J. Pharm. Pharmacol. 1993, 45: 632–635
Received September 23, 1992



# Effect of Local Anaesthetics on the Stimulus-secretion Coupling in Bovine Adrenal Chromaffin Cells*

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**Abstract**—This study was carried out to determine the relative potencies of local anaesthetics to inhibit the cholinergic synaptic transmission using cultured bovine adrenal chromaffin cells, and to clarify if the inhibitory action would correlate with biophysical and pharmacological properties. Local anaesthetics (bupivacaine, etidocaine, tetracaine, lignocaine and procaine; 0·02–2 mM) inhibited carbachol-induced catecholamine release from the cells in a concentration-dependent manner. This inhibition was completely reversible. IC50 (concentration of 50% inhibition) of each anaesthetic showed no correlation with the lipid solubility. The local anaesthetics showed greater inhibitory potency at a higher extracellular pH. The results suggest that clinically relevant concentrations of local anaesthetics inhibit the stimulus-secretion coupling in the chromaffin cells. The un-ionized base form plays a major role, and the inhibitory potency does not depend on the lipid solubility of the anaesthetics.

The primary pharmacological activity of local anaesthetic drugs involves inhibition of the excitation-conduction process in peripheral nerves. However, local anaesthetics have marked effects on central nervous system (CNS) function. Circulating lignocaine reduces minimum alveolar concentration (MAC) of cyclopropane in rats, MAC of halothane in dogs and MAC of nitrous oxide in man. There is no detailed information on whether the reduction of MAC results from similar effects to inhalation anaesthetics, i.e. depression of synaptic transmission. Within the CNS, it has proved difficult to assign particular transmitters to specific pathways which can be studied readily in-vitro (Pocock & Richards 1988). The adrenal medullary chromaffin cells have become widely used as models of neurosecretion (Baker & Knight 1984). They are readily isolated and maintained in culture, will extend processes similar to neurites, and will secrete catecholamines in response to nicotinic stimulation (Chern et al 1987).

The present study was carried out to determine the relative potencies of local anaesthetics to inhibit the stimulus-secretion coupling in chromaffin cells, and to clarify if the inhibitory action would correlate to biophysical and pharmacological properties of local anaesthetics. pH-dependency of local anaesthetic action on the chromaffin cells was also examined to clarify which of the protonated and neutral forms would exert actions.

## Materials and Methods

*Cell cultures*

Fresh bovine adrenal glands were obtained from a local slaughterhouse. Adrenal chromaffin cells were isolated by collagenase digestion of slices of the adrenal medulla as described previously (Wada et al 1985; Matsumoto et al 1986). In brief, the adrenal vein was cannulated and perfused with $Ca^{2+}$-free Krebs-Ringer phosphate (KRP) buffer at 37°C for 10 min to flush out the gland. The medullary tissues were sliced using a Stadie-Rigg's slicer. The slices were subjected to stepwise digestion with collagenase. The isolated cells were filtered through nylon mesh, collected by centrifugation and washed three times with KRP buffer. (Further purification of the chromaffin cells using a Percoll gradient was not performed, as there was no apparent effect on the experiments of carbachol on potassium-induced catecholamine release (Holz et al 1982).) Subsequently, the cells were suspended in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated foetal calf serum, aminobenzyl penicillin (60 μg $mL^{-1}$), streptomycin (100 μg $mL^{-1}$) and amphotericin B (0·3 μg $mL^{-1}$), and plated at a density of $4 \times 10^6$ cells per 2 mL per culture dish (Falcon, 35 mm diam.). After three days at 37°C in 5% $CO_2$–95% air, the chromaffin cells had formed monolayers containing 44·5 ± 2·7 μg (n = 8, mean ± s.e.) of catecholamines per dish. The culture medium was replaced at three-day intervals, and experiments were performed between days 3 and 10. The viability of the cells was more than 95% as judged by the trypan blue exclusion test.

*Catecholamine release*

In this experiment, adrenaline and noradrenaline were measured, and the sum of these two amines was expressed as catecholamines.

The incubation medium used for the catecholamine-release experiment was a warmed (37°C) modified Locke's solution. The composition of the standard solution was as follows (mM): NaCl 154, KCl 5·6, $CaCl_2$ 2·2, glucose 10 and Tris-HCl buffer (pH 7·4) 40. The cells were washed once with 2 mL ice-cold Locke's solution. To obtain equilibrium of local anaesthetic partition between medium and cells, before

* This manuscript was presented in part at 2nd America-Japan Anesthesia Congress, Hawaii, USA, 1991.

