ropivacaine (RPV), mepivacaine (MPV), and lidocaine (LDC) (2.3 mM for all) on 100 mM KCl-evoked (*A*) or 1 mM CAB-evoked (*B*) $[Ca^{2+}]_i$ transients. Values presented are expressed as mean ± SEM (n = 3–59) and expressed as percentage of the control group (-) TTX (the first bar from the left). The significance of the differences among different groups was tested using Student-Neuman-Keuls test and labeled as follows: #$P < 0.05$ *versus* the control group without LA and TTX (the first bar from the left); \$$P < 0.05$ *versus* the TTX-control group without LA (the second bar from the left); n.s. = no significant difference was found between groups (±) TTX.

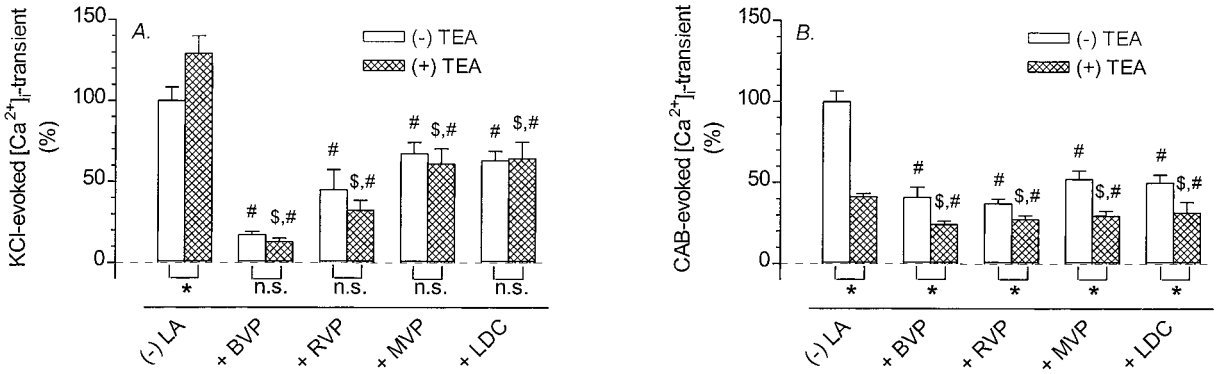

Fig. 5. Effects of K^+ channel blocker tetraethylammonium (TEA, 10 mM) and local anesthetics (LAs) bupivacaine (BPV), ropivacaine (RPV), mepivacaine (MPV), and lidocaine (LDC) on evoked $[Ca^{2+}]_i$ transients. In the presence of 1.5 mM $CaCl_2$ in the reaction buffer, 100 mM KCl (*A*) or 1 mM CAB (*B*) was applied. The LA concentrations applied were as follows: (*A*) 0.25 mM for BPV, 0.5 mM for RPV and LDC, 1 mM for MPV, respectively; (*B*) 0.25 mM for all four LA. Values are shown as mean ± SEM (n = 2–17) and expressed as percentage of the control group (-) TEA (the first bar from the left). The significance of the difference among different groups was tested using Student-Neuman-Keuls test and labeled as follows: #*P* < 0.05 *versus* the control group without LA and TEA (the first bar from the left); $*P* < 0.05 *versus* the TEA-control group without LA (the second bar from the left). Between groups (±) TEA: *significance at *P* < 0.05; n.s. = no significant difference.

affected by TEA-sensitive K^+ channels, but not tetrodotoxin-sensitive Na^+ channels.

Intracellular Ca^{2+} Stores Modulate LA Action on KCl-evoked and Carbachol-evoked $[Ca^{2+}]_i$ Transients

Previously, in SH-SY5Y cells, we have shown that there are two major intracellular Ca^{2+} stores, the caffeine-sensitive and the IP_3-sensitive, which are functionally connected. The KCl-evoked $[Ca^{2+}]_i$ transients are dependent on Ca^{2+} release from the caffeine-sensitive stores, while the carbachol-evoked $[Ca^{2+}]_i$ transients are mainly attributed to Ca^{2+} release from the IP_3-sensitive stores.[7] Moreover, the carbachol-evoked $[Ca^{2+}]_i$ transients were largely (~90%) inhibited by a muscarinic antagonist atropine (1 μM), indicating that the carbachol-evoked $[Ca^{2+}]_i$ transients in our SH-SY5Y cells were predominantly mediated by activation of muscarinic receptors (data not shown). Using a sequential stimulation protocol, we investigated potential LA targets associated with both intracellular Ca^{2+} stores.

