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Insensitivity of P-Type Calcium Channels to Inhalational and Intravenous General Anesthetics

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Background: **Voltage-gated Ca^{2+} channels long have been considered plausible targets for general anesthetics. Previous anesthetic studies have focused on L-, T-, or N-type channels, but there have been no studies on channels identified as P-type. Since P-type channels may be the most important voltage-gated Ca^{2+} channels involved in synaptic transmission in mammalian brain, it is important to establish their sensitivity to clinically relevant concentrations of general anesthetics.**

Methods: **Acutely dissociated cerebellar Purkinje neurons were obtained from 7–14-day-old Sprague-Dawley rats. P-type currents were measured using the whole-cell version of the patch-clamp technique, with Ba^{2+} as the current carrier. General anesthetics were applied to the neurons in aqueous solution at room temperature (20–23°C).**

Results: **P-type Ca^{2+} channels were found to be very insensitive to a variety of general anesthetics and ethanol. Inhibitions of less than 10% were produced by 0.35 mM halothane, 0.35 mM isoflurane, 32 μM thiopental, 50 μM pentobarbital, 2 μM propofol, and 200 mM ethanol. Substantial anesthetic inhibition was found only at free aqueous concentrations much greater than those that are clinically relevant. For halothane, the dose-response curve showed an IC_{50} concentration of 1.17 ± 0.02 mM and a Hill coefficient of 2.02 ± 0.04 (mean ± SEM).**

Conclusions: **The relatively small inhibitions of P-type Ca^{2+} channels produced by volatile and intravenous anesthetics at their free aqueous EC_{50} concentrations for general anesthesia in mammals suggest that these channels do not play a major role in the induction of general anesthesia. (Key words: Anesthetics, intravenous: pentobarbital; propofol; thiopental. Anesthetics, volatile: halothane; isoflurane. Brain, cerebellar Purkinje neurons: P-type calcium channels. Ethanol.)**

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VOLTAGE-GATED Ca^{2+} channels in neuronal plasma membranes are intimately involved in signal transmission in the central nervous system. These channels represent, therefore, obvious and plausible targets for general anesthetics. However, it recently became clear that there are many different types of voltage-gated Ca^{2+} channels. On the basis of their electrical and pharmacologic properties, a convenient classification[1–4] (which is undoubtedly oversimplified) groups them into four types: T, L, N, and P. While T- and L-type channels are present in a wide variety of neuronal and nonneuronal cells, N- and P-type channels have so far been found mainly in neurons.[1–3] Because of their ready accessibility (in cardiac cells, for example), most anesthetic studies to date have been on channels that appear to be of the T- and L-types.[5–11] Only a few anesthetic studies[12,13] have been performed on channels identified as N-type, whereas none have been conducted on channels identified as P-type, which are found throughout the nervous system and may be the major class of voltage-gated Ca^{2+} channel involved in synaptic transmission in the mammalian central nervous system.[3,14]

P-type channels get their name from their initial discovery in cerebellar Purkinje cells,[15] the type of central neuron used in the current investigation. Antibody labeling, however, shows that these channels are distributed widely in the mammalian central nervous system.[16] Based on inhibition by specific toxins of depolarization-induced Ca^{2+} uptake by rat brain synaptosomes, P channels appear to constitute as much as 80% of the voltage-gated Ca^{2+} channels in mammalian brain nerve terminals.[3,17,18] Furthermore, the specific P-channel blocker ω-Aga-IVA has been shown to inhibit by more than 80% excitatory postsynaptic currents in hippocampal neurons and inhibitory postsyn-

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aptic currents in cerebellar and spinal neurons.[14] Therefore, it is important for our understanding of anesthetic mechanisms to determine the sensitivity of P-type Ca^{2+} channels to clinically relevant concentrations of various classes of general anesthetic agents.

In the current study, we investigated the effects on voltage-gated P-type Ca^{2+} channels of four clinically used general anesthetics (halothane, isoflurane, propofol, and thiopental), a barbiturate often used in experimental studies (pentobarbital), and the nontherapeutic drug ethanol.

Materials and Methods

Animals

Sprague-Dawley rats (7–14 days old) were used and handled in compliance with U.K. regulations.

