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THE INTERACTION OF AMINE LOCAL ANAESTHETICS WITH MUSCARINIC RECEPTORS

W.J. TAYLOR, A. WOLF & J.M. YOUNG

Department of Pharmacology, University of Cambridge, Hills Road, Cambridge CB2 2QD

1 Amine local anaesthetics inhibited the binding of (−)-[^{3}H]-quinuclidinyl benzilate ((−)-[^{3}H]-QNB) to muscarinic receptors in crude synaptosomal preparations from guinea-pig brain. The order of potency was SKF 525A > tetracaine > procaine ≃ quinidine > procainamide > bupivacaine > lignocaine > prilocaine.

2 The concentration of tetracaine or prilocaine causing 50% inhibition of the receptor-specific binding of [^{3}H]-QNB varied linearly with the concentration of [^{3}H]-QNB present for the range of concentrations of prilocaine used and at lower concentrations of tetracaine, thus providing evidence for a competitive interaction. The affinity constant for tetracaine was $2.6 \pm 0.2 \times 10^5$ M^{-1} and that for prilocaine $2.6 \pm 0.8 \times 10^3$ M^{-1}. At higher concentrations of tetracaine the interaction appears to diverge from simple competitive kinetics.

3 The log dose-response curve for the contractile response of longitudinal muscle strips from guinea-pig intestine to carbachol was shifted in a parallel fashion by low concentrations of tetracaine, but flattened by higher doses. A similar effect was observed for both lignocaine and prilocaine. The affinity constants for tetracaine and prilocaine calculated from the parallel shifts, 1×10^5 M^{-1} and 4×10^3M^{-1}, respectively, were in reasonable accord with the binding data.

4 The curve for the inhibition of [^{3}H]-QNB binding by carbachol was not significantly altered, either in position or shape, in the presence of 1 mM prilocaine. Thus there is no evidence that prilocaine, which increases the affinity of nicotinic acetylcholine receptors for agonists, has any similar effect on agonist binding to muscarinic receptors.

Introduction

There have been a number of studies of the effects of local anaesthetics on responses mediated by muscarinic acetylcholine receptors in a variety of tissues. While some of these have described only non-competitive effects (Feinstein & Paimre, 1967; Bury & Mashford, 1976) and have drawn the conclusion that the primary site of action is on some membrane constituent other than the receptor, others have observed competitive effects at low doses (Fleisch & Titus, 1973; Richelson, Prendergast & Divinetz-Romero, 1978; Weinstock & Weiss, 1979). However, a recent study has shown that the binding of [^{3}H]-scopolamine to neuroblastoma cells is inhibited in a non-competitive manner by tetracaine (Burgermeister, Klein, Nirenberg & Witkop, 1978).

The nature of the interaction between local anaesthetics and the muscarinic receptor is of some interest, since in the case of the nicotinic acetylcholine receptor, which can exist in more than one conformational state, certain local anaesthetics, such as prilocaine, stabilize a conformation which has a high affinity for agonists (Cohen, Weber & Changeux, 1974) and which may represent the desensitized receptor (Heidmann & Changeux, 1978).

In an attempt to clarify the nature of the interaction of tetracaine and prilocaine with the muscarinic receptor, we have examined their effect and that of certain other amine anaesthetics on the binding of [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) (Yamamura & Snyder, 1974) to muscarinic receptors in a membrane-fraction from guinea-pig brain. The results of this study, which indicate a competitive action of both tetracaine and prilocaine at low doses, are described here.

Methods

Measurement of binding of [^{3}H]-QNB

A crude synaptosomal fraction was prepared from guinea-pig cerebral cortex essentially following the method of Hebb & Whittaker (1958). The cortex was homogenized in 10 volumes of 320 mM sucrose con-

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taining 5 mM Na-K phosphate buffer, pH 7.6, and then centrifuged at 1,000 *g* for 5 min. The pellet was discarded and the supernatant centrifuged at 17,000 *g* for 30 min. The pellet, the P2 fraction of Hebb & Whittaker (1958), was resuspended in 320 mM sucrose: 5 mM Na-K phosphate buffer and stored at −10°C.

For measurement of the inhibition of the binding of [^{3}H]-QNB each incubation in 4.1 ml Krebs-bicarbonate or Krebs-phosphate solution contained 0.96 nM (±)-[^{3}H]-QNB (i.e. 0.48 nM of the active (−)-isomer), 0.15 to 0.20 mg protein and an appropriate concentration of inhibitor. Incubation at 30°C was for 1 h. At the end of this period a 1 ml sample was filtered rapidly under vacuum (approx. 200 mmHg) through a Whatman GF/B glass-fibre filter and the filter then washed 3 times with 5 ml of ice-cold Krebs-bicarbonate or Krebs-phosphate solution. Triplicate measurements were made on duplicate incubations at each inhibitor concentration. The filters were transferred to a scintillation vial, 10 ml of scintillator (0.6% butyl PBD in ethoxyethanol-toluene, 1:2, v/v) added, the vial shaken vigorously and then allowed to stand overnight before counting in a liquid scintillation spectrometer.

For the series of experiments with tetracaine and prilocaine where the IC_{50} (concentration of inhibitor giving 50% inhibition of the methylatropinium-sensitive [^{3}H]-QNB binding) was measured at different concentrations of [^{3}H]-QNB), the volume of the incubation medium was increased proportionately as the final concentration of [^{3}H]-QNB decreased, while maintaining the amount of protein added constant, in order to minimize problems of depletion of the free[^{3}H]-QNB at low concentrations. The size of the sample filtered was increased in proportion to the increase in the total incubation volume.

