INIBSA/Instituto de Farmacología Teófilo Hernándo
Pharmacological profile of IQB-9302, a new local anaesthetic

REPORT 99/3

EFFECTS OF IQB-9302 ON $\alpha_3\beta_4$ NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS EXPRESSED IN OOCYTES

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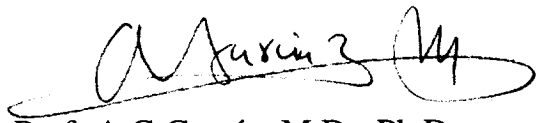
The present study was performed in compliance with the rules and regulations of Good Laboratory Practices published by OECD (1981) and according to the Real Decreto 822/1993 BOE (May 1993). There were no incidences that could affect reliability of data.

Director of the study:



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Date: July 14th 1999

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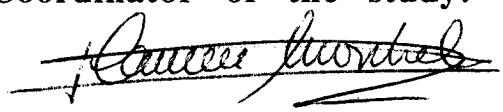

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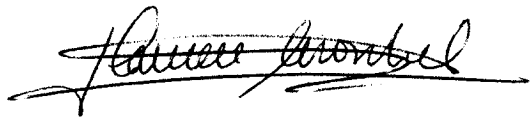
Date: July 14th 1999

Realization of the experiments:



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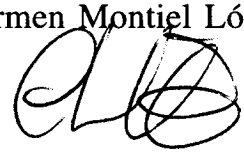

Carlos J. Herrero

INTRODUCTION

As mentioned in previous reports, local anaesthetics like IQB-9302 or bupivacaine bind to a specific receptor inside the pore of the Na^+ channel in the nerves, blocking the entry of Na^+ ions. However, additional effects of local anaesthetic drugs on nicotinic receptors have been described in the literature. Thus, we think that in the context of the study of the pharmacological profile of this new compound, it would be of interest to study a possible additional blocking effects of IQB-9302 on a pure population of nicotinic acetylcholine receptors. To address this question, we have expressed the neuronal rat brain $\alpha_3\beta_4$ nicotinic acetylcholine receptors (nAChRs) in oocytes of the frog *Xenopus laevis*.

With this purpose, we studied the effects of IQB-9302 and bupivacaine on $\alpha_3\beta_4$ currents induced by acetylcholine (ACh) applied to oocytes injected with the corresponding mRNAs.

MATERIALS AND METHODS

Techniques for the *in vitro* transcription of nicotinic AChR subunits cDNAs, oocytes injection and electrophysiological recordings of the expressed foreing receptors have been described previously by our group (Montiel et al., 1997; López et al., 1998; Herrero et al., 1999).

Preparation of RNA and injection of Xenopus oocytes

The plasmids pPCA48E, pZPC13, containing the entire coding regions of rat brain nicotinic AChR α_3 and β_4 subunits were linearized with the restriction enzymes *Eco*RI and *Xho*I, respectively. Linearized plasmids were transcribed with SP6 (α_3) and T3 (β_4) RNA polymerases using a mCAP RNA capping Kit (Stratagene C.S. La Jolla, CA. USA).

Mature female *Xenopus laevis* frogs obtained from a commercial supplier (CRBM du CNRS, Montpellier, France) were anesthetized with tricaine solution (0.125%) and ovarian lobes were dissected out. Then, follicle-enclosed oocytes were manually stripped from the ovary membranes and incubated overnight at 16ºC in a modified Barth's solution containing (in mM): NaCl 88, KCl 1, $NaHCO_3$ 2.4, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 10, buffered to pH 7.4 and supplemented with gentamycin (0.1 mg ml^{-1}) and sodium pyruvate (5 mM). Next day, healthy follicle-enclosed oocytes were injected with 50 nl (25:25 ng) of α_3:β_4 RNAs using a nanoject automatic injector (Drummond Scientific Co., Broomall, PA, USA). Electrophysiological recordings were made 2-5 days after RNA injections.

Electrophysiolgical recordings

Experiments were carried out at room temperature (22-25^{o}C) in Ringer's solution containing (in mM): NaCl 115, KCl 2, $CaCl_2$ 1.8, HEPES 5, buffered to pH 7.4 with NaOH. Membrane currents were recorded with a two-electrode voltage clamp amplifier (OC-725-B Warner Instrument Corporation, Hamden, CT, USA) using microelectrodes with resistances of 0.5-5 M? made from borosilicate glass (GC100TF-15, Clark Electromedical, Pangbourne, UK) and filled with KCl (3 M). The holding potential in all experiments was -60 mV.

