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Depolarizing Effect of Various Local Anaesthetics on the *Helix aspersa* Neurons: Dose-response Relationship

P. CARDI, M. LARTIGUE* AND J. M. MEUNIER

*Laboratoire de Physiologie et de *Pharmacologie Pharmacie, UER de Médecine et de Pharmacie, 2 rue du Dr Marcland—87025 Limoges Cedex, France*

Abstract—The depolarizing effect of various local anaesthetics (LA) on the membrane potential of *Helix* central neurons has been examined. There is a relation between depolarizing effect and concentration of LA in the bath that is linear over a range of concentrations. The slope of the curve is significantly higher for amethocaine (tetracaine) than for procaine while for dibucaine the dose-response relation is not linear. The blockade of a response to acetylcholine (ACh) is about two fold higher for dibucaine and amethocaine than for procaine. These results suggest that both amethocaine and procaine act at the ACh-site in addition to their binding with specific sites located within the ionic channel lumen; dibucaine appears to act through another mechanism.

A depolarizing response is obtained by local application of procaine on certain types of snail neurons (D. type depolarized by ACh). This effect is interpreted as an interaction of procaine with the cholinergic receptor (Israel & Meunier 1979). We set out to determine if other local anaesthetic (LA) drugs possess this property and to study the relation between the amplitude of this effect and the dose response to different LAs. The antagonistic effect of LAs on the cholinergic response, widely described in other systems such as the neuromuscular (Katz & Miledi 1975; Adams 1977; Gage & Wachtel 1984) and *Electrophorus* and *Torpedo* electric organs (Podleski & Bartels 1963; Bartels & Nachmansohn 1965; Cohen et al 1974; Heidmann & Changeux 1979), has also been considered. The interaction of LA molecules with specific membrane sites (Koblin & Lester 1979) does not exclude a binding of LAs to (or close to) the cholinergic site which would result in an agonist-type effect (Weber & Changeux 1974; Aracava & Albuquerque 1984; Waksman et al 1980).

Materials and Methods

The perioesophageal ring of the nervous system of *Helix aspersa* was removed and placed in an experimental chamber of internal volume of 4 mL. The preparation was continuously perfused with fresh Ringer with the following ionic concentrations (mM) NA^+, 120; K^+, 5; Ca^{2+}, 6; Mg^{2+}, 3·5; Cl^-, 134; Tris HCl, 10; pH = 7·3.

Nerve cells in ganglia were easily visible under a binocular microscope. The outer connective sheath around the brain was removed and a double microelectrode was inserted into the cell of the visceral ganglia. Conventional glass microelectrodes having resistances of 5–20 MΩ and filled with KCl 3M were used. They led via a cathode follower to an oscilloscope and a pen recorder. Membrane potential and action potentials were recorded.

Correspondence: M. Lartigue, Laboratoire de Pharmacologie-Pharmacie, UER de Médecine et de Pharmacie, 2 rue du Dr Marcland—87025, Limoges Cedex, France

ACh was applied iontophoretically. Procaine was tested by dissolving it in the bathing medium and was delivered via a pneumatic ejection system which allowed rapid administration (0·05 to 1 s) of small volumes of solution from micropipette. According to the classification of Tauc & Gerschenfeld (1961), D neurons are excited by ACh and H neurons are inhibited. This work was carried out on D cells which also respond to procaine application with depolarization. These neurons are named D_1 cells (Israel & Meunier 1979). Before the beginning of a recording session, the cell was hyperpolarized (−80 mV) to prevent spontaneously firing action potentials. Membrane resistance was systematically monitored by measuring the voltage deflection produced by constant transmembrane current pulses.

The experimental procedure was as follows: first, the neuron received an iontophoretic application of ACh to obtain a control depolarizing response (15 mV), the preparation was then superfused with an anaesthetic solution until a maximum depolarization was reached. Before the ganglia were bathed with a new concentration or a new anaesthetic solution, the extracellular medium was exchanged for the standard solution until the complete recovery of the initial membrane potential, and the amplitude of the control ACh response. Since this recovery could take a long time, the number of tests on one cell were insufficient to draw a dose-response curve. Consequently, curves were plotted with mean values obtained from several different cells (individual variability was compensated for by a large number of tests: 38).