In all experiments on the local anaesthetics the level of non-specific (non-receptor) binding was determined from three incubations containing 1 μM methylatropinium bromide. In most cases the binding of [^{3}H]-QNB insensitive to inhibition by the local anaesthetic was determined independently from the inhibition curve (see below).

The Krebs-bicarbonate (Krebs-Henseleit) solution contained (mM): NaCl 116, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and D-glucose 5.5. The Krebs-phosphate solution contained (mM): NaCl 116, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, Na_2HPO_4 5. The Na_2HPO_4 stock solution (100 mM) was adjusted to pH 7.4 with HCl prior to addition.

Analysis of inhibition curves

Curves of the percentage of the uninhibited binding of [^{3}H]-QNB versus concentration of inhibitor, were analyzed by assuming that the binding of the inhibitor followed a Hill equation, fractional occupancy of antagonist, $AR = A^n.K/(A^n.K + 1)$, where A is the concentration of inhibitor, n is the Hill coefficient and K is the equilibrium association constant (affinity constant). The actual equation fitted was:

$$\%\text{ of uninhibited binding of [}^3\text{H]-QNB} = \frac{100 - NS}{A^n.K + 1} + NS$$

where NS is the level of binding insensitive to the inhibitor and K_{app} is apparent affinity constant, since competition with the [^{3}H]-ligand shifts the inhibition curve to higher concentrations of inhibitor by a factor $D.K_{QNB} + 1$, where D is the concentration of (−)-[^{3}H]-QNB and K_{QNB} its affinity constant, from the position it would occupy if it represented binding of the inhibitor alone. The best-fit values of n, K_{app} and NS and their estimated standard errors were obtained by a Marquardt non-linear regression technique, as implemented in the Harwell library programme VB01A on the Cambridge IBM 370/165. All points were weighted according to the reciprocal of the variance associated with them.

The affinity constant of the inhibitor, K_a was determined from the IC_{50} using the relationship $K_a = (D.K_{QNB} + 1)/IC_{50}$. K_{QNB} was taken to be 1.3×10^{10} M^{-1}, the mean of the two determinations made.

Variation of IC_{50} with [^{3}H-QNB]

Rearrangement of the relationship above leads to the expression:

$$IC_{50} = \frac{D.K_{QNB}}{K_a} + \frac{1}{K_a}$$

where D is the concentration (−)-[^{3}H]-QNB. Thus for a competitive interaction, which the derivation of the relationship assumes, if IC_{50} is plotted against D a straight line should result from which K_a is obtained as the reciprocal of the intercept on the ordinate and K_{QNB} from slope/intercept.

Organ-bath experiments

Longitudinal muscle strips from guinea-pig small intestine, prepared by the method of Rang (1964), were suspended in 10 ml Krebs-bicarbonate (Krebs-Hanseleit) solution at 30°C in a conventional organ-bath. Contractions to carbachol were recorded isotonically. Doses of the agonist were added every 3 min and allowed to act for 20 to 30 s. Where appropriate, tetracaine, prilocaine or lignocaine was present in the Krebs solution. The affinity constant of the antagonist was determined from the parallel shift of the log dose-

response curve, using the relationship: Dose-ratio = $A.K_a + 1$, where the dose-ratio is the ratio of the dose of carbachol required for a given response in the presence of antagonist to the dose needed for the same response in the absence of antagonist.

pK_a values

Values of pK_a for the amines were obtained from potentiometric titration of the amine salt (1.4 to 8.6 mM) in 110 mM NaCl at 22 to 24°C with 50 mM NaOH using a Radiometer automatic titrator. The pK_a was taken to be the pH at half-equivalence. The free-base of SKF-525A was insufficiently soluble, even at 0.4 mM, for accurate determination by this method. Bupivacaine was not measured.

Drugs

[^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB), specific activity 13 or 16 Ci/mmol, was obtained from the Radiochemical Centre, Amersham, and its radiochemical purity checked by high-voltage electrophoresis (50 V/cm) on Whatman No. 1 paper in 60 mM citric acid-phosphate buffer, pH 3.0. As supplied [^{3}H]-QNB is a racemate, only one isomer of which has appreciable antimuscarinic activity (Kloog & Sokolovsky, 1977). Concentrations of [^{3}H]-QNB given throughout are those calculated for the active (−)-isomer.

Drugs were obtained from the sources indicated: lignocaine hydrochloride (Pharmaceutical Manufacturing Co.); tetracaine hydrochloride, methylatropinium bromide and procaine hydrochloride (Sigma) and quinidine sulphate (B.D.H.). Bupivacaine hydrochloride was kindly donated by Duncan-Flockhart, SKF-525A (Proadifen, 2-diethylaminoethyl diphenylpropylacetate hydrochloride) by Smith, Kline & French and prilocaine hydrochloride and procainamide hydrochloride by Astra Pharmaceuticals.

