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INACTIVATION OF BRAIN CORTEX MUSCARINIC RECEPTORS BY 4-DIPHENYLACETOXY-1-(2-CHLOROETHYL) PIPERIDINE MUSTARD

MAGALI WAELBROECK,* ANNA-RITA RENZETTI,† MICHÈLE TASTENOY,* R. B. BARLOW‡ and J. CHRISTOPHE*§

*Department of Biochemistry and Nutrition, Medical School, Université Libre de Bruxelles, CP 611, 808 Route de Lennik, B-1070 Brussels, Belgium; †Department of Pharmacology, Laboratori Guidotti S.P.A., via Livornese 402, I-56122 Pisa, Italy; and ‡Ash Lea Cottage, Ravenstonedale, Kirby Stephen, Cumbria CA17 4NG, U.K.

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Abstract—We demonstrated in this study that 4-DAMP [4-diphenylacetoxy-1-(2-chloroethyl) piperidine] mustard, which cyclizes to the aziridinium ion, behaved as a non-selective, non-competitive inhibitor of muscarinic receptors in rat brain cortex. It inactivated to the same extent the M_1, M_2 and M_4 muscarinic receptors present in this tissue, as well as receptors accessible or not accessible to quarternary anti-muscarinic drugs. Under mild incubation conditions, the muscarinic receptors in a state with super high affinity for agonists (SH receptors) were less affected by preactivated 4-DAMP mustard than the receptors in the states with lower affinity for agonists (H and L receptors).

Muscarinic receptors have been subdivided according to several criteria:

1. Five messenger RNAs, encoding different muscarinic receptor proteins, have been cloned from cardiac or brain cDNA libraries, sequenced, and expressed in mammalian cells (for review, see Ref. 1).

2. Up to four muscarinic receptor subtypes can be distinguished in binding and pharmacological studies using selective antagonists [2–4]. M_1 receptors are defined as the receptors with a high affinity for pirenzepine [5]; M_2 receptors show high affinity for AF-DX 116 (11-({2-[(diethylamino)methyl]-1-piperidinyl}acetyl)-5,11-dihydro-6*H*-pyrido(2,3-*b*)-(1,4)benzodiazepin-6-one‖) [6], and M_3 and M_4 receptors, which have a high affinity for 4-DAMP (4-diphenylacetoxy-1-(2-chloroethyl) piperidine) methobromide [7] and for hexahydro-sila-difenidol [8], are discriminated by methoctramine or himbacine [3, 4]. it is likely, but not unambiguously proven, that among mRNA sequences the m1 sequence corresponds to that of the M_1 receptor, m2 to the M_2 receptor, m3 to the M_3 receptor, and m4 to the M_4 receptor [2–4].

3. All brain cortex muscarinic receptors can be further subdivided into three subclasses with, respectively, super high (SH), high (H) or low (L) affinities for agonists [9]. There is no correspondence between the SH/H/L and the $M_1 \rightarrow M_4$ classification, since each of the M_1, M_2, M_3 and M_4 subtypes has been found in different agonist binding states. It is generally believed that the SH, H and L receptor states reflect receptor interaction with effector proteins [9].

4. Some of the receptors labeled by [^{3}H]-quinuclidinyl benzilate (a tertiary muscarinic antagonist) in rat cerebral cortex homogenates cannot be recognized by quarternary muscarinic antagonists [10]. These subpopulations are not correlated with the existence of different receptor subtypes [10, 11] and those muscarinic receptors recognized by amines and not by quarternary ammonium derivatives are thought to be in a very hydrophobic environment or facing inside closed vesicles.

"Mustard" (2-chloroethyl amino) derivatives of several muscarinic agonists and antagonists have been synthesized and tested in pharmacological and biochemical studies [12–14]. Recently, a mustard derived from 4-DAMP has been described [15]. It cyclizes to form an aziridinium ion and its effects on muscarinic receptors in intact guinea pig ileum are much higher than those on guinea pig atria (dose-ratios over 100 on ileum compared with around 2 on atria).

