Effects of intravenous and local anesthetic agents on ω-conotoxin $MVII_A$ binding to rat cerebrocortex

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Purpose: The cellular target site(s) for anesthetic action remain controversial. In this study we have examined any interaction of *iv* anesthetics (thiopental, pentobarbital, ketamine, etomidate, propofol, alphaxalone), local anesthetics (lidocaine, prilocaine, procaine and tetracaine), and the non anesthetic barbiturate, barbituric acid with the ω-conotoxin $MVII_A$ binding site on N-type voltage sensitive Ca^{2+} channels in rat cerebrocortical membranes.

Methods: [^{125}I] ω-conotoxin $MVII_A$ binding assays were performed in 0.5 ml volumes of Tris.HCl buffer containing BSA 0.1% for 30 min at 20°C using fresh cerebrocortical membranes (5 μg of protein). Non-specific binding was defined in the presence of excess (10^{-8} M) ω-conotoxin $MVII_A$. The interaction of *iv* (alphaxolone, etomidate, propofol, pentobarbitone, ketamine and thiopentone), local (lidocaine, prilocaine, procaine and tetracaine) anesthetics and barbituric acid was determined by displacement of [^{125}I] ω-conotoxin $MVII_A$ (~1pM).

Results: The binding of [^{125}I] ω-conotoxin was concentration-dependent and saturable with B_{max} and K_d of 223 ± 15 fmol/mg protein and 2.13 ± 0.14 pM, respectively. Unlabelled ω-conotoxin $MVII_A$ displaced [^{125}I] ω-conotoxin $MVII_A$ yielding a pK_d of 11.04 ± 0.04 (9.2 pM). All *iv* and local anesthetics at clinically relevant concentrations did not show any interaction with the ω-conotoxin $MVII_A$ binding site.

Conclusion: The present study suggests that ω-conotoxin $MVII_A$ binding site on N-type voltage sensitive Ca^{2+} channels may not be a target for *iv* and local anesthetic agents.

Objectif : L'existence de sites cellulaires cibles pour l'action des anesthésiques demeure controversée. La présente étude a examiné toutes les interaction des anesthésiques *iv* (thiopental, pentobarbital, kétamine, étomidate, propofol, alphaxalone), et locaux (lidocaïne, prilocaïne, procaïne et tétracaïne), des barbituriques non anesthésiques et de l'acide barbiturique avec le site de fixation de la T-conotoxine $MVII_A$ sur les canaux Ca^{2+}voltage-dépendants de type N, localisés sur des membranes cérébrocorticales de rats.

Méthode : Des essais de fixation avec la [^{125}I] T-conotoxine $MVII_A$ ont été réalisés dans 0,5 ml de tampon Tris.HCL contenant de l'albumine de sérum de bœuf (ASB) à 0,1 % pendant 30 min à 20 °C en utilisant des membranes cérébrocorticales fraîches (5 μg de protéine). La fixation était jugée non spécifique en présence d'un excès (10^{-8} M) de T-conotoxine $MVII_A$. L'interaction des anesthésiques *iv* (alphaxolone, étomidate, propofol, pentobarbital, kétamine et thiopental), locaux (lidocaïne, prilocaïne, procaïne et tétracaïne) et de l'acide barbiturique a été déterminée par le déplacement de la [^{125}I]T-conotoxine $MVII_A$ (~1pM).

Résultats : La fixation de la [^{125}I]T-conotoxine était dépendante de la concentration et saturable avec B_{max} et K_d de 223 ± 15 fmol/mg de protéine et 2,13 ± 0,14 pM, respectivement. La T-conotoxine $MVII_A$ non marquée a déplacé la [^{125}I]T-conotoxine $MVII_A$ fournissant un pK_d de 11,04 ± 0,04 (9,2 pM). Tous les anesthésiques *iv* et locaux en concentrations applicables en clinique n'ont pas montré d'interaction avec le site de fixation de la T-conotoxine $MVII_A$.

Conclusion : L'étude suggère que le site de fixation de la T-conotoxine $MVII_A$ sur les canaux Ca^{2+} voltage-dépendants de type N, ne serait peut-être pas une cible pour les anesthésiques *iv* et locaux.

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THE role of voltage sensitive Ca^{2+} channels (VSCC) in anesthesia is controversial.[1,2] We have shown that intravenous[3] and local[4] anesthetics interact with dihydropyridine binding sites on L-type VSCC. Moreover, there was a correlation between anesthetic potency and interaction with the dihydropyridine binding sites.[3,4] Despite the observation that central transmission is not usually under the control of L-VSCC these data might implicate L-VSCC as anesthetic target sites.