Results

Inhibition of [^{3}H]-QNB binding by amine anaesthetics

Curves for the inhibition of the binding of 0.48 nM (−)-[^{3}H]-QNB by tetracaine, procainamide and prilocaine are shown in Figure 1. Similar curves were obtained for the other amine anaesthetics examined and the values of the Hill coefficient, obtained by weighted non-linear regression and the affinity constants calculated from the concentration required for 50% inhibition are set out in Table 1, with the values for methylatropinium for comparison. For tetracaine, procainamide, lignocaine and SKF-525A the Hill coefficients are close to unity, the value expected for a simple mass-action equilibrium between inhibitor and receptor. In contrast for procaine, quinidine and, most notably, prilocaine the values appear to be sig-

Table 1 Hill coefficients, affinity constants and levels of inhibitor-insensitive binding for the inhibition of (−)-[^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding by local anaesthetics

	pKa	*Hill coefficient*	*n*	*Affinity constant* (M^{-1})	*Binding (%) insensitive to* *Inhibitor*	*Me atr*‡
Methylatropinium	—	1.04 ± 0.14	(24)	1.5×10^9	18 ± 2	17 ± 5
Methylatropinium*	—	0.91 ± 0.07	(16)	1.5×10^9	13 ± 1	14 ± 1
SKF 525A*	§	0.98 ± 0.12	(7)	2.3×10^6	8 ± 4	20 ± 1
Tetracaine	8.5	1.01 ± 0.19	(13)	1.1×10^5	8 ± 4	22 ± 2
Procaine	9.0	1.18 ± 0.05	(21)	(8.8×10^4)	12 ± 1	17 ± 1
Quinidine	8.7	1.25 ± 0.09	(20)	(6.7×10^4)	7 ± 2	14 ± 1
Procainamide	9.2	1.03 ± 0.04	(21)	1.2×10^4	6 ± 1	17 ± 3
Bupivacaine*	8.2¶		(4)	9.7×10^3	†	23 ± 2
Lignocaine	7.9	1.00 ± 0.15	(14)	3.7×10^3	11 ± 3	19 ± 2
Prilocaine	8.0	1.45 ± 0.09	(19)	(1.6×10^3)	9 ± 2	19 ± 2

Hill coefficients and the % of the binding of 0.48 nM (−)-[^{3}H]-QNB insensitive to inhibitor were obtained by weighted non-linear regression analysis as described under Methods. Affinity constants were calculated from the concentration of inhibitor giving 50% inhibition of the inhibitor-sensitive binding, using the formula given under Methods. The values in parentheses are derived from inhibition curves where the Hill coefficient is significantly > 1. *n* is the number of points on each curve.

*Measured in Krebs-phosphate. All other measurements were made in Krebs-bicarbonate. ‡1 μM methylatropinium bromide. §Not determined (see Methods & Discussion). ¶Value cited by Bury & Mashford (1976). †Limited solubility prevented measurement of the whole of the inhibition curve.

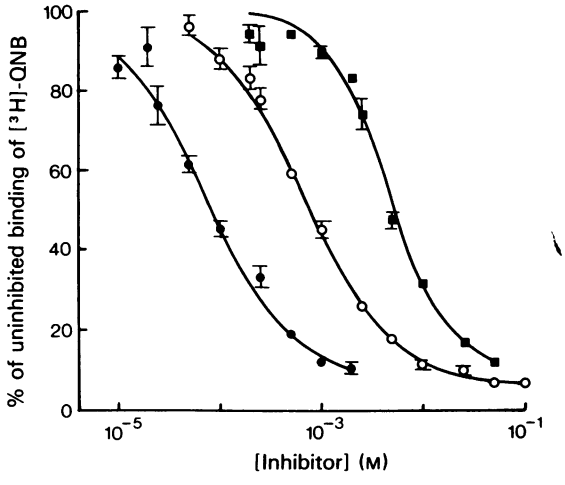

Figure 1 Inhibition of [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding by tetracaine, procainamide and prilocaine. Measurements of the inhibition of the binding of 0.48 nM (−)-[^{3}H]-QNB were made in Krebs-bicarbonate solution. For the sake of clarity where more than one measurement was made at a given concentration the weighted mean ± s.e. has been plotted. The curves drawn are the best-fit lines obtained from weighted non-linear regression (see Methods). (●) Tetracaine; (○) procainamide; (■) prilocaine.

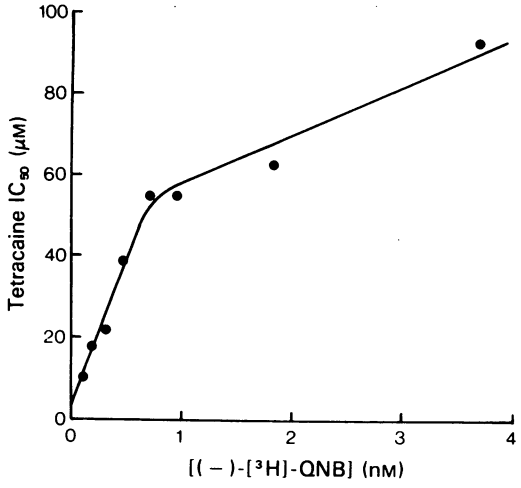

Figure 2 The variation of IC_{50} for tetracaine with [(−)-[^{3}H]-QNB]. Measurements in Krebs-phosphate solution were made as described under Methods. IC_{50} is the concentration of tetracaine which gives 50% inhibition of the binding of [^{3}H]-QNB sensitive to inhibition by 1 μM methylatropinium.

nificantly greater than unity. However, for all the amines the best-fit value for the percentage of inhibitor-insensitive binding was less (Table 1) than the level of binding in the presence of 1 μM methylatropinium, a concentration sufficient to reduce receptor-specific binding to negligible levels. In part the inhibition of the non-receptor binding may reflect the high concentrations of the amines necessary for maximum inhibition, but even with SKF-525A, the most potent of the series examined, the discrepancy was apparent at 10^{-4}M.

In the first series of experiments Krebs-bicarbonate solution was generally used, in order to have a salt solution as close to physiological as possible, but in subsequent experiments measurements were made in Krebs-phosphate, where there is a better control of pH. There was no significant difference in the binding of methylatropinium measured in Krebs-bicarbonate or Krebs-phosphate (Table 1).