Single oocytes were held in a 0.3 ml volume chamber and constantly superfused with Ringer's solution by gravity (4 ml min^{-1}). The volume in the chamber was maintained constant using the reverse suction of one air pump. Solutions containing the physiological nicotinic receptor agonist acetylcholine (ACh), or the local anaesthetics IQB-9302 or bupivacaine were applied with the use of a set of 2-mm diameter glass tubes located close to the oocyte. Voltage protocols, ACh pulses and data adquisition were controlled using a Digidata 1200 Interface and CLAMPEX software (Axon Instruments, Foster City, CA, USA).

Materials and solutions

All products not specified were purchased from SIGMA (Madrid, Spain), including bupivacaine.HCl. IQB-9302.HCl batch nº 9454.001 was obtained from LEBSA. All chemicals were reagent grade. IQB-9302 and bupivacaine were dissolved in distilled water at a concentration of 10^{-2} M and at the moment of each experiment diluted in Ringer's solution to the desired concentrations.

Statistical analysis

Values of antagonist concentration eliciting 50% blockade of maximal current (IC_{50}) were estimated through non-linear regresion analysis of ISI software for a PC computer from the concentration-response curves for antagonists (IQB-9302 and bupivacaine). Differences between groups were compared by Student's t test with the statistical program Statworks TM; a value of $p<0.05$ was taken as the limit of statistical significance.

RESULTS

Blockade by IQB-9302 and bupivacaine of $\alpha_3\beta_4$ currents

Oocytes expressing $\alpha_3\beta_4$ nicotinic AChRs were stimulated with pulses of ACh (100 µM, 20 s) applied at 2 min intervals. This interval between pulses was chosen in order to avoid nicotinic receptor desensitisation. Using this protocol, after a few initial ACh pulses, $\alpha_3\beta_4$ - mediated peak currents stabilised and they were quite reproducible over a 30 min period. Averaged amplitude of these currents at the beginning of the experiment amounted to 3 ± 0.3 µA (n= 5 oocytes). Figure 1 shows three original traces of the currents elicited by three successive ACh pulses in a typical oocyte expressing $\alpha_3\beta_4$ AChRs and the blockade exerted by 10 µM IQB-9302 perfused 1 min before and during the ACh pulse; such effect was exerted quickly and it was completely reversed during the first ACh pulse applied after whashout the drug.

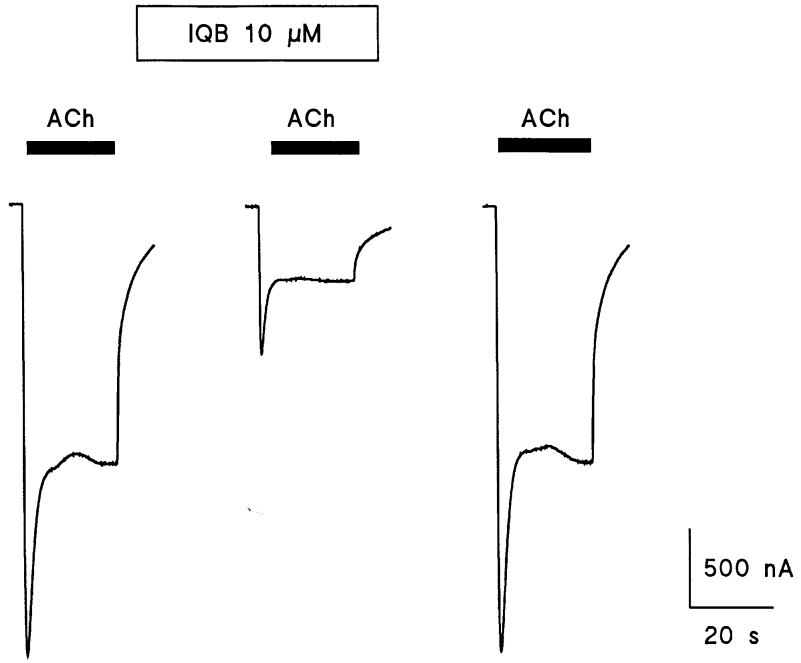

Fig.1.- Effects of IQB-9302 (IQB) on $\alpha_3\beta_4$ nicotinic currents. Original traces of currents elicited by three successive ACh pulses (100 µM, 20 s) applied every 2 min, in the absence, in the presence of IQB-9302, or after washout of the drug. Figure shows a typical experiment (out of 5).

When the same protocol was repeated, but this time studing the effect of bupivacaine, the blockade of $\alpha_3\beta_4$ current was similar to that obtained with IQB-9302. Figure 2 shows the original traces of the currents induced by successive ACh pulses applied to a typical oocyte expressing $\alpha_3\beta_4$ nicotinic AChRs, in control conditions and upon the addition of 10 µM bupivacaine .