N-type VSCC are distributed widely in the nervous system and play a far more important role in the control of neurotransmitter release.[2] Electrophysiological (functional) studies suggest that clinically relevant concentrations of propofol,[5] several barbiturates[6] and local anesthetic agents[7] significantly depress ω-conotoxin sensitive N-channel currents. The precise site of this interaction is unclear.

As part of a broader pharmacological examination of the role of VSCC in anaesthesia (and to allow comparison L-VSCC data) we have examined whether a range of intravenous and local anesthetic agents interact with ω-conotoxin binding sites on N-type VSCC. ω-Conotoxin VII_A was used as the binding is fully reversible and ideally suited to radioligand binding studies.

Methods

Tissue preparation

Female Wistar rats (250-300g) were stunned then decapitated. The brain was rapidly removed, the cerebrocortex detached from its internal structures, placed in 50 mM Tris.HCl, pH7.4 at 4°C, and homogenized using an ultra turrax (T25). The resulting homogenate was centrifuged at 18000 g for 10 min and the pellet resuspended in Tris.HCl buffer. This procedure was repeated three times.

Measurement of [^{125}I] ω-conotoxin $MVII_A$ binding.

All binding assays were performed in 0.5 ml volumes of Tris.HCl buffer containing 0.1% bovine serum albumin for 30 min at 20°C using 5 μg fresh membranes. Saturation analyses to determine the equilibrium dissociation constant (K_d) and the maximal binding capacity (B_{max}) were performed using increasing concentrations of [^{125}I] ω-conotoxin $MVII_A$ (0.1-10 nM). Non-specific binding was defined with 10^{-8}M ω-conotoxin $MVII_A$. Bound and free radioactivity were separated using a Brandell cell harvester onto Whatman GFB filters (treated with 0.1% polyethylenimine) with bound estimated using a γ-counter.

In order to determine if an agent interacted with ω-conotoxin binding sites on N-VSCC, these were labeled with a fixed concentration of [^{125}I] ω-conotoxin $MVII_A$. Increasing concentrations of unlabeled displacers (anesthetic agents) were added, and displacement of [^{125}I] ω-conotoxin $MVII_A$ binding indicates an interac-