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starting the reaction, incubation for 1 min was carried out with 2 mL of the medium at 37°C in the presence or absence of local anaesthetics. The reaction was started by replacement of the pre-incubation medium with the reaction medium (2 mL) containing carbachol (0·3 mM) and various concentrations of local anaesthetics (0·02–2 mM bupivacaine, etidocaine, tetracaine, lignocaine or procaine), and was carried out for 1 min at 37°C. After the reaction, the medium was transferred to test tubes for catecholamine assay.

To examine the influence of medium pH on the effect of local anaesthetics, the same reaction was carried out using lignocaine and procaine at pH 6·8, 7·4 or 7·6. The reversibility of the effect of local anaesthetics was examined as follows. The first incubation was carried out for 1 min in the manner described above with various concentrations of local anaesthetics, except that the medium contained no carbachol. After incubation the cells were washed twice, and the reaction was carried out for 1 min in the medium containing carbachol.

For assay of catecholamines, 1 mL of the medium was acidified by addition of 0·5 mL 2·5% perchloric acid to precipitate protein. Catecholamines in the deproteinized sample were determined by an automated double-column high-performance liquid chromatography system (Yamatodani & Wada 1981) (Model CA825, Tosoh Co. Ltd, Tokyo). This assay system is based on the trihydroxyindole reaction, and has a limit of sensitivity of 5 pg $mL^{-1}$ for adrenaline and noradrenaline and the inter- and intra-assay variations are less than 3%.

*Statistical analysis*

All data are reported as mean ± s.e.m. Data were analysed using one-way analysis of variance with a Scheffe test to determine differences among the three groups. A $P$ value $< 0.05$ was considered statistically significant.

## Results

Basal release of catecholamines from the cells during a 1 min incubation period with no stimulation was less than 0·1 μg/$10^6$ cells (0·9% of the total catecholamines in the cells). Carbachol at 0·3 mM (the concentration that caused maximal response of the cells to release catecholamines) increased the release of catecholamines to 1·21 ± 0·15 μg/$10^6$ cells (n = 6) corresponding to 10·9% of the total catecholamines in the cells.

Fig. 1 shows the concentration-effect curves with respect to the inhibitory effects of local anaesthetics on the carbachol-induced catecholamine release from the chromaffin cells. Each anaesthetic inhibited the catecholamine release in

Table 1. Concentration of anaesthetics for 50% inhibition (IC50) of catecholamine release and lipid solubility of anaesthetics.

| Anaesthetics | IC50 (μM) | Lipid solubility* |
|---|---|---|
| Bupivacaine | 40 | 28 |
| Etidocaine | 120 | 141 |
| Tetracaine | 180 | 4·1 |
| Lignocaine | 210 | 2·9 |
| Procaine | 300 | 0·02 |

* Heptane/buffer partition coefficient at pH 7·4 (Covino 1986).

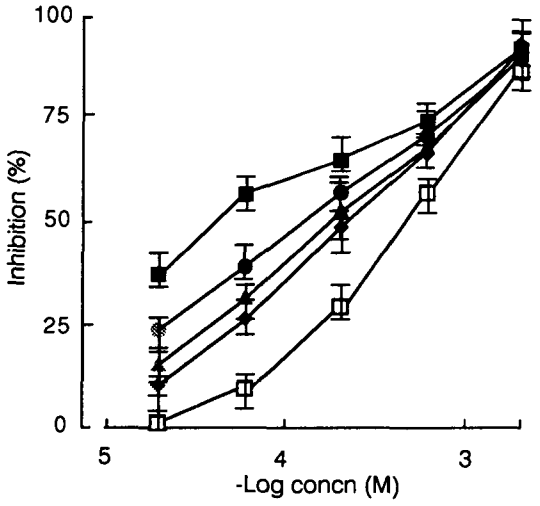


FIG. 1. Concentration-effect curves with respect to inhibitory effects of local anaesthetics on carbachol-induced catecholamine release from chromaffin cells. Chromaffin cells were incubated for 1 min in modified Locke's solution containing carbachol and various concentrations of anaesthetics (mean ± s.e., each point represents 6–8 experiments). ■ Bupivacaine, ● etidocaine, ▲ tetracaine, ◆ lignocaine, □ procaine.