Variation of IC_{50} for amine anaesthetics with [[^{3}H]-QNB]

Hill coefficients near unity do not necessarily mean that the inhibition of [^{3}H]-QNB binding is competitive. To test for competition a series of experiments was carried out with two of the amines, in which the variation of the concentration of inhibitor for 50% inhibition of the receptor-specific binding of [^{3}H]-QNB was measured as a function of the concentration of [^{3}H]-QNB. For a competitive interaction a plot of IC_{50} against [^{3}H]-QNB should give a straight line from which K_a is obtained as the reciprocal of the intercept on the ordinate and K_{QNB}, the affinity of [^{3}H]-QNB, from slope/intercept (see Methods).

The two amines which were examined in this way were tetracaine, since this compound had been found to inhibit [^{3}H]-scopolamine binding non-competitively in neuroblastoma cells (Burgermeister *et al.*, 1978), and prilocaine, which showed the biggest deviation from a Hill coefficient of unity (Table 1) and which was one of the amines shown to have allosteric effects on the nicotinic receptor (Cohen *et al.*, 1974). The need to obtain only the IC_{50} value has the particular advantage in the case of prilocaine of avoiding the very high concentrations of the drug which were necessary to define the foot of the inhibition curve (Figure 1). However, the difficulty which was encountered with both tetracaine and prilocaine in applying this IC_{50} method was that when the concentration of (±)-[^{3}H]-QNB was increased much beyond about 1 nM (i.e. beyond c. 0.5 nM (−)-[^{3}H]-QNB) the increasing proportion of binding insensitive to 1 μM methylatropinium, but still largely sensitive to inhibition by the amine, increased the uncertainty in the determination of the IC_{50}.

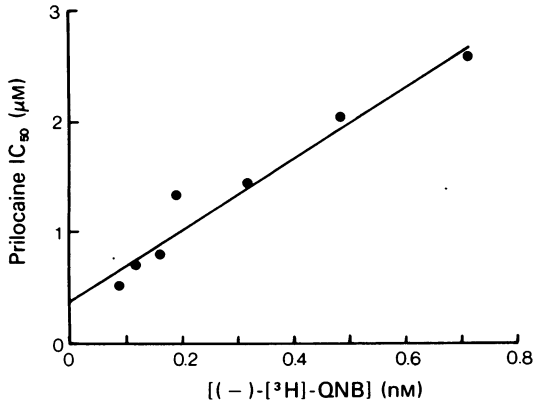

Figure 3 The variation of IC_{50} for prilocaine with [(−)-[^{3}H]-QNB]. Measurements in Krebs-phosphate solution were made as described under Methods. IC_{50} is the concentration of prilocaine which gives 50% inhibition of the binding of [^{3}H]-QNB sensitive to inhibition by 1 μM methylatropinium. The line drawn was fitted by linear regression analysis.

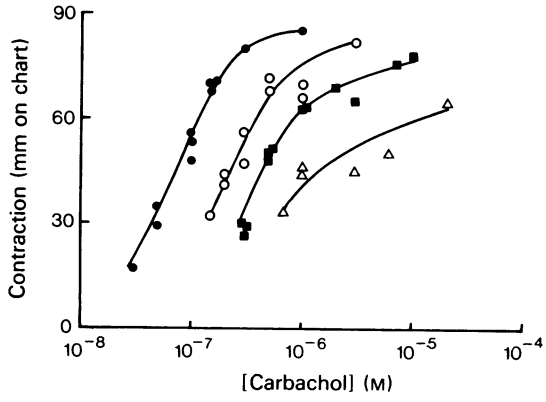

Figure 4 Inhibition by tetracaine of the contractile response of the longitudinal muscle from guinea-pig small intestine to carbachol. Tetracaine concentration: (●) nil; (○) 2×10^{-5} M; (■) 4×10^{-5} M; (△) 8×10^{-5} M.

The variation of the IC_{50} for tetracaine, defined as the concentration of tetracaine required for 50% inhibition of the methylatropinium-sensitive binding, as a function of the concentration of [^{3}H]QNB is shown in Figure 2. There is a linear relationship up to circa 0.8 nM (−)-[^{3}H]-QNB, but thereafter the curve flattens suggesting a transition from competitive to more complex kinetics. For prilocaine there was no indication of anything other than a linear relationship in the concentration range studied (Figure 3), implying a competitive interaction.

The affinity constants for the competitive interaction, obtained from linear regression analysis of the data at concentrations of [^{3}H]-QNB below 0.8 nM, were $2.6 \pm 0.2 \times 10^5$ M^{-1} for tetracaine and $2.6 \pm 0.8 \times 10^3$ M^{-1} for prilocaine. The corresponding values of the affinity constant for [^{3}H]-QNB were $1.8 \pm 1.2 \times 10^{10}$ M^{-1} and $0.8 \pm 0.3 \times 10^{10}$ M^{-1}, the much larger uncertainty arising from the fact that K_{QNB} comes from the ratio slope/intercept, both of which have an error associated with them.

Inhibition by amine anaethetics of the contractile response of longitudinal muscle strips

To seek further evidence for a competitive interaction at lower concentration of the amines, measurements were made of the inhibition of the contractile response to carbachol of longitudinal muscle strips from guinea-pig small intestine. In addition to tetracaine and prilocaine, lignocaine was also included in this study since the value determined for the affinity constant (Table 1) differs markedly from those of Richelson *et al.* (1978) and Fields, Roeske, Morkin & Yamamura (1978).

The dose-response curves obtained in the presence of increasing concentrations of tetracaine are shown in Figure 4. The initial shift with 2×10^{-5}M tetracaine appears to be parallel, but increasing the concentration of tetracaine to 4×10^{-5}M and then 8×10^{-5}M causes an increasing flattening of the curves. All the shifts were readily reversible and the control curve reobtained on washing. The affinity constant for teracaine calculated from the first parallel shift was 1×10^5 M^{-1}, in good agreement with the values obtained from the other two methods.