Results

Drug selection

The following LA molecules were tested by replacing the bathing medium via a perfusion system with 10^{-4} M amethocaine (tetracaine), procaine, lignocaine, butacaine, stovaine, procainamide, benzocaine, dibucaine, dimethisoquin. Results are summarized in Table 1 which gives the mean (±s.e.m.) of depolarization and the number of experiments

Table 1. Effect of different LA on membrane potential of D_1 cells.

Drug 10^{-4} M	mV	N
Amethocaine	27·4 ± 1·2	(20)
Procaine	19·7 ± 1·6	(23)
Lignocaine	2·67 ± 1·11	(9)
Butacaine	0·25 ± 0·25	(4)
Stovaine	1·00 ± 1	(4)
Procainamide	3·50 ± 1·85	(4)
Benzocaine	2·25 ± 1·32	(4)
Dibucaine	2·9 ± 0·6	(28)
Dimethisoquin	0·86 ± 0·59	(7)

The results are the mean depolarization ± s.e.m. of the number of experiments indicated.

for each LA. This Table shows that amethocaine is the most potent drug in terms of cell depolarization (27·4 ± 1·2 mV). While procaine also induced a notable depolarization, other LAs for example, butacaine had a weak depolarizing effect (0·25 ± 0·25 mV). For those LAs that were weakly acting at 10^{-4} M, higher concentrations were also used. Only dibucaine and dimethisoquin then showed a significant effect; at $0·5 \times 10^{-3}$ M the depolarization obtained was large (about 25 to 30 mV) and was accompanied by firing of the cell. After these preliminary studies three LAs were chosen to determine the dose-response curve.

Dose-response relations

The range of LA concentrations was between 10^{-5} and 5×10^{-4} M (dibucaine) and 10^{-5} to 10^{-4} M (amethocaine, procaine). The results from a typical experiment are shown in Fig. 1 (for procaine and amethocaine) and in Fig. 2 (for dibucaine).

The effects of each LA at various doses were recorded on the same cell. The amplitude of depolarization increases with the dose. Data from 38 experiments are summarized in Table 2 which gives the mean amplitude of depolarizing responses (± s.e.m.) and number of experiments.

The amplitude of the depolarizing response is plotted against LA concentration in Fig. 2B. For amethocaine and procaine, the relation seems to be linear over the middle part of the curve (from $0·25 \times 10^{-4}$ to 10^{-4} M). Moreover,

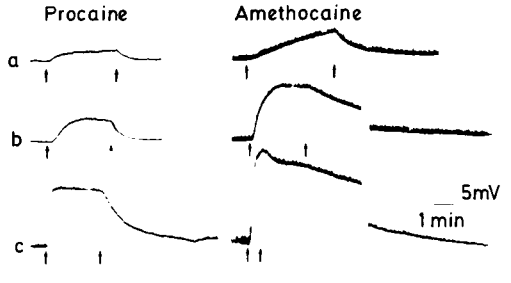

FIG. 1. Depolarizing responses induced by two LA on the membrane of D_1 cells. Procaine and amethocaine are added to the Ringer solution in concentrations of (a) 10^{-5}, (b) 5×10^{-5} and (c) 10^{-4} M. The cells were held hyperpolarized throughout at −80 mV. The amplitude of the effects was dose dependent and lasted for several minutes before the potential gradually returned to 'normal'. Arrows indicate the beginning and the end of each perifusion. The apparent noise on the traces is spontaneous synaptic activity.