Preparation of Cerebellar Purkinje Cells

The vermis of the cerebellum was dissected from decapitated rats and cut into chunks (~1 mm^3). Cells were dissociated using the solutions and enzymes described by Mintz *et al.*[19] Briefly, the chunks of vermis were incubated for 7–8 min under an oxygen atmosphere at 37°C in 82 mM Na_2SO_4, 30 mM K_2SO_4, 5 mM $MgCl_2$, 2 mM HEPES, 10 mM glucose, and 0.001% phenyl red indicator (titrated to p*H* 7.4 with NaOH) containing 3 mg/ml protease (type XXIII). The protease solution was decanted and the tissue suspended in 82 mM Na_2SO_4, 30 mM K_2SO_4, 5 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, and 0.001% phenyl red indicator (titrated to p*H* 7.4 with NaOH) containing 1 mg/ml trypsin inhibitor (type II-O, chicken egg white) and 1 mg/ml bovine serum albumin (fraction V) at 37°C, and then left to cool to room temperature (20–23°C) under an oxygen atmosphere. A few chunks were withdrawn and gently triturated by 10–15 passages through the tip of a fire-polished Pasteur pipette, and the cell suspension was deposited uniformly in a glass-bottomed recording bath (bath volume ~ 100 μl) containing Tyrode solution: 150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (titrated to p*H* 7.4 with NaOH). Most of the cells adhered to the glass within 5–10 min, and the Purkinje neurons were identified easily by their comparatively large size (15–25 μm diameter) and their distinctive dendritic stump (identification was confirmed by staining with propidium iodide[20] in separate experiments). After the cells had adhered, they were continually superfused (~1 ml/min) with Tyrode solution until patch formation (see below). After each recording, the bath was cleaned and a fresh aliquot of cells added. Cells were used within 4 h of dissection. All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. (Poole, Dorset, United Kingdom).

Recording Technique

P-type Ca^{2+} channel currents were recorded using the standard whole-cell voltage-clamp technique.[21] Pipettes were fabricated from thin-walled filamented borosilicate glass (GC150TF, Clark Electromedical Instruments, Reading, Berkshire, United Kingdom) using a two-stage pull (Narishige PB-7 micropipette puller, Tokyo, Japan), fire-polished and filled with 108 mM cesium methanesulfonate, 4 mM $MgCl_2$, 9 mM EGTA, 9 mM HEPES, 4 mM Mg-ATP, 14 mM phosphocreatine (Tris salt), and 1 mM Tris-GTP (titrated to p*H* 7.4 with CsOH). Typical electrode resistances were 5–10 MΩ. Upon establishing the whole-cell configuration, the cells were routinely voltage-clamped at −84 mV. The Tyrode solution was exchanged for the Ba^{2+} recording solution: 5 mM $BaCl_2$, 160 mM TEA-Cl, 0.1 mM EGTA, and 10 mM HEPES (titrated to p*H* 7.4 with TEA-OH). These solutions were designed to eliminate Na^+ and K^+ currents and to isolate currents through Ca^{2+} channels, using Ba^{2+} ions as the current carrier. (Ba^{2+} often is used in place of Ca^{2+} to eliminate Ca^{2+}-dependent inactivation, minimize second-messenger effects, prevent activation of Ca^{2+}-activated K^+ currents, and/or to increase the magnitude of the evoked currents.) Inward currents were recruited by 60-ms voltage steps to (typically) −25 mV delivered every 20 s. Consecutive current records were observed to increase during the initial few minutes, presumably because of exchange of the intracellular medium with the pipette solution. Subsequent records generally reached a consistent level, with current rundown being minimal during the time-course of an experiment. All current traces were corrected for small leak components, calculated from the currents observed during 10-mV hyperpolarizing pulses. Inhibitions were monitored by evoking currents every 20 s, and percentage inhibition was calculated from the steady-state block achieved after adequate (~1 min) exposure to anesthetic solutions. Inhibitions almost always were reversed upon washout; data from the rare instances of irreversibility were excluded from analysis. All experiments were performed at room temperature (20–23°C). Currents

were filtered at 1 kHz (−3 dB, 8-pole Bessel filter) and sampled at 4 kHz. All voltages have been compensated for series resistance and corrected for a liquid junction potential of −4 mV between the pipette and the Ba^{2+} recording solution. Ca^{2+} channel currents were identified as P-type using synthetic ω-Aga-IVA (Peptides International, Louisville, KY). The synthetic toxin has been shown[22] to have the same high degree of selectivity as the native toxin purified from the funnel-web spider *Agelenopsis aperta.* The toxin was dissolved with 1 mg/ml cytochrome C (from horse heart; Sigma) to minimize nonspecific binding.