With both lignocaine and prilocaine (Figure 5 a and b) the lowest dose applied, 2×10^{-4} M, produced a parallel shift of the log dose-response curve, but increasing the concentration to 5×10^{-4} M caused a flattening of the curves with both amines, although with prilocaine it seemed that the maximum response was still obtained. The higher concentration, 5×10^{-4}M, of both lignocaine and prilocaine induced small spontaneous contractions of the muscle strip. This effect was reversed rapidly on washing. The affinity constants calculated from the first parallel shift were 3×10^3 M^{-1} for lignocaine and 4×10^3 M^{-1} for prilocaine. The value for prilocaine is in reasonable agreement with that, 2.6×10^3 M^{-1}, determined from the variation of IC_{50} with [^{3}H-QNB] (Figure 3).

Effect of prilocaine on the inhibition of [^{3}H]-QNB binding by carbachol

The interaction between prilocaine and the muscarinic antagonist [^{3}H]-QNB appears to be competitive, but the possibility remains open that the binding of

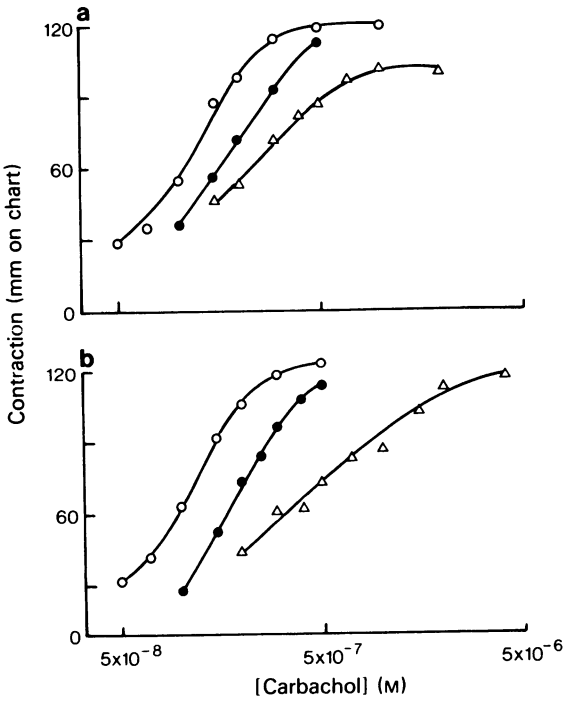

Figure 5 Inhibition by lignocaine and prilocaine of the contractile response of the longitudinal muscle from guinea-pig small intestine to carbachol. (a) Lignocaine, (b), prilocaine. Concentration of local anaesthetic present: (○) nil; (●) 2×10^{-4} M; (△) 5×10^{-4} M.

muscarinic agonists, which in general appear to distinguish at least two independent binding sites (Birdsall & Hulme, 1976; Birdsall, Burgen & Hulme, 1978), might be affected in a more complex manner by prilocaine, in analogy with its effects on the nicotinic acetylcholine receptor (Cohen *et al.*, 1974; Heidmann & Changeux, 1978). To test this, the inhibition of the binding of 0.49nM (−)-[^{3}H]-QNB by carbachol was measured in Krebs-phosphate solution in the presence and absence of 1 mM prilocaine. This concentration of prilocaine on its own produced a 24% inhibition of [^{3}H]-QNB binding, but had no significant effect on the shape or position of the inhibition curve for carbachol.

The mean Hill coefficient for carbachol binding was 0.41 ± 0.04. Analysis of the combined data as binding to two independent sites using the weighted non-linear regression technique (see Methods) yielded affinity constants of 1.4 ± 0.7 × 10^6 and 8.2 ± 1.5 × 10^3 M^{-1}, in reasonable agreement with previous measurements on rat brain (Birdsall & Hulme, 1976; Birdsall *et al.*, 1978) and guinea-pig ileum (Ward & Young, 1977). The percentages of the high and low affinity sites were 25 ± 3 and 67 ± 3, respectively.

Discussion

On the evidence of the linear relationship between the concentration of tetracaine and prilocaine required to reduce the receptor-specific binding of [^{3}H]-QNB by 50% and the concentration of [^{3}H]-QNB present, it seems very probable that the interaction between these amines and the muscarinic receptor at lower concentrations of the amines is competitive. This conclusion is in accord with that of Richelson *et al.* (1978) who investigated the effect of tetracaine and other local anaesthetics on muscarinic receptor-mediated cyclic guanosine 3', 5'-monophosphate (cyclic GMP) formation by N1E-115 neuroblastoma cells. In contrast, Burgermeister *et al.* (1978), who used the same neuroblastoma clone, found that the inhibition by tetracaine of [^{3}H]-scopolamine binding by these cells was non-competitive. Their method of analysis was by double reciprocal plots of the binding of the [^{3}H]-ligand in the presence of 0, 10^{-5} and 5 × 10^{-5}M tetracaine, but examination of their data for 10^{-5}M tetracaine suggests that the divergence from the pattern expected for a competitive interaction is probably not statistically significant. Consequently at low concentrations of the amine there may be no discrepancy. At the higher concentration of tetracaine the interaction with [^{3}H]-scopolamine was more clearly not competitive , but this concentration, 5 × 10^{-5}M, is only slightly less than that at which we observed a divergence from competitive kinetics, so that there is quite probably no real difference in the conclusions to be drawn from the two binding studies. The difficulty in making a more detailed investigation of the kinetics at higher concentrations is that tetracaine, in common with the other amines, causes a significant inhibition of the methylatropinium-insensitive, and therefore presumably non-receptor, binding of [^{3}H]-QNB. Burgermeister *et al.* (1978) noted a similar effect on the binding of [^{3}H]-scopolamine. The higher the concentration of [^{3}H]-QNB present, the greater the proportion of non-receptor binding and consequently the greater the uncertainty in the determination of the IC_{50} for the receptor-specific component.