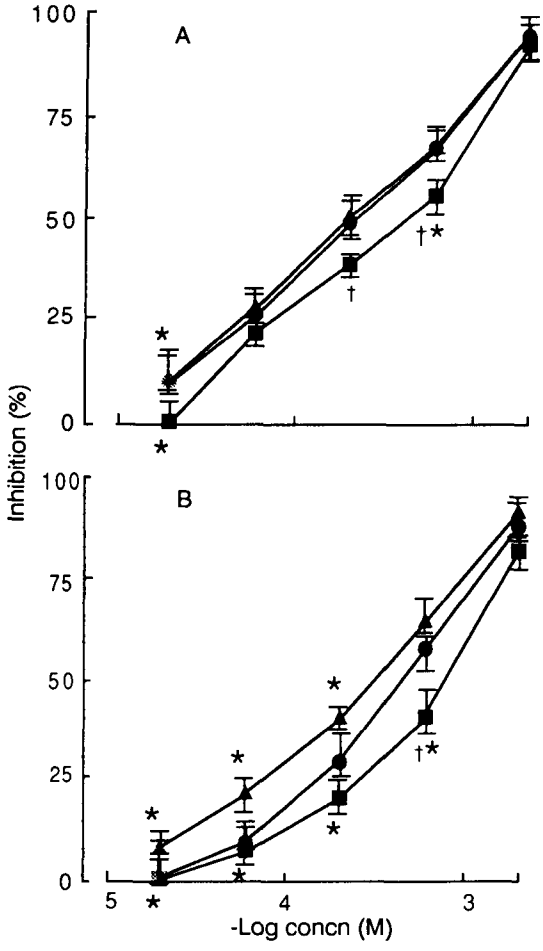


FIG. 2. Influence of extracellular pH on the inhibitory effect of lignocaine (A) and procaine (B) on carbachol-induced catecholamine release. Chromaffin cells were incubated for 1 min in modified Locke's solution at pH 6·8 (■), 7·4 (●) or 7·6 (▲) (mean ± s.e., each point represents 6–8 experiments). *$P < 0.05$ compared with pH 7·4, †$P < 0.05$ compared with pH 7·6.

Table 2. Reversibility of the inhibitory effects of local anaesthetics on carbachol-induced catecholamine release from chromaffin cells (mean ± s.e.; number of experiments is indicated in parentheses).

| Anaesthetic (2 mM) | Catecholamine release (% of control) | | | |
|---|---|---|---|---|
| | Experimental | (n) | After washout | (n) |
| Control | 99·2 ± 5·1 | (8) | 100·1 ± 6·2 | (4) |
| Bupivacaine | 10·5 ± 1·8 | (8) | 93·4 ± 7·0 | (3) |
| Etidocaine | 9·7 ± 1·1 | (6) | 99·5 ± 8·1 | (3) |
| Tetracaine | 10·6 ± 1·5 | (6) | 94·1 ± 5·0 | (3) |
| Lignocaine | 10·5 ± 1·2 | (8) | 100·5 ± 4·5 | (4) |
| Procaine | 11·0 ± 0·8 | (8) | 92·1 ± 7·1 | (4) |

a concentration-dependent manner over a concentration range of 0·02–2 mM. Table 1 shows the concentrations needed for 50% inhibition (IC50) of the catecholamine release and lipid solubility of each local anaesthetic (Covino 1986).

Influence of extracellular pH on the inhibitory effect of local anaesthetics was examined as shown in Fig. 2. Both procaine and lignocaine showed a greater potency at a higher pH. As shown in Table 2, the inhibitory effect of local anaesthetics was reversible; after its removal from the medium, the ability of the cells to release catecholamines was restored to the control level.

## Discussion

Besides effects on peripheral nerve fibres, local anaesthetics have marked effects on CNS functions (Bernards et al 1989; Scott et al 1989), and a number of investigators have described the use of lignocaine as a part of a general anaesthetic combination. It has been shown that lignocaine at plasma concentrations of 4·7–23·5 μM causes a mean decrease of cyclopropane MAC of 42% in rats (DiFazio et al 1976), and that lignocaine at plasma concentrations above 47·4 μM causes a decrease of halothane MAC by 45% in dogs (Himes et al 1977). In man, plasma lignocaine also showed a potentiation of general anaesthetic action; lignocaine (13·5 μM) reduced MAC of nitrous oxide by 30% (Himes et al 1977). The action of local anaesthetics on the CNS might involve a similar mechanism to that of general anaesthetics. However, there has been no detailed examination of this possible mechanism.

General anaesthetics have been shown to affect chemical transmission at anaesthetic concentrations rather than impulse conduction in the nerve fibres (Richards 1973; Richards & White 1975; Richards et al 1975; Zorychta et al 1975). The principal object of our experiments was to determine whether local anaesthetics could affect chemical transmission at concentrations that lie within the range encountered in clinical settings. For this purpose, we have used adrenal chromaffin cells, because chromaffin cells offer a suitable experimental model for the analysis of the action of anaesthetics on neurosecretion (Chern et al 1987). Indeed, Pocock & Richards (1988) examined the effects of volatile anaesthetics on the chromaffin cells and showed that volatile anaesthetics at clinical concentrations inhibited catecholamine release induced by carbachol, and indicated that the effect of volatile anaesthetics on the chromaffin cells would be closely related to that on the CNS.