Anesthetic Solutions

Anesthetics were dissolved in the Ba^{2+} recording solution. The volatile agents halothane (ICI, Macclesfield, Cheshire, United Kingdom) and isoflurane (Abbott, Queenborough, Kent, United Kingdom) were diluted from saturated solutions (halothane 17.5 mM,[23] isoflurane 15.3 mM[24]). Thiopental (formulated as Intraval: 92% thiopental, 8% Na_2CO_3; May and Baker, Dagenham, Essex, United Kingdom), ethanol (BDH, Poole, Dorset, United Kingdom), and sodium pentobarbital (Sigma) were weighed out and dissolved. Propofol (supplied as pure 2,6-diisopropylphenol by Zeneca, Macclesfield, Cheshire, United Kingdom) was made up to 10 mM in ethanol before dilution in the recording solution. The 2-μM propofol solution therefore contained a final concentration of ethanol of 3.4 mM. This ethanol concentration did not cause any significant current inhibition. Nonetheless, in the experiments with propofol, the control recording solutions also contained 3.4 mM ethanol. Cells were continuously superfused (~1 ml/min) throughout the experiment; losses of volatile agents from the perfusion system were found to be negligible as measured by gas chromatography.

Anesthetic Concentrations

All anesthetic concentrations in this paper are expressed as free concentrations in aqueous solution. To discuss the results of our *in vitro* experiments in the context of anesthetic mechanisms, it is essential to use anesthetic concentrations that are close to free aqueous concentrations present during surgical general anesthesia. For halothane and isoflurane, the free aqueous concentrations at minimum alveolar concentration (lack of response to a painful stimulus) are, on average, about 0.25 mM and 0.32 mM, respectively, for a variety of mammals, including humans.[25] Although minimum alveolar concentration values for halothane and isoflurane expressed as partial pressures decrease markedly with temperature,[25,26] there is a compensating, but slightly smaller, increase in water/gas partition coefficients.[25] Consequently, when expressed as aqueous concentrations, minimum alveolar concentration values change relatively little with temperature. The values used here are for 37°C, but are reasonable approximations for *in vitro* experiments at room temperature.[25] They are close to the EC_{50} concentrations needed to induce anesthesia in tadpoles at room temperature (0.23 mM halothane[27] and 0.29 mM isoflurane[28]). For intravenous agents, complications arise from their relatively rapid redistribution and metabolism, and explicit account must be taken of their (often) tight binding to plasma proteins and blood constituents. (For example, about 98% of plasma propofol is bound to protein,[29] so that the free aqueous concentration of propofol is only about 2% of the total plasma concentration.) Recent estimates of the free aqueous EC_{50} concentrations for general anesthesia in mammals (lack of response to a painful stimulus) are 25 μM thiopental, 50 μM pentobarbital, and 0.4 μM propofol.[30] (In our experiments, to test the sensitivity of P-type Ca^{2+} channels, we used 32–100 μM thiopental, 50 μM pentobarbital, and 2 μM propofol.) Whereas the values for the inhalational agents are reasonably precise, the EC_{50} concentrations for the intravenous anesthetics are less secure because of their complex pharmacokinetics.

Results

Ca^{2+} channel currents (1–6 nA) were readily evoked from cerebellar Purkinje neurons by stepping the membrane potential from a holding value of −84 mV to voltages more positive than −50 mV. Control currents were fast activating (peak at ~5 ms) and essentially noninactivating (less than 10% inactivation) during the time course (60 ms) of the test potential (figs. 1 and 2). The currents were completely blocked by 500 μM Cd^{2+} (not shown). More importantly, they were inhibited 91 ± 1% (mean ± SEM, n = 6 cells) by 100 nM ω-Aga-IVA (fig. 1), showing the currents to be almost entirely P-type.[18,19,22] The current-voltage curves (fig. 2) show that the maximum channel current was evoked at a test potential of about −25 mV.

At a halothane concentration relevant to general anesthesia (0.35 mM ≈ 1.4 MAC, see materials and methods), the Ca^{2+} channel current was only slightly inhibited (table 1 and fig. 2 inset), and there was little