Because 4-DAMP is a selective antagonist with a preference for forebrain M_1 and M_4 receptors [3] and for ileum M_3 receptors [7] over M_2 receptors, we anticipated that the 4-DAMP mustard would have similar selectivity.

Our results indicate that the compound inactivated M_2 as well as M_1 and M_4 receptors in rat cerebral cortex but affected the super high affinity state of all receptors for agonists less efficiently than the two lower affinity states.

MATERIALS AND METHODS

Materials. 1-[*N-methyl*-^{3}H]Scopolamine ([^{3}H]-

§ Corresponding author. Tel: (32) 2-555-62-28; FAX (32) 2-555-62-30.

‖ Abbreviations: 4-DAMP, 4-diphenylacetoxy-1-(2-chloroethyl) piperidine; AF-DX 116, 11-({2-[(diethylamino)methyl] - 1 - piperidinyl} acetyl) - 5,11 - dihydro - 6*H*-pyrido(2,3-*b*)(1,4)benzodiazepin-6-one; K_D, equilibrium dissociation constant of the tracer or unlabeled drug; IC_{50}, concentration of unlabeled drug required to inhibit 50% of tracer binding at equilibrium.

NMS (1-[*N-methyl*-]^{3}H]scopolamine methylchloride, 85 Ci/mmol) was obtained from Amersham International (Bucks, U.K.). Pirenzepine and AF-DX 116 were gifts from Boehringer-Ingelheim (Brussels, Belgium). Carbamylcholine and *N*-methyl atropine (atropine methyl bromide) were, respectively, from Federa (Brussels, Belgium) and the Sigma Chemical Co. (St Louis, MO, U.S.A.). The preparation of 4-DAMP mustard has been described elsewhere [15].

Methods. Wistar albino rats were killed by decapitation. The heart was dissected, rinsed in NaCl (9 g/L) and homogenized as previously described [16]. The brain cortex was quickly dissected and homogenized in 30 mL of 10 mM sodium phosphate buffer (pH 7.4). Crude membranes were centrifuged 30 min at 30,000 *g*, 4°; the supernatant discarded and the pellet resuspended at 2 mg protein/mL (the protein concentration was determined according to Lowry *et al.* [17], using bovine serum albumin as standard). These membranes were used to compare preactivated 4-DAMP mustard competition curves, using a filtration procedure, as described in Ref. 16. The [^{3}H]NMS concentration used was 200 pM. In other experiments, the membranes were pretreated with preactivated 4-DAMP mustard, as detailed below.

A 10 mM stock solution of 4-DAMP mustard was prepared daily, in water, and 5% of 5 N acetic acid immediately added to this solution. Prior to membrane treatment, 4-DAMP mustard was diluted to 1 mM (nominal concentration) in 10 mM sodium phosphate buffer and the pH adjusted to 7.0 using sodium hydroxide. The solution clarified in about 1 hr at 25° reflecting cyclization of the (insoluble) 4-diphenylacetoxy-1-(2-chloroethyl) piperidine to soluble 4-diphenylacetoxy-1-aziridinium piperidine.

Crude cortex membranes (1.5–2.0 mg protein/mL) were incubated for 1 hr, at 25°, in the absence or presence of 10 nM to 1 μM pretreated 4-DAMP mustard (nominal concentration). This incubation was followed by centrifugation and two washing steps (30 min centrifugation at 30,000 *g*, 4°, using 10 mM sodium phosphate buffer pH 7.0 to homogenize the pellet).

[^{3}H]NMS and [^{3}H]QNB binding were measured by a filtration procedure, as described in Ref. 17. Each assay tube contained 1.2 mL of 50 mM sodium phosphate buffer (pH 7.4) enriched with 2 mM $MgCl_2$, 1% bovine serum albumin, [^{3}H]NMS or [^{3}H]QNB and 0.03 mg (for [^{3}H]NMS binding) or 0.06 mg (for [^{3}H]QNB binding) of membrane protein. Non-specific binding was defined as tracer binding in the presence of 1 μM atropine.