The present results show that local anaesthetics inhibit carbachol-induced catecholamine release in a dose-dependent manner. The inhibitory action of lignocaine appears at 20 μM and caused 20% reduction of catecholamine release at 50 μM. In man, it has been reported that the CNS symptom of lignocaine appears at plasma levels above 21·4–42·7 μM (Klein et al 1968; Grossman et al 1969; Lie et al 1974; Viegas & Stoelting 1975). Etidocaine showed 22% inhibition of catecholamine release at 20 μM, and it was reported that etidocaine caused CNS symptoms in man at about 10 μM. Procaine was reported to cause convulsions in man at 150 μM (Usubiaga et al 1966; Wikinski et al 1970), which is the concentration showing 15% inhibition of catecholamine release. Scott et al (1989) in 12 volunteers, studied the relationship between plasma levels of bupivacaine and CNS symptoms and reported that the majority of the CNS symptoms occurred at plasma concentrations of 4–8 μM, which is the concentration causing 15% reduction of catecholamine release. The plasma concentrations of local anaesthetics during usual clinical usage, such as epidural anaesthesia and intercostal nerve block, lie in the range of 21–29·4 μM with lignocaine, 7·2–10·9 μM with etidocaine, and 3·6–5·4 μM with bupivacaine (Asada 1979). These data indicate that the concentrations of these anaesthetics to inhibit catecholamine release would not deviate from those encountered in clinical settings, and are similar to those which cause CNS depression in man or in animals.

Gissen et al (1980) investigated the differential sensitivities of mammalian nerve fibres to various local anaesthetics, and reported that the concentrations causing 50% inhibition of impulse conduction (ED50) in B fibres, which showed intermediate values between A and C fibres, were between 13 μM (tetracaine) and 375 μM (lignocaine). These concentrations were very similar to the IC50 values reported here. Thus in CNS, local anaesthetics could possibly block synaptic transmission as well as impulse conduction of nerve fibres.

Göthert et al (1979) reported that inhibition of receptor-mediated noradrenaline release from the sympathetic nerves by general anaesthetics and alcohols was proportional to their hydrophobic property. This tendency was seen in the inhibitory effect of volatile anaesthetics on carbachol-induced catecholamine release from bovine adrenal chromaffin cells (Pocock & Richards 1988). There was no correlation between the inhibitory potency of local anaesthetics and hydrophobicity, suggesting that local anaesthetics might affect the stimulus-secretion coupling in the chromaffin cells by a different mechanism from general anaesthetics. It is possible that local anaesthetics have a specific binding site on the voltage-gated $Na^+$ channel resulting in inhibition of $Na^+$ current (Butterworth & Strichartz 1990). Two kinds of $Na^+$ channels are believed to exist in the cultured chromaffin cells—$Na^+$ channels associated with the nicotinic receptor–ionophore complex and voltage-sensitive $Na^+$ channels (Livett 1984). These $Na^+$ channels are considered to play significant roles and could possibly be direct targets of the action of local anaesthetics.

Local anaesthetics may exist as the free base or as the cation. The degree of ionization has an important influence on drug distribution and action, as only the free base readily crosses cell membranes. The degree of ionization of a substance depends on the dissociation constant ($pK_a$) (Stri-

chartz et al 1990) and the pH of the medium in which it is present. It has been reported that decreasing pH of blood and brain will decrease the amount of local anaesthetic crossing the blood-brain barrier, but will increase the activity of the local anaesthetic that has already entered the brain. The important factor is therefore the ratio of the un-ionized to the ionized form in the brain (Englesson & Grevsten 1974). We examined the influence of medium pH on the IC50 of local anaesthetics. The results show that local anaesthetics exhibited a greater inhibitory potency at a higher medium pH, suggesting that the base form of local anaesthetics plays a major role in this inhibitory effect.

In conclusion, local anaesthetics at clinical concentrations exert inhibitory effects on the stimulus-secretion coupling in the chromaffin cells. The base form of local anaesthetics would play a major role in this inhibition, and there is no correlation between the inhibitory potency and the lipid solubility.

*Acknowledgements*

The authors wish to thank Ms T. Konishi, Ms M. Kobayashi and Ms T. Uemura for their assistance throughout this study.

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