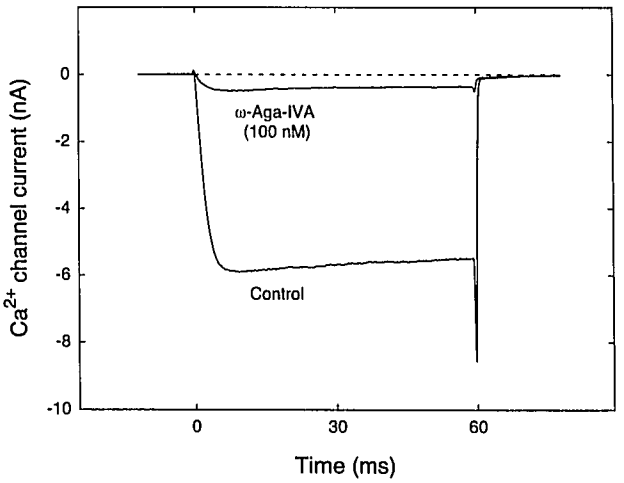

Fig. 1. P-type Ca^{2+} channel current from rat cerebellar Purkinje neurons. Currents through the channel were blocked (91 ± 1%, mean ± SEM, n = 6 cells) by 100 nM ω-Aga-IVA. In this example, the current was elicited by a 60-ms depolarization to a test potential of −20 mV. The dashed line indicates zero current. The spike after the end of each depolarization is a "tail current," which is caused by the increased driving force through the open Ca^{2+} channels that subsequently close rapidly after the return to the holding potential.

change in either the time-course of the current or the shape of the I-V curve (fig. 2). Only at much higher concentrations was there substantial inhibition of the peak current, with somewhat more inactivation of the inhibited current (~30% at 1.40 mM halothane) during the voltage step (not shown).

Table 1. Inhibition of P-type Calcium Channel Peak Currents by General Anesthetics and Ethanol

Anesthetic Agent	Concentration	% Inhibition (mean ± SEM)	Cells (n)	EC_{50} for General Anesthesia*
Halothane	0.35 mM	8.1 ± 1.6	7	0.25 mM
Isoflurane	0.35 mM	8.1 ± 1.5	6	0.32 mM
Thiopental	32 μM	5.4 ± 0.4	6	25 μM
Thiopental	100 μM	19.2 ± 2.5	5	25 μM
Propofol	2 μM	2.8 ± 0.4	6	0.4 μM
Pentobarbital	50 μM	6.4 ± 1.1	6	50 μM
Ethanol	200 mM	4.7 ± 1.9	5	—

* See materials and methods.

The percentage inhibition of the current as a function of halothane concentration is shown in figure 3. The data were fitted (by the method of least-squares, with weights equal to the reciprocals of the squares of the standard errors) to a Hill equation of the form

$$y = c^h/(c^h + IC_{50}{}^h),$$

where y is the fractional inhibition, c is the halothane concentration, and h is the Hill coefficient. The best fit gave an IC_{50} (mean ± SEM) of 1.17 ± 0.02 mM halothane (>4 times MAC) with h = 2.02 ± 0.04.

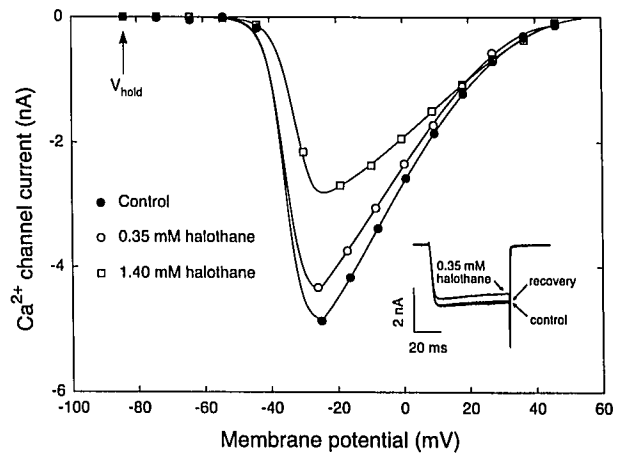

Fig. 2. Current-voltage curves for peak P-type Ca^{2+} channel currents in control saline (●) and in the presence of 0.35 mM (○) and 1.40 mM (□) halothane. V_{hold} indicates the holding potential (−84 mV). (*Inset*) Currents elicited by 60-ms depolarizations to −25 mV, the standard test potential, in the presence and absence of 0.35 mM halothane (~1.4 MAC). Note the small inhibition of peak current and the recovery upon washout.