Mathematical analysis. The total concentration of muscarinic receptors was measured by analysis of [^{3}H]NMS saturation curves using the computer-assisted curve fitting program described by Richardson and Humrich [18]. The [^{3}H]NMS/pirenzepine, [^{3}H]NMS/AF-DX 116 and [^{3}H]QNB/*N*-methyl atropine competition curves were adequately described by a two sites model [10, 16]; the [^{3}H]NMS/carbamylcholine competition curve was best described by a three sites model [9, 19]. All competition curves were analysed by a computer-assisted curve fitting program [18]. To compare the carbamylcholine competition curves in control and treated membranes, we assumed that the IC_{50} values of the three "binding sites" were unchanged after 4-DAMP mustard treatment.

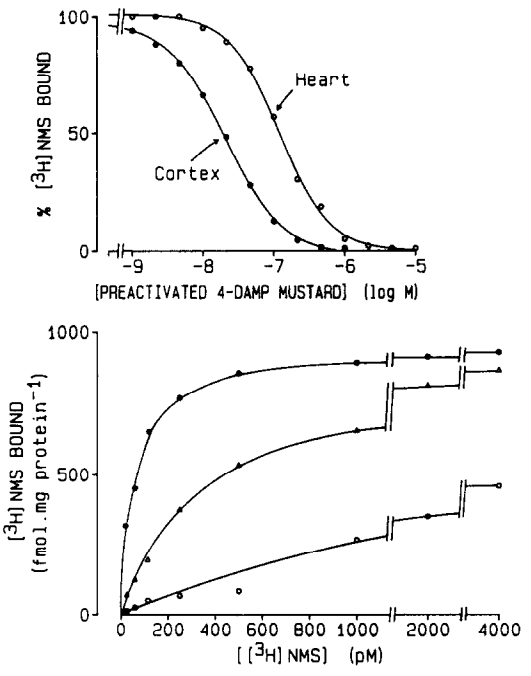

Fig. 1. Top panel: rat cerebral cortex membranes (●) and rat heart homogenates (○) were incubated for 1 hr in the presence of 200 pM [^{3}H]NMS and in the presence or absence of the indicated preactivated 4-DAMP mustard concentrations. Representative of three experiments in duplicate. Bottom panel: rat cerebral cortex membranes were incubated in the presence of 25 pM to 4 nM [^{3}H]NMS and in the absence (●) or presence of 3 nM (△) or 30 nM (○) preactivated 4-DAMP mustard. The total receptor concentrations, estimated as explained in Ref. 18, were (in fmol/mg protein): 980 (control), 950 (with 3 nM 4-DAMP mustard) and 1000 (with 30 nM 4-DAMP mustard). The apparent [^{3}H]NMS K_D values were 90 pM (control), 360 pM (in the presence of 3 nM 4-DAMP mustard) or 3 nM (in the presence of 30 nM 4-DAMP mustard). Representative of three experiments.

RESULTS

[^{3}H]*NMS binding to rat brain cortex and rat heart membranes in the presence of* 4-*DAMP mustard*

[^{3}H]NMS saturation curves were obtained in the absence or presence of preactivated 4-DAMP mustard (Fig. 1, bottom). The apparent K_D value of [^{3}H]NMS was increased and the total receptor concentration unchanged in the presence of 3 or 30 nM 4-DAMP mustard, a result compatible with competitive inhibition of [^{3}H]NMS binding. Preactivated 4-DAMP mustard inhibited [^{3}H]NMS binding to crude cortex membranes at lower concentrations than binding to heart homogenates (Fig. 1, top). Assuming competitive binding, we