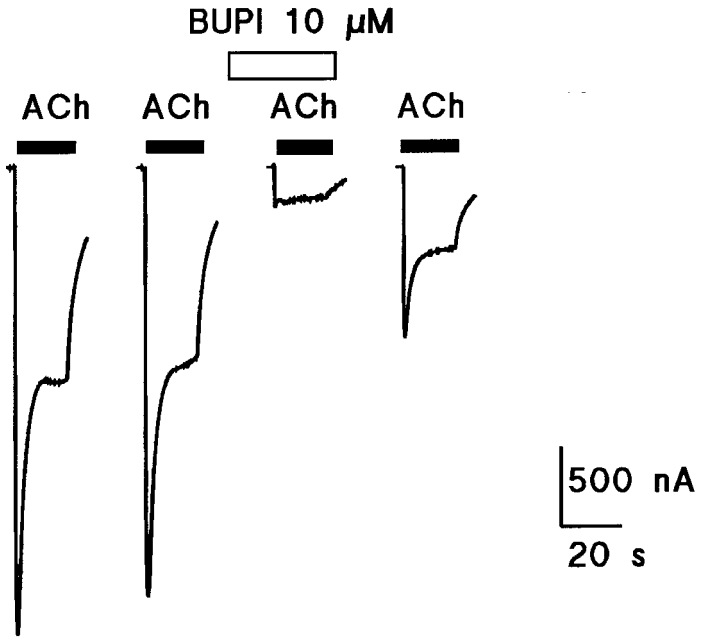

Fig.2.- Effects of bupivacaine (BUPI) on $\alpha_3\beta_4$ nicotinic currents. Original traces of currents elicited by successive ACh pulses (100 µM, 20 s) in the absence, in the presence of bupivacaine, or after washout of the drug. Figure shows a typical experiment (out of 4).

Recovery of blockade exerted by IQB-9302 and bupivacaine on $\alpha_3\beta_4$ currents

To study the rate of recovery of IQB-9302 and bupivacaine effect on nicotinic currents the following experiments were carried out. Oocytes expressing $\alpha_3\beta_4$ nAChRs were stimulated with brief ACh pulses (100 µM, 1s), at 1 min intervals. Reproducible responses were obtained under these experimental conditions. The perfusion of IQB-9302 or bupivacaine produced an inmediate maximum blockade during the first ACh pulse applied in the presence of both drugs; however while the recovering of the IQB-9302 effect was complete 2 min after remove the drug, the blockade by bupivacaine was only partial upon 6 min of washout; also the rate of recovering was slower with bupivacaine compared with IQB-9302 (Fig 3).

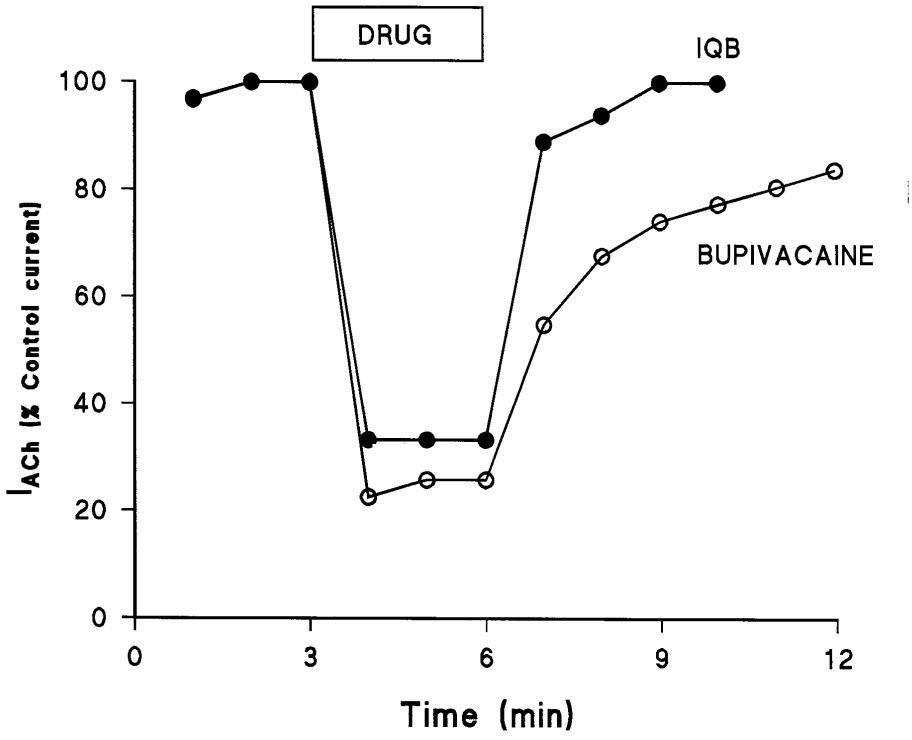

Fig.3.- Recovery of the IQB-9302 and bupivacaine blockade on $\alpha_3\beta_4$ nicotinic currents upon washout of the drug. Original traces of currents elicited by successive ACh pulses (100 µM, 1 s) at 1 min intervals. Pulses were applied in the absence, in the presence of IQB-9302 or bupivacaine, or after washout of the drug. Figure shows a typical experiment (out of 4).

Concentration-response curves of IQB-9302 and bupivacaine on $\alpha_3\beta_4$ currents

In order to compare the potency of IQB-9302 and bupivacaine to block the neuronal nAChRs, concentration-response curves for both anaesthetic drugs were performed. Oocytes expressing $\alpha_3\beta_4$ nicotinic AChRs were stimulated with pulses of ACh (100 µM, 20 s), applied at 2 min intervals. The peak amplitude of the stabilised ACh-induced current (I_{ACh}), just preceding the addition of IQB-9302 or bupivacaine, was used as control response (100%). Then, the effect of increasing concentrations of IQB-9302 or bupivacaine (range from 100 nM to 300 µM) on $\alpha_3\beta_4$ currents was determined. No more than two different concentrations per single oocyte were assayed. Figure 4 shows the results obtained with each of these drugs added 1 min before and during the ACh pulse. Each point in the figure represents means$\pm$ SEM of 4-6 oocytes tested. The IC_{50s} values obtained for IQB-9302 and bupivacaine were $1.4x10^{-6}$ M and $3x10^{-6}$ M, respectively; no statistical differences were found between these two values.

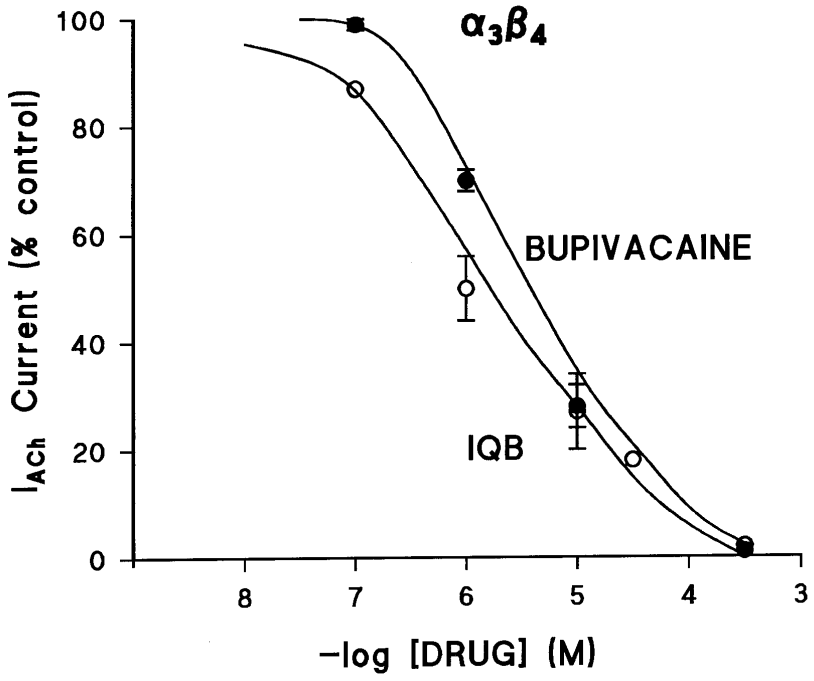

Fig.4.- Effects of increasing concentrations of IQB-9302 (IQB) and bupivacaine on $\alpha_3\beta_4$ nicotinic currents. Currents were evoked by ACh pulses (100 µM, 20 s) applied every 2 min. Anaesthetic drugs were perfused 1 min before and during the ACh pulse. Values are expressed as percentage of control current elicited by ACh in the absence of drugs. Each point represents means±SEM of the values obtained in 4-6 different oocytes tested.

DISCUSSION AND CONCLUSIONS

According to the results presented above, we can conclude that both IQB-9302 and bupivacaine block the $\alpha_3\beta_4$ neuronal nicotinic acetylcholine receptors at concentrations in the low micromolar range (the IC_{50s} were $1.4x10^{-6}$ M and $3x10^{-6}$ M respectively for IQB-9302 and bupivacaine). Thus, both compounds show similar potency blocking this nicotinic receptor subtype, although IQB-9302 effect was reversed faster and completely while bupivacaine effect was not. These actions are unlikely contributing to the mechanism of the local anaesthetic actions of these compounds, neither to adverse drug reactions when injected topically. However, if given systemically by accident they might induce hypotension by interfering transmission at sympathetic ganglia where neuronal nicotinic acetylcholine receptors are present.

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