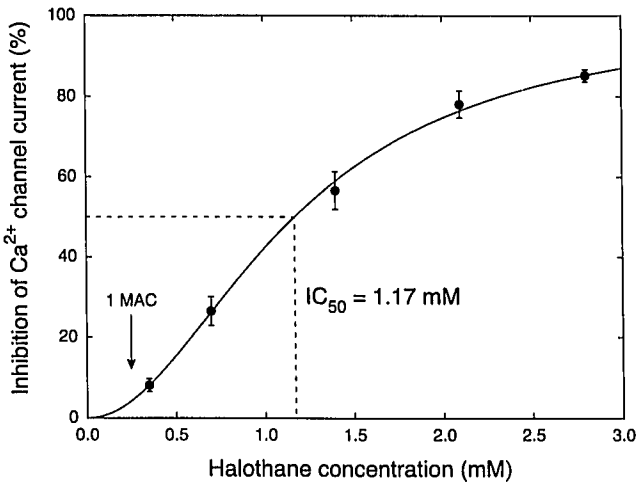

Fig. 3. Dose-response curve for the inhibition of the peak P-type Ca^{2+} channel current by halothane. The error bars represent the standard errors (for at least five cells). The solid line was calculated using a Hill equation with IC_{50} of 1.17 mM and a Hill coefficient (h) of 2.02.

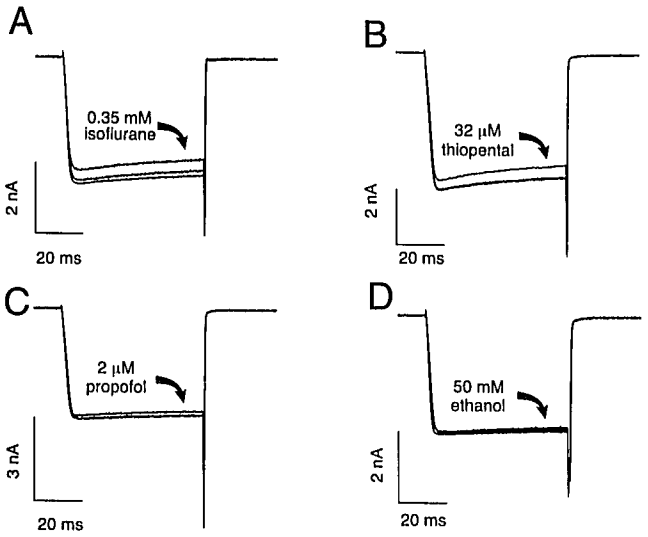

Fig. 4. The P-type Ca^{2+} channel current is very insensitive to a variety of clinical general anesthetics and ethanol. This figure shows representative data of the small inhibitions observed with (*A*) 0.35 mM isoflurane, (*B*) 32 μM thiopental, (*C*) 2 μM propofol, and (*D*) 50 mM ethanol. In each set, the inhibited current is shown with the control before application of the anesthetic (or ethanol) and the recovery after washout. See also table 1.

The P-type Ca^{2+} channel current was also remarkably insensitive (fig. 4) to clinically relevant levels of other anesthetics, as well as to relatively high concentrations (up to 200 mM) of ethanol. These results are summarized in table 1.

Discussion

Our major finding is that P-type voltage-gated Ca^{2+} channels in cerebellar Purkinje neurons are very insensitive to inhibition by surgically relevant concentrations of inhalational and intravenous general anesthetics (table 1). For the volatile anesthetics halothane and isoflurane, concentrations equivalent to about 1 MAC inhibited the peak current by less than 10%. The intravenous agents thiopental and propofol, at levels (32 μM and 2 μM, respectively) in excess of EC_{50} concentrations for a lack of response to a painful stimulus, produced even smaller inhibitions. For ethanol, a concentration of 50 mM, which causes numerous behavioral effects in humans, caused no measurable inhibition (fig. 4). Even a concentration as high as 200 mM, which usually would be lethal,[31] inhibited peak channel current by only about 5%.

The effects of halothane were studied further, over a range of higher concentrations. The halothane dose-response curve (fig. 3) showed that a concentration (1.17 mM) well in excess of the clinically relevant range was required to half-inhibit peak currents. The steepness of the dose-response curve (Hill coefficient ≈ 2) is consistent with the existence of more than one low-affinity binding site for halothane on the channel protein. Our observed halothane IC_{50} concentration for the P-type channel peak current falls within the range found in previous electrophysiologic studies of other (mainly L-type) high-voltage-activated Ca^{2+} channels. For example, Bosnjak *et al.*[11] determined an IC_{50} for halothane of ~0.75 mM for inhibition of L-type currents in dog heart ventricular cells, and Terrar and Victory[6] reported that 1.57 mM halothane inhibited a largely L-type Ca^{2+} current in myocytes from guinea-pig ventricle by only ~33%. Furthermore, L-type currents in dog cardiac Purkinje cells would be inhibited by ~50% at 0.70 mM halothane.[8] Finally, Herrington *et al.*[9] measured a halothane IC_{50} of 0.85 mM for L-like currents in clonal GH_3 pituitary cells, and Takenoshita and Steinbach[10] found IC_{50} of 1.5 mM halothane for an unclassified high-voltage-activated Ca^{2+} current in rat dorsal root ganglion cells. Similar insensitivity has been found for the effects of isoflurane,[5,8,11] in line with our observations (table 1) at 0.35 mM isoflurane (~EC_{50} for general anesthesia). It thus appears that P-type channels are not alone among high-voltage-activated channels in being insensitive to volatile general anesthetics.