The effect of tetracaine on the contractile response of longitudinal muscle strips from intestine is consistent with an initial competitive interaction. The effect on the log dose-response curve observed, a parallel shift at low dose but flattening at higher concentrations, is very similar to that reported by Fleisch & Titus (1973) for inhibition of the contractile response to carbachol of rat spiral tracheal smooth muscle

strips. Further, the affinity constant calculated from the initial parallel shift in our experiments, 1×10^5 M^{-1}, is closely similar to that, 1.3×10^5 M^{-1}, determined on tracheal muscle. The parallel shift of the log dose-response curve alone is not conclusive evidence for a competitive interaction, since in the intact guinea-pig ileum and isolated longitudinal muscle carbachol needs to occupy only a small fraction of the receptors to produce a response and consequently an antagonist which is effectively irreversible on the time scale on which the response is obtained, circa 15 s, will still produce a parallel shift. The affinity constant calculated on the assumption of equilibrium kinetics is still correct (Rang, 1966), although the interaction need not be at the agonist site. However, the similarity of the value for tetracaine obtained from the shift of the log dose-response curve, 1×10^5 M^{-1}, with that obtained from the [^{3}H]-QNB inhibition study and with that, 1.6×10^5 M^{-1}, from the inhibition of the cyclic GMP response in neuroblastoma cells (Richelson *et al.*, 1978) gives some confidence that the interaction is indeed competitive. This conclusion is strengthened by the observation of Fleisch & Titus (1973), using the test of Paton & Rang (1965), that the combined dose-ratio with both atropine and tetracaine present was consistent with the values determined for the two antagonists acting alone.

The depression of the log dose-response curve with 8×10^{-5}M and possibly to some extent with 4×10^{-5}M could well represent antagonism of the function of depolarization-sensitive calcium channels on the smooth muscle membrane, as observed by Feinstein & Paimre (1967), who demonstrated that the action of tetracaine at this site was competitive with an affinity constant of 3.8×10^4 M^{-1}. These authors, observed only flattening of the log dose-response curve for carbachol by tetracaine even at low concentrations in the guinea-pig ileum, even though in guinea-pig taenia coli a parallel shift was observed. It is possible that the difference between this observation and our own could be explained by the fact that whereas Feinstein & Paimre (1967) used whole ileal segments, where electrical coupling between the longitudinal and circular muscle layer is intact (Connor, Kreulen & Prosser, 1977), we used longitudinal muscle strips that are essentially free of circular muscle. This might similarly account for the discrepancy between our observation of an initial parallel shift with lignocaine and the report of Bury & Mashford (1976) that 1.7×10^{-4}M lignocaine depressed the maximum response of segments of guinea-pig ileum to acetylcholine.

The other amine, in addition to tetracaine, which we examined in detail, prilocaine, also seems to act competitively at lower concentrations as indicated by the linear plot of IC_{50} versus [^{3}H-QNB]. For this amine the very high concentrations necessary for inhibition of higher concentrations of [^{3}H]-QNB, coupled with the problem of inhibition of non-receptor binding discussed above, have discouraged us from making any attempt to see whether non-competitive effects eventually appear. The initial parallel shift of the log dose-response for carbachol on longitudinal muscle strips is again consistent with a competitive antagonism and the values for the affinity constant obtained by the two methods, $2.6 \pm 0.8 \times 10^3$ and 4×10^3 M^{-1}, are in satisfactory agreement.

The Hill coefficient for the inhibition of [^{3}H]-QNB binding by prilocaine measured in Krebs-bicarbonate solution was significantly greater than unity, but some caution is necessary in interpreting measurements made in this medium, particularly where very high concentrations of inhibitor are present. Krebs-bicarbonate solution has the advantage of having an ionic composition similar to that of extracellular fluid in man, but has the disadvantage that if it is not gassed continuously with 95% CO_2 and 5% O_2, as is the case in the capped vials in our incubations, it is difficult to ensure that the pH has not drifted to more alkaline values during the course of an incubation. Since several of the tertiary amines have pK_a values not greatly different from pH 7.4 (Table 1) it is possible that the proportions of protonated and uncharged amine present vary. However, unless there were uneven variation through the experiment this should not affect the shape of the curve but would decrease the potency, on the assumption that the protonated form binds with higher affinity than the uncharged amine. The data for both prilocaine and tetracaine appear to support this supposition, since the IC_{50} values against 0.49 nM (−)-[^{3}H]-QNB in Krebs-bicarbonate (Figure 1) are greater than in Krebs-phosphate, where there is effective pH control (Figures 2 and 3). It is possible that prilocaine does show apparent positive cooperativity with the very high concentrations required to obtain high levels of inhibition in Krebs-bicarbonate but it would require a more extensive investigation to be sure that this is not simply a pH effect. In the case of procaine, which is a substrate for cholinesterase, the Hill coefficient >1 might be accounted for by the absence of an anti-cholinesterase in the incubation medium.

One point which may be noted is that none of the amines give Hill coefficients significantly <1 and thus show no evidence of any selectivity between the two states of the muscarinic receptor which are apparently distinguished by the binding of muscarinic agonists (Birdsall & Hulme, 1976; Ward & Young, 1977; Birdsall *et al.*, 1978).