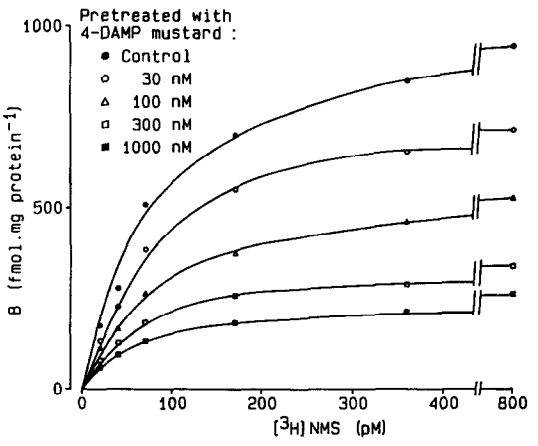

Fig. 2. Rat cerebral cortex membranes were pretreated without or with preactivated 4-DAMP mustard [control (●); 30 nM (○); 100 nM (△); 300 nM (□) or 1 μM (■)] and extensively washed. [^{3}H]NMS binding was then measured at different tracer concentrations (25–1000 pM). The total receptor concentrations, estimated as explained in Ref. 18 were (in fmol/mg protein): 1052 (control), 803 (after 30 nM preactivated 4-DAMP mustard), 590 (after 100 nM preactivated 4-DAMP mustard), 389 (after 300 nM preactivated 4-DAMP mustard) and 291 (after 1 μM preactivated 4-DAMP mustard). The [^{3}H]NMS K_D values were: 83.7 pM (control), 85.7 pM (after 30 nM preactivated 4-DAMP mustard), 95.7 pM (after 100 nM preactivated 4-DAMP mustard), 77.8 pM (after 300 nM preactivated 4-DAMP mustard) and 76.9 pM (after 1 μM preactivated 4-DAMP mustard). Representative of five experiments.

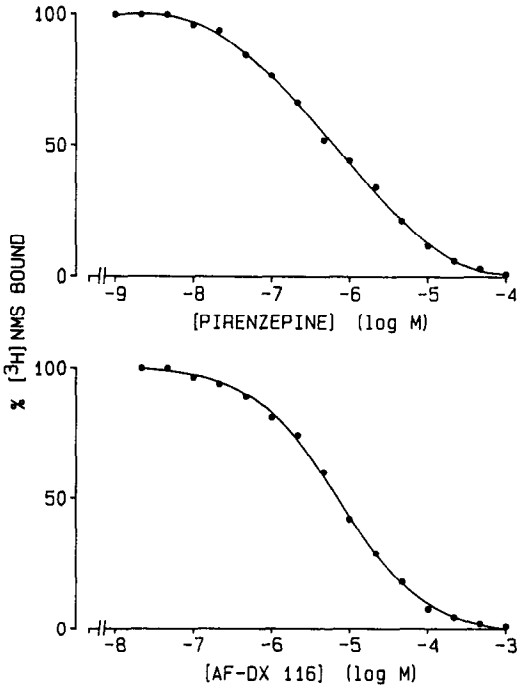

Fig. 3. [^{3}H]NMS (1.0 nM) specific binding to rat cerebral cortex membranes was measured in the absence or presence of the indicated pirenzepine (top) or AF-DX 116 (bottom) concentrations. The standard deviation of each point, expressed as per cent control binding (in the absence of unlabelled drug) was 2–3%. The competition curves obtained using control membranes and membranes treated with 10, 30, 100, 300 or 1000 nM preactivated 4-DAMP mustard were within 2% of each other when expressed as per cent binding in the absence of pirenzepine or AF-DX 116: one symbol is presented rather than four overlapping symbols. Pirenzepine inhibited [^{3}H]NMS binding to 40% of the sites with an IC_{50} of 90 nM and had an average IC_{50} ov 2.5 μM for the remaining 60% of non-M_1 sites. AF-DX 116 inhibited [^{3}H]NMS binding to 15% of its binding sites with an IC_{50} of 300 nM, and had an average IC_{50} of 11 μM for the remaining 85% non-M_2 sites. Average of three experiments in duplicate.

obtained K_i values of 4 and 30 nM, respectively, in cortex and heart.

Total receptor concentration

In a second set of experiments, we pretreated rat cerebral membranes with or without preactivated 4-DAMP mustard. The treated membranes were then extensively washed to remove the free 4-DAMP mustard. We observed a dose-dependent decrease of the total muscarinic receptor concentration following this pretreatment with 4-DAMP mustard 10 nM to 1 μM. The affinity of [^{3}H]NMS for the remaining sites was unchanged by the pretreatment (Fig. 2).