The LAs attenuated the KCl-evoked $[Ca^{2+}]_i$ transients in a concentration-dependent manner. Prestimulation with carbachol *did not alter* the potency of bupivacaine and ropivacaine in inhibiting the KCl-evoked $[Ca^{2+}]_i$ transients, suggesting that the observed inhibitory effect was unrelated to an action on the IP_3-sensitive stores. However, the potency of mepivacaine and lidocaine was altered by carbachol prestimulation, suggesting an *additional* effect of these two drugs on the carbachol-depletable Ca^{2+} store/IP_3-sensitive pathway. Following carbachol prestimulation, the mepivacaine potency in inhibiting the KCl-evoked $[Ca^{2+}]_i$ transients was increased, while lidocaine potency decreased (table 1), suggesting that mepivacaine and lidocaine either affected an additional target of the carbachol-depletable Ca^{2+} store differently or they were acting at different sites on that store.

All four LAs reduced the carbachol-evoked $[Ca^{2+}]_i$ transients in a concentration-dependent fashion. The KCl prestimulation *decreased the potency* of ropivacaine, mepivacaine, and lidocaine in inhibiting the carbachol-evoked $[Ca^{2+}]_i$ transients. This suggests that part of the action of LA might involve prevention of a contribution of Ca^{2+} from the caffeine-sensitive store to the IP_3-sensitive Ca^{2+} response, as was reported previously with halothane and isoflurane.[7] When the caffeine-sensitive store is depleted, the LAs appear to be less potent. The Ca^{2+} contribution of the caffeine-sensitive store could result from either a direct Ca^{2+} release through the ryanodine receptors[18] into the cytoplasm or a more direct Ca^{2+} translocation within the endoplasmic reticulum from the caffeine- to the IP_3-sensitive stores.[7]

A comparison of the slopes of the LA concentration–response curves (the Hill coefficients) for inhibition of evoked $[Ca^{2+}]_i$ transients prior to or following a prestimulation (table 1) reveals that predepletion of a Ca^{2+} store may alter the cooperative interaction between LAs and/or their potential target. This appears especially true for ropivacaine in carbachol-evoked $[Ca^{2+}]_i$ transients following KCl prestimulation, where the cooperativity seems to be significantly increased (higher Hill coefficients, table 1).

In *Xenopus* oocytes, LAs were also shown to inhibit muscarinic receptor-mediated signaling through an action on the receptors and the coupled G-proteins, but not on the downstream events involving the phospholipase C-IP_3-Ca^{2+} pathway.[19–23] In the present study, since a direct LA-action on the sites associated with the IP_3-sensitive Ca^{2+} store was not apparent for bupivacaine and ropivacaine (see the previous paragraph), the elements upstream of the store, such as the muscarinic receptors and/or coupled G_q-protein(s), need to be considered as the possible sites for the LA action. Mepivacaine and lidocaine, however, seem to affect the IP_3-

sensitive Ca^{2+} store. It is important to mention that our data only indicate an LA target associated with the IP_3-sensitive Ca^{2+} store, but not the exact nature of this site. We cannot rule out that the IP_3-mediated Ca^{2+} signaling pathway in oocytes is controlled differently than in neuronal cells and, therefore, responds to LAs differently.

Since the potency of bupivacaine in inhibiting both the KCl-evoked and the carbachol-evoked $[Ca^{2+}]_i$ transients remained unchanged with prestimulation (table 1), bupivacaine appears to act mostly on other sites, possibly the sites associated with the plasma membrane, to inhibit the evoked $[Ca^{2+}]_i$ transients rather than on sites associated with either caffeine- or IP_3-sensitive stores, or other intracellular compartment. However, the possibility that bupivacaine equally suppresses the evoked $[Ca^{2+}]_i$ transients mediated through these two Ca^{2+} stores cannot be ruled out.

Roles of Voltage-dependent Na^+ and K^+ Channels on the LA Action on Carbachol- and KCl-evoked $[Ca^{2+}]_i$ Transients

Activation of Na^+ currents, which occurs during KCl-induced depolarization, is not essential for the generation of the KCl-evoked and carbachol-evoked $[Ca^{2+}]_i$-transients. In these neuronal cells, the LA-block of Na^+ currents does not contribute to the LA inhibition of the evoked $[Ca^{2+}]_i$ transients.