The pK_a values for the amines given in Table 1 are in good agreement with other determinations recorded in the literature or quoted by manufacturers (see e.g. Bury & Mashford, 1976; Hille, 1977). How-

ever, the low solubility of the free base of SKF-525A prevented us from making any accurate determination with this compound. Suarez-Kurz & Bianchi (1970), from a titration curve with 40 mM SKF-525A, reported a value of 5.7 for the pK_a which seems much too low for this type of structure. In our measurements, even at 0.4 mM SKF-525A the titration curve was clearly not parallel to that of ligocaine or the other amines and consequently the estimate of pK_a from this determination, 7.6, is probably still low. More concentrated solutions of SKF-525A, where insolubility on partial neutralization was very apparent, gave lower estimates and it seems very likely that this is the reason for the low value of Suarez-Kurz & Bianchi (1970).

The number of determinations of affinity constants for the amines available in the literature for comparison with our own values is limited, coming as they do from those studies in which a competitive action has been proposed (Fleisch & Titus, 1973; Richelson *et al.*, 1978; Weinstock & Weiss, 1979). The generally good agreement for tetracaine is noted above. Procaine was observed by Tjioe & Bianchi (1969) to have a potent anti-muscarinic action on the frog isolated ventricle and the apparent affinity in Table 1, 8.8×10^4 M^{-1}, is in reasonable agreement with the value of Richelson *et al.* (1978), 1.6×10^5 M^{-1}, although rather higher than the value of circa 7×10^3 M^{-1} found by Weinstock & Weiss (1979) as an antagonist of the contractile response of rat fundic strips to acetylcholine. The affinity for procainamide, 1.2×10^4 M^{-1}, compares with the value of 2.1×10^4 M^{-1} reported by Fields *et al.* (1978) from the inhibition of the binding of [^{3}H]-QNB to rabbit heart, but their values for quinidine, 4.9×10^5 M^{-1}, and lignocaine, 5.5×10^4 M^{-1}, were both much higher than our own. This discrepancy may in part be due to the different medium they employed, 50 mM Na-K phosphate buffer, pH 7.4, which contains no divalent cations, in particular no Ca^{2+}. Differences in the characteristics of muscarinic receptor binding in phosphate buffer and Krebs have been observed by Hedlund & Bartfai (1979), who emphasised the importance of using solutions approximating to physiological. In addition, the higher affinity constant for [^{3}H]-QNB, 3.7×10^{10} M^{-1} determined by Fields *et al.* (1978), nearly 3 fold greater than our mean value, will serve to give higher affinities for inhibitors, since the correction to the IC_{50} to take account of competition with the [^{3}H]-ligand will be larger. This assumes, of course, that the interaction of these amines is simply competitive, which neither study tested. However, even bearing these factors in mind and the problem of pH control in Krebs, it does seem possible that for lignocaine there is some discrepancy, notwithstanding the consistency of our binding and organ-bath determinations, since the value of Fields *et al.* (1978) for lignocaine is similar to that (5×10^4 M^{-1}) of Richelson *et al.* (1978), who measured the antagonism of the muscarinic agonist stimulated formation of cyclic GMP.

The very high affinity of (−)-QNB for the muscarinic receptor, with the consequent ease with which depletion of the free drug concentration can occur, is probably responsible for the wide spread of values for the affinity constant which have appeared in the literature. The values from the IC_{50} studies, 8.3×10^9 M^{-1} (Figure 3) and 1.8×10^{10} M^{-1} (Figure 2) are in the range of recently reported values (all M^{-1}): 5.6×10^9 in rat brain (Kloog & Sokolovsky, 1977), 7.1×10^9 in rat brain (Hulme, Birdsall, Burgen & Mehta, 1978), 1.0×10^{10} and 1.7×10^{10} in clones of neuroblastoma cells (Burgermeister *et al.*, 1978), 2.3×10^{10} in rat striatum (Kruk & Smith, 1978), 2.5×10^{10} in rat striatum and 4×10^{10} in rat hippocampus (Gilbert, Hanley & Iversen, 1979), 2.5×10^{10} in mouse neuroblastoma cells (Strange, Burgen & Birdsall, 1978), 3.7×10^{10} in rat brain and 5.0×10^{10} in rat striatum (Fields *et al.*, 1978).

One of our aims in commencing this work was to look for possible effects on muscarinic acetylcholine receptors analogous to those observed on the nicotinic acetylcholine receptor with histrionicotoxin, prilocaine and certain other local anaesthetics (Cohen *et al.*, 1974; Heidmann & Changeaux, 1978; Krodel, Beckman & Cohen, 1979). Burgermeister *et al.* (1978) looked for such an effect of dihydrohistrionicotoxin on acetylcholine inhibition of [^{3}H]-scopolamine binding, but failed to find any evidence of an enhancement of agonist affinity. We have similarly investigated the effect of prilocaine, which seemed the most likely candidate as an allosteric effector, on the binding of carbachol, as mirrored by the inhibition of the binding of [^{3}H]-QNB. The lack of any significant effect of 1 mM prilocaine on the position or shape of the carbachol inhibition curve is in accord with the observations of Burgermeister *et al.* (1978) and suggests that prilocaine, and by extension probably other amine anaesthetics, cannot readily induce an interconversion between low- and high-affinity forms of the muscarinic receptor.

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References

BIRDSALL, N.J.M., BURGEN, A.S.V. & HULME, E.C. (1978). The binding of agonists to brain muscarinic receptors. *Mol. Pharmac.*, **14**, 723–736.

BIRDSALL, N.J.M. & HULME, E.C. (1976). Biochemical studies on muscarinic acetylcholine receptors. *J. Neurochem.*, **27**, 7–16.