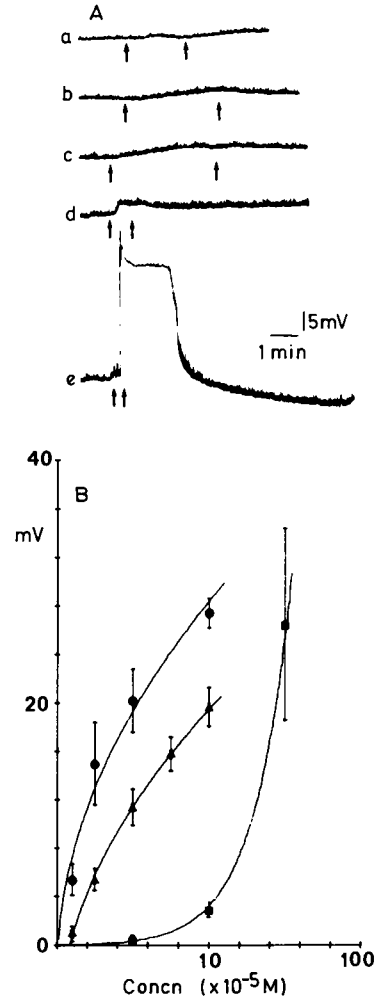

FIG. 2. (A) Effects of dibucaine on membrane potential of the same D_1 cell in Fig. 1. Dibucaine was added to the medium at (a) 10^{-5}, (b) $0·5 \times 10^{-4}$, (c) 10^{-4}, (d) $0·25 \times 10^{-3}$ and (e) $0·5 \times 10^{-3}$ M. Up to concentration of $0·25 \times 10^{-3}$ M, the membrane potential was relatively unaffected; at the highest concentration ($0·5 \times 10^{-3}$ M) however, the depolarization was abrupt and intense with recovery taking less than 2 min. Arrows indicate the beginning and the end of LA perfusion. (B) Amplitude of depolarization induced by perfusion of three anaesthetic solutions: amethocaine (●), procaine (▲) and dibucaine (■). The amplitude of the voltage deflection was measured and plotted against the concentration of various anaesthetic solutions ($\times 10^{-5}$ M). Each point is a mean value of 6 to 28 measurements; vertical lines are s.e.m.

Table 2. Blockade of ACh iontophoretic response.

Drug 10^{-4} M	% ACh blockade	N
Amethocaine	66·70 ± 5·10	(10)
Dibucaine	66·22 ± 5·47	(9)
Procaine	31·85 ± 6·40	(10)
Stovaine	32·51 ± 6·81	(5)
Procainamide	36·40 ± 7·90	(5)
Lignocaine	37·50 ± 7·51	(4)

The blockade of ACh iontophoretic response by various LA is given as a percentage (± s.e.m.) of inhibition of a control response induced at the same potential; the number of experiments is in parentheses.

amethocaine appears to have a significantly greater depolarizing action ($20{\cdot}2 \pm 2{\cdot}6$ mV at $0{\cdot}5 \times 10^{-4}$ M) than procaine ($11{\cdot}4 \pm 1{\cdot}5$ mV) at the same concentration. Conversely, the depolarizing effect of dibucaine was weak or absent in the range of concentrations used for procaine or amethocaine but was marked at $0{\cdot}5 \times 10^{-3}$ M ($26{\cdot}5 \pm 7{\cdot}9$ mV). Thus, the hypothesis of a unique regression curve for both amethocaine and procaine cannot be considered in this case.

In the presence of an ACh antagonist, atropine or hexamethonium 10^{-4} M, the depolarizing responses of procaine, amethocaine and dibucaine were abolished. Partial or total recovery appeared after the ganglia had been washed in the case of procaine and amethocaine but not dibucaine.

To compare the depolarizing potency of LAs with that of ACh, we also studied the response induced by various concentrations of ACh in the bath. At a concentration of $0{\cdot}75 \times 10^{-5}$ M a mean depolarization of $15{\cdot}6 \pm 2{\cdot}1$ mV was obtained ($n = 5$).

Antagonist effect

In parallel with the study of depolarizing effects we explored the potency of LAs in inhibiting ACh iontophoretic control response. Since LAs in the bath caused membrane depolarization, the amplitude of the ACh response after washing was compared with that induced at the same membrane potential level. Results are expressed as percentage of inhibition of this control response (Table 2).

Amethocaine and dibucaine at 10^{-4} M were the most potent antagonists ($66{\cdot}7 \pm 5{\cdot}1$ and $66{\cdot}2 \pm 5{\cdot}5$%, respectively) whereas the blockade induced by procaine and the other drugs was about two-fold weaker. Reversal of the depolarizing effect with washing out of the LA was much more rapid than the removal of an ACh blockade. Reversal of the blocking effect lasted 3–4 min with procaine and took much longer with both amethocaine and dibucaine (15 to 20 min at 10^{-4} M).

Discussion

In *Helix* neurons, procaine and amethocaine had two effects at the same doses (10^{-5} to 10^{-4} M): 1) a depolarizing ACh-agonist effect, and 2) a blocking ACh-antagonistic effect. Israel & Meunier (1979) have shown that the depolarization due to procaine is the result of the activation of an ACh-receptor, since ionic (Na^+ dependence), electrical (voltage dependence) and pharmacological properties (+)-tubocurarine and atropine blockade) are identical with features of the depolarization obtained with ACh.