Proportion of M_1 or M_2 sites

M_2 muscarinic receptors have a lower affinity for [^{3}H]NMS as compared to the other subtypes [3, 16]. To ensure that they were adequately labeled by the tracer in the following experiments, we chose a high (1.0 nM) [^{3}H]NMS concentration to perform pirenzepine and AF-DX 116 competition curves. At 1.0 nM [^{3}H]NMS, the IC_{50} values of pirenzepine for M_2, M_3 and M_4 sites are very similar (higher than its IC_{50} value for M_1 sites [3, 16]. A "two sites" model is therefore adequate to determine the proportions of M_1 and non-M_1 sites. The IC_{50} values of AF-DX 116 for M_1, M_3 and M_4 sites are also similar and higher than its IC_{50} value for M_2 sites [3, 16] so that a "two sites" binding model was used to determine the proportions of M_2 and non-M_2 sites (M_1 + M_3 + M_4). The results, shown in Fig. 3, indicated that the proportions of M_1 sites (40 ± 5%) and of M_2 sites (15 ± 5%) were identical in control membranes and in membranes pretreated with preactivated 4-DAMP mustard.

Proportion of sites accessible to quaternary antagonists

Only 85% of the [^{3}H]QNB was bound to muscarinic binding sites with high affinity for *N*-methyl atropine (Fig. 4, top panel). The remaining 15% had a high affinity for atropine, a normal stereoselectivity for the benzetimide enantiomers (dexetimide and levitimide: over 10,000-fold) (not shown) but were inaccessible to high affinity quarternary ligands (Fig. 4). The proportion of accessible/inaccessible sites was not changed by mustard pretreatment (Fig. 4).

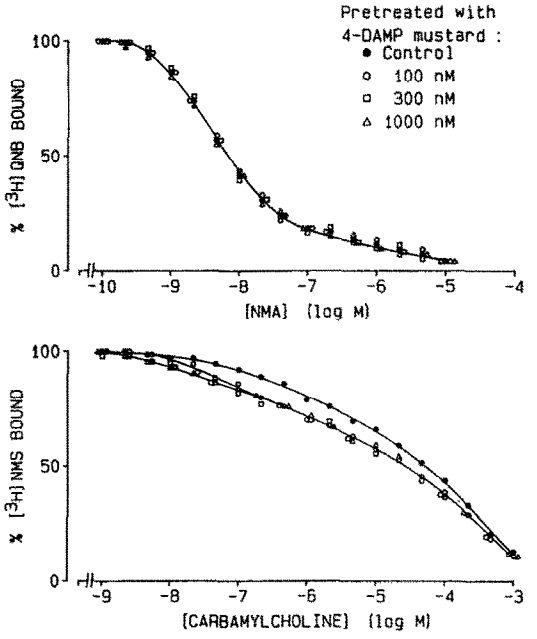

Fig. 4. Top panel: [^{3}H]QNB specific binding to rat cerebral cortex membranes was measured in the absence or presence of the indicated *N*-methyl atropine concentrations (non-specific binding was defined using 1 μM atropine). The tracer was used at a saturating (1 nM) concentration. The standard deviation of each point was 2–3%. The results obtained using control (●) membranes and membranes pretreated with 100 nM (○), 300 nM (□) or 1 μM (△) preactivated 4-DAMP mustard were within 2% of each other. Average of three experiments performed in duplicate. Bottom panel: specific [^{3}H]NMS binding was measured in the absence or presence of the indicated concentrations of carbamylcholine, using 200 pM [^{3}H]NMS as tracer and control (●) membranes or membranes pretreated with 100 nM (○), 300 nM (□) or 1 μM (△) preactivated 4-DAMP mustard. The standard deviation of each point was 2–3%. The proportion of SH, H and L sites and their IC_{50} values are indicated in the text. Average of three experiments performed in duplicate, with a standard deviation below 2%.