BURGERMEISTER, W., KLEIN, W.L., NIRENBERG. M. & WITKOP, B. (1978). Comparative binding studies with cholinergic ligands and histrionicotoxin at muscarinic receptors of neural cell lines. *Molec. Pharmac.*, **14**, 751–767.

BURY, R.W. & MASHFORD, M.L. (1976). Interactions between local anaesthetics and spasmogens on the guinea-pig ileum. *J. Pharmac. exp. Ther.*, **197**, 633–640.

COHEN, J.B., WEBER, M. & CHANGEUX, J.-P. (1974). Effects of local anaesthetics and calcium on the interaction of cholinergic ligands with the nicotinic receptor protein from Torpedo marmorata. *Molec. Pharamc.*, **10**, 904–932.

CONNOR, J.A., KREULEN, D., PROSSER, C.L. & WEIGEL, R. (1977). Interaction between longitudinal and circular muscle in intestine of cat. *J. Physiol.* **273**, 665–689.

FEINSTEIN, M.B. & PAIMRE, M. (1967). Mode of anticholinergic action of local anaesthetics. *Nature*, **214**, 151–153.

FIELDS, J.Z., ROESKE, W.R., MORKIN, E. & YAMAMURA, H.I. (1978). Cardiac muscarinic receptors. *J. biol. Chem.*, **253**, 3251–3258.

FLEISCH, J.H. & TITUS, E. (1973). Effect of local anaesthetics on pharmacologic receptor systems in smooth muscle. *J. Pharmac. exp. Ther.*, **186**, 44–51.

GILBERT, R.F.T., HANLEY, M.R. & IVERSEN, L.L. (1979). [^{3}H]-quinuclidinyl benzilate binding to muscarinic receptors in rat brain: comparison of results from intact brain slices and homogenates. Br. J. Pharmac. **65**, 451–456.

HEBB, C.O. & WHITTAKER, V.P. (1958). Intracellular distribution of acetylcholine and choline acetylase. *J. Physiol.* **142**, 187–196.

HEDLUND, B. & BARTFAI, T. (1979). The importance of thiol- and disulfide groups in agonist and antagonist binding to the muscarinic receptor. *Molec. Pharmac.*, **15**, 531–544.

HEIDMANN, T. & CHANGEUX, J.-P. (1978). Structural and functional properties of the acetylcholine receptor protein in its purified and membrane bound states. *A. Rev. Biochem.*, **47**, 317–357.

HILLE, B. (1977). The pH-dependent rate of action of local anaesthetics on the node of Ranvier. *J. gen. Physiol.*, **69**, 457–496.

HULME, E.C. BIRDSALL, N.J.M., BURGEN, A.S.V. & MEHTA, P. (1978). The binding of antagonists to brain muscarinic receptors. *Molec. Pharmac.*, **14**, 737–750.

KLOOG, Y. & SOKOLOVSKY, M. (1977) Muscarinic acetylcholine receptor interactions: competition binding studies with agonists and antagonists. *Brain Res.*, **134**, 167–172.

KRODEL, E.K. BECKMAN, R.A. & COHEN, J.B. (1979). Identification of a local anaesthetic binding site in nicotinic post-synaptic membranes isolated from *Torpedo marmorata* electric tissue. *Molec. Pharmac.*, **15**, 294–312.

KRUK, Z.L. & SMITH, L.A. (1978). A simple and cheap vacuum filtration apparatus. *Br. J. Pharmac.*, **62**, 469–470P.

PATON, W.D.M. & RANG, H.P. (1965). The uptake of atropine and related drugs by intestinal smooth muscle of the guinea-pig in relation to acetylcholine receptors. *Proc. R. Soc. B*, **165**, 1–44.

RANG, H.P. (1964). Stimulant actions of volatile anaesthetics on smooth muscle. *Br. J. Pharmac. Chemother.*, **22**, 356–365.

RANG, H.P. (1966). The kinetics of action of acetylcholine antagonists in smooth muscle. *Proc. R. Soc. B*, **164**, 488–510.

RICHELSON, E., PRENDERGAST, F.G. & DIVINETZ-ROMERO, S. (1978). Muscarinic receptor-mediated cyclic GMP formation by cultured nerve cells.—Ionic dependence and effects of local anaesthetics. *Biochem. Pharmac.*, **27**, 2039–2048.

STRANGE, P.G. BIRDSALL, N.J.M. & BURGEN, A.S.V. (1978). Ligand-binding properties of the muscarinic acetylcholine receptor in mouse neuroblastoma cells. *Biochem. J.*, **172**, 495–501.

SUAREZ-KURTZ, G. & BIANCHI, C.P. (1970). Sites of action of SKF 525A in nerve and muscle. *J. Pharmac. exp. Ther.*, **172**, 33–43.

TJIOE, S. & BIANCHI, C.P. (1969). Effects of local anaesthetics on muscarinic sites of the isolated frog ventricle. *Eur. J. Pharmac.*, **7**, 143–151.

WARD, D. & YOUNG, J.M. (1977). Ligand binding to muscarinic receptors in intact longitudinal muscle strips from guinea-pig intestine. *Br. J. Pharmac.* **61**, 189–197.

WEINSTOCK, M. & WEISS, C. (1979). Effects of procaine and extracellular calcium concentration on response of rat stomach fundus muscle to acetylcholine and 5-hydroxytryptamine. *Br. J. Pharmac.*, **65**, 593–599.

YAMAMURA, H.I. & SNYDER, S.H. (1974). Muscarinic cholinergic binding in rat brain. *Proc. natn. Acad. Sci. U.S.A.*, **71**, 1725–1729.

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