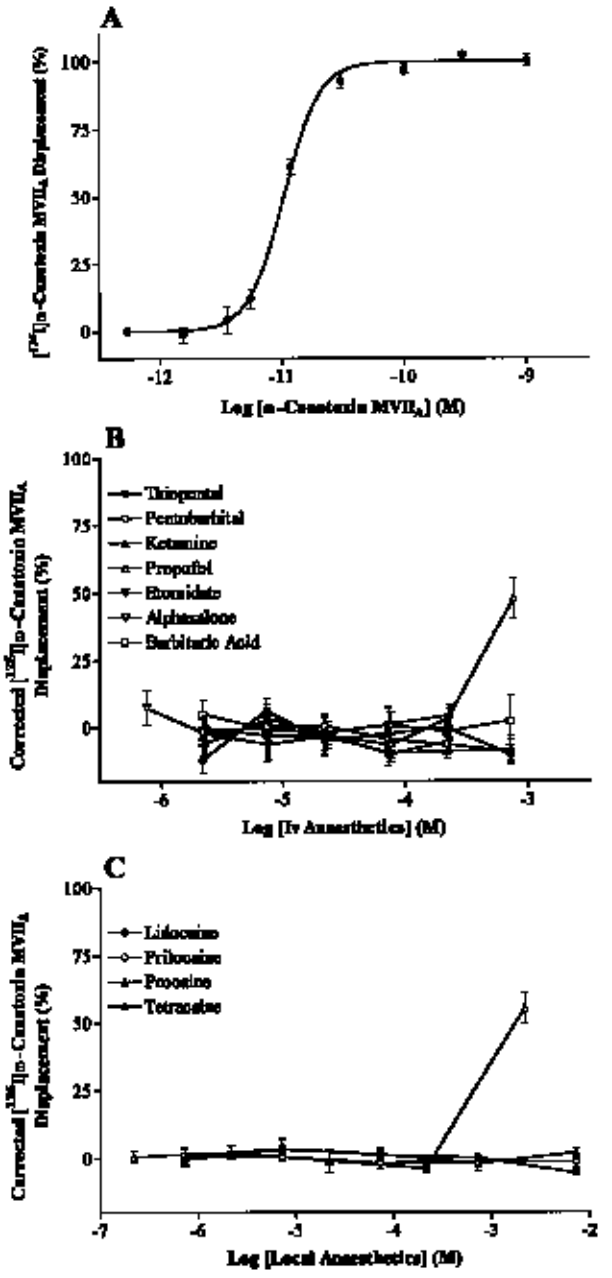

FIGURE Unlabeled ω-conotoxin $MVII_A$ (A) but not Iv (B) or local (C) anesthetic agents at clinically relevant concentrations displaced [^{125}I] ω-conotoxin $MVII_A$ binding to N-type voltage sensitive Ca^{2+} channels. Data are mean ± SEM (n=6) and expressed as displacement where the curves were corrected for the competing mass of [^{125}I] ω-conotoxin $MVII_A$.

tion at the same site as the radiolabel. [^{125}I] ω-conotoxin $MVII_A$ (~1pM) was incubated with various concentrations of the following unlabelled intravenous anesthetic agents: thiopental, pentobarbital, ketamine, etomidate, propofol and alphaxalone, and local anesthetic agents: lidocaine, prilocaine, procaine and tetracaine. A non-anesthetic barbiturate, barbituric acid was also included. Unlabeled ω-conotoxin $MVII_A$ was included as a positive control. Agents were dissolved in Tris.HCl buffer from stocks as follows; thiopental (100 mM stock in 0.1M NaOH), pentobarbital and barbituric acid (50 mM stock in distilled water), propofol (100 mM stock in DMSO), alphaxalone (50 mM stock in DMSO), etomidate (50 mM stock in 0.1M HCl), ketamine and local anesthetic agents (500 mM stock in distilled water), and ω-conotoxin $MVII_A$ (10^{-6}M in distilled water). Anesthetic concentration used was limited by solubility.

Data analysis

B_{max} and K_d were obtained from Scatchard transformation of the specific binding data. The log concentration unlabelled conotoxin producing 50% displacement of labelled conotoxin binding (i.e., isotope dilution), pK_d was obtained by computer assisted curve fitting (GraphPad-PRISM). All data are expressed as mean ± SEM, n refers to the number of individual full saturation or displacement curves constructed.

Results

The binding of [^{125}I] ω-conotoxin was concentration-dependent and saturable with B_{max} and K_d of 223 ± 15 fmol·mg^{-1} protein and 2.13 ± 0.14 pM, respectively. Unlabelled ω-conotoxin $MVII_A$ displaced [^{125}I] ω-conotoxin $MVII_A$ yielding a pK_d of 11.04 ± 0.04 (9.2 pM), in reasonable agreement with the value obtained by direct saturation. These K_d and B_{max} values for [^{125}I] ω-conotoxin $MVII_A$ binding were similar to those previously reported by Yamada *et al.*[8] More importantly, all anesthetic agents at clinically relevant concentrations failed to displace [^{125}I] ω-conotoxin $MVII_A$ (Figure).

Discussion

We have failed to detect any interaction between a range of intravenous and local anesthetic agents with the ω-conotoxin $MVII_A$ binding site on N-VSCC. These negative findings have important implications in that they cast doubt on the suggestion that N-VSCC may represent a target for, particularly, *iv* anesthetic agents.

In the search for a common target site for anesthetic agents, voltage and ligand gated ion channels have been extensively studied. It has been suggested that $GABA_A$ receptors and voltage sensitive Na^+ channels may be the most important target sites for general and local anesthesia, respectively.[1,9] However, Ca^{2+} is also likely to play an important role in the mechanism of general and local anesthesia, as this cation regulates neuronal excitability and neurotransmitter release through VSCC.[2]

Several reports[5–7] suggest that intravenous and local anesthetics at clinical concentrations inhibit N-VSCC. These data implicate N-VSCC in the mechanism of anesthesia. However, in the present study, clinically relevant concentrations of intravenous and local anesthetic agents did not displace ω-conotoxin $MVII_A$ binding to N-VSCC. Nacif-Coelho and colleagues[10] failed to report any involvement of N-VSCC in the hypnotic response to dexmedetomidine as the hypnotic effects of this agent were not modified by ω-conotoxin $MVII_A$. However, nifedipine (L-VSCC blocker) and ω-conotoxin $MVII_C$ (P+Q-VSCC blocker) produced a loss of righting reflex in the presence of a subhypnotic dose. The clinical significance of these data are difficult to state as this is a mechanistic study. Collectively our data suggest that ω-conotoxin $MVII_A$ binding sites on N-VSCC may not be an important anesthetic target. To test this hypothesis in man is not at present feasible as there are currently no N-VSCC blockers available for clinical use.

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