We also found that P-type Ca^{2+} channels were relatively insensitive to the intravenous anesthetics propofol, thiopental, and pentobarbital (table 1). This is consistent with most previous studies on other voltage-gated Ca^{2+} channels. Puttick and Terrar[32] found that $IC_{50} \approx 100$ μM propofol (more than 2 orders of magnitude above the EC_{50} for general anesthesia) for inhibition of L-like Ca^{2+} current in guinea-pig ventricular myocytes. Little work on Ca^{2+} currents has been carried out using thiopental, but other barbiturates have been shown to inhibit a number of voltage-gated Ca^{2+} channels; however, once again, the IC_{50} concentrations are generally high.[12,33,34] For example, N-type Ca^{2+} channels expressed in *Xenopus* oocytes injected with human brain RNA were inhibited by pentobarbital with IC_{50} concentrations of ~1,000 μM for peak currents and 400 μM for sustained currents.[12] Conversely, voltage-gated Ca^{2+} channels of unknown type(s) in guinea-pig hippocampal CA1 neurons have been reported[35] to

be exceptionally sensitive to pentobarbital (IC_{50} = 16 μM for peak currents).

Our use of Ba^{2+} as a current carrier rather than Ca^{2+} (for the reasons outlined in materials and methods) raises one possible caveat that should be addressed. When Ca^{2+} ions are used, a Ca^{2+}-dependent inactivation of the inward current is sometimes observed with Ca^{2+} channels[36]; anesthetics, in principle, could affect this process. It also has been observed with both halothane[9] and pentobarbital[12,13] that the responses following long depolarizations are somewhat more sensitive to anesthetic inhibition than the peak currents. However, voltage-gated Ca^{2+} channels present at synaptic nerve terminals (usually considered to be most relevant to general anesthesia) open only briefly (~ a millisecond[37]) in response to depolarization by an invading action potential. Therefore, the peak, rather than sustained, currents are probably more important when considering mechanisms of general anesthesia.

The P-type channels we have studied in cerebellar Purkinje neurons may turn out to consist of a heterogeneous population of Ca^{2+} channels, and we cannot rule out the possibility that a small subpopulation has a higher sensitivity to anesthetics. In addition, anesthetic sensitivity might be different *in vivo* than in our acutely dissociated neurons, because of, for example, modulation of Ca^{2+} channel properties by neurotransmitters acting on second-messenger systems.[38] Furthermore, there have been isolated reports[10,35] of Ca^{2+} channels in other neurons being exceptionally sensitive to certain anesthetics. However, the bulk of the evidence available,[25,30] including the results of the current study, suggests that most Ca^{2+} channels (and voltage-gated channels in general) are relatively insensitive to inhibition by anesthetic EC_{50} concentrations that produce general anesthesia. We believe an opposite conclusion was drawn often in the past mainly because of the uncritical use of anesthetic concentrations much higher than the free aqueous EC_{50} concentrations for general anesthesia (table 1 and materials and methods).

It is conceivable that the small inhibitions of P-type Ca^{2+} channels we have observed at surgically relevant anesthetic concentrations might be amplified in some way (for example, there is a steep dependence of neurotransmitter release on Ca^{2+} entry at nerve terminals[39]) and hence lead to significant disruption of neuronal function. (One can always postulate that an anesthetic effect, however small, may have some profound physiologic consequence.) Recent evidence on the effects of anesthetics on neurotransmitter release, however, suggests the chain of events underlying presynaptic neurotransmitter release can be relatively insensitive to anesthetics.[30] Overall, we believe, the contrasting, very much greater anesthetic sensitivity of certain ligand-gated ion channels[30] makes these, rather than voltage-gated Ca^{2+} channels, the most attractive candidates for major anesthetic targets. In conclusion, inhibition of voltage-gated P-type Ca^{2+} channels plays, at most, a supporting role, as opposed to a primary role, in the induction of general anesthesia.

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