In SH-SY5Y cells, the TEA-sensitive K^+ channel has been shown to be predominantly responsible for the delayed rectifier K^+ currents.[15,17] During depolarization, opening of these K^+ channels will oppose the depolarizing effect on inward Ca^{2+} currents. Therefore, blocking of TEA-sensitive K^+ channels will be expected to increase the Ca^{2+} channel activity and in turn, the KCl-evoked $[Ca^{2+}]_i$ transients, as demonstrated in figure 5A. In the presence of either of the four LAs, the level of the $[Ca^{2+}]_i$ transients was similar with or without TEA. This suggests that the TEA-sensitive K^+ channels are blocked by LAs. One possibility is that LAs inhibited TEA-sensitive K^+ channels and hence an additional blockade by TEA did not further affect the LA-action on the KCl-evoked $[Ca^{2+}]_i$ response. However, blockade of K^+ channels does not explain the overall inhibition of the KCl-evoked $[Ca^{2+}]_i$-response, since TEA alone increased this response (fig. 5A). Most likely, LA caused reduction of the KCl-evoked $[Ca^{2+}]_i$-response was mediated through LA action on voltage-dependent Ca^{2+} channels, since we have previously shown that the generation of the KCl-evoked $[Ca^{2+}]_i$ transients are dependent on the extracellular Ca^{2+} and functional voltage-dependent Ca^{2+} channels,[7] which have been shown to be blocked by LA.[4,5,24–26]

The carbachol-evoked $[Ca^{2+}]_i$ transients were significantly lower in the presence of TEA, suggesting that K^+ channels contribute to the carbachol-evoked $[Ca^{2+}]_i$ transients. In the presence of LA, the inhibition by TEA was still visible in addition to the LA effect on the carbachol-evoked $[Ca^{2+}]_i$ transients (fig. 5B). Moreover, the sum of the individual LA inhibition and TEA inhibition was larger than the inhibition caused by a coapplication of both drugs. These results suggest that LA and TEA were acting on different and overlapping sites, such as the same TEA-sensitive K^+ channels. The K^+ channels might modulate the carbachol-evoked $[Ca^{2+}]_i$ transients through direct or regulatory action on the muscarinic receptor and/or coupled G-proteins.

Hydrophobicity and Local Anesthetic Action

In general, the higher hydrophobicity is attributed to the larger size of the alkyl substituents in the molecule of an LA and correlates with a higher local LA.[9] Comparison of the partition coefficients (total drug/ml octanol ÷ total drug/ml buffer, pH 7.4,[8] a parameter for the hydrophobicity) of the LAs shows a descendent potency order: bupivacaine (346/560) > ropivacaine (115/n.d.) > lidocaine (43/110) > mepivacaine (21/42) (values represent the partition coefficient determined at 25/36°C; n.d. = not determined), which not only resembles the potency order of these drugs in blocking conduction but also in inhibiting the KCl-evoked $[Ca^{2+}]_i$ transients (see Results).[9] The presence of a secondary potential LA target on the carbachol-sensitive Ca^{2+} store might be correlated with the smaller hydrophobic amine domain in the molecules of mepivacaine and of lidocaine. In contrast, the LA potency for inhibition of the carbachol-evoked $[Ca^{2+}]_i$ transients was similar for four LAs, showing little correlation to their very different partition coefficients.

In summary, this study has demonstrated inhibitory effects of several LAs on the KCl-evoked and carbachol-evoked $[Ca^{2+}]_i$ transients mediated through different pathways. The major LA inhibition seems to occur at sites associated with the caffeine-sensitive Ca^{2+} store (such as ryanodine receptor) and/or sites associated with plasma membrane, such as voltage-dependent Ca^{2+} channels, muscarinic receptor, and/or coupled G-protein. A direct action of LA on the IP_3-sensitive store is not apparent for bupivacaine and ropivacaine, but LA may affect the IP_3-sensitive Ca^{2+} releases indirectly through their inhibitory effect on the muscarinic receptor and/or coupled G-protein. In contrast, mepivacaine and lidocaine might act on both the caffeine- and IP_3-sensitive stores. Although a blockade of Na^+ currents did not affect the evoked $[Ca^{2+}]_i$ transients or the LA effects on them, the K^+ currents seem to modulate both the KCl-evoked and carbachol-evoked $[Ca^{2+}]_i$ transients through different pathways, and their blockade may affect the LA action on intracellular $[Ca^{2+}]_i$ responses.

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