In other preparations, *Electrophorus electricus* electroplaques (Podleski & Bartels 1963; Bartels & Nachmansohn 1965) or in-vitro, application of LAs to receptor-rich membrane fragments obtained from *Torpedo marmorata* (Cohen et al 1974; Aracava & Albuquerque 1984), have shown that LAs may bind to the ACh receptor site but at concentrations significantly greater than those blocking the response to agonists. On the other hand, Cohen & Changeux (1973) have already suggested from biochemical evidence that dansylcholine (a fluorescent analogue of ACh) may have both anaesthetic and agonist properties, while decamethonium (Adams & Sakmann 1978) opens and blocks end-plate channels in frog muscle fibres.

Our study on the agonistic action of LAs shows a dose dependency (Fig. 2) for both procaine and amethocaine that is similar but of a different efficacy. For example a 15 mV depolarization was obtained by adding amethocaine $2{\cdot}5 \times 10^{-5}$ M, whereas for the same effect to be induced by procaine required a three-fold higher concentration ($7{\cdot}5 \times 10^{-5}$ M). In the case of dibucaine, only a weak depolarization was obtained in the above range of concentrations. However, at higher concentrations, a sudden maximal response was observed. It was not possible to obtain depolarization of variable amplitude by adding intermediate doses.

The relationship between ACh dose and response amplitude resembles that of amethocaine and procaine but with a greater slope of the dose-response curve. Indeed, to induce the same 15 mV depolarization with ACh a dose of $0{\cdot}75 \times 10^{-5}$ M was sufficient.

To measure the effects of different doses successively, on the same cell without lengthening or disturbing (by firing or high depolarization) the return to initial conditions, doses higher than 10^{-4} M were generally avoided so we were unable to determine precisely the amplitude of maximal effects. This prevented us from establishing the DE50 value and comparing the efficiency of several drugs and their affinity for receptor sites. Despite this limitation, a graphic extrapolation (Fig. 2) shows a procaine maximum depolarization lower than that of amethocaine which itself is lower than that of ACh. Furthermore, these results provide a reference dose-response curve for an homogeneous cell population of the visceral ganglion (depolarized by ACh and LA).

By studying the antagonistic effect of LAs on the ACh response, another order of efficacy appears since dibucaine and amethocaine are more potent in antagonizing ACh depolarization: at 10^{-4} M they blocked 60% of the ACh response while procaine blocked only 30%. Thus, the presence of two types of effects (ACh agonist and antagonist) and two orders of potency of these effects (amethocaine and procaine being more potent in depolarizing effects while dibucaine and amethocaine were more potent in blocking ACh) support the conclusion that different binding sites are involved in the action of LAs, as already proposed by Earnst et al (1984). Moreover, a study of reversibility showed a dissociation into at least two types of binding sites since membrane repolarization upon washing always occurred before the disappearance of ACh blocking effect. Indeed, recent work on *Electrophorus* and *Torpedo* electric organs or receptor rich membrane fragments (reviewed by Changeux et al 1984) provides evidence for the binding of LAs to three categories of sites: i) high affinity sites, sensitive to histrionicotoxine present as a unique copy per receptor pentamer (Cohen et al 1980; Krodel et al 1979) and located within the channel itself; ii) low affinity sites, insensitive to histrionicotoxine, but much more numerous and lipid dependent (these sites are most likely to be located at the interface of the receptor with the membrane lipids (Koblin & Lester 1979; Adams 1977)); iii) finally LA may bind to the ACh receptor site; this occurs in a range of concentrations greater than that for which LAs block the response to agonists. Our work on *Helix* shows that both the agonist and antagonist action of LAs may occur within the same range of concentrations. The similarity of depolarizing effects of procaine and ametho-

caine with those of ACh suggests a similar mechanism involving binding to the same sites. Whatever the mechanism of the dibucaine depolarization, its blockade by ACh antagonists (as for procaine and amethocaine) indicates that ACh sites are also necessary for its effect.

For dibucaine blockade, it is necessary to envisage a different mechanism in which perturbation of the membrane arrangement may occur as seen in platelet membranes (Peerschke 1986) and from spin labelling on protein conformation in membrane components (proteins and lipids) of frog sciatic nerve (Grop & Belagy 1983). Antagonistic effects can be explained by the classical mechanism of action of non-competitive blockers which block ion translocation sterically and accelerate allostericaly some of the transitions of the ACh receptor (Gage et Wachtel 1984).

Thus although both the described effects are in opposition they are not mutually exclusive since different types of binding sites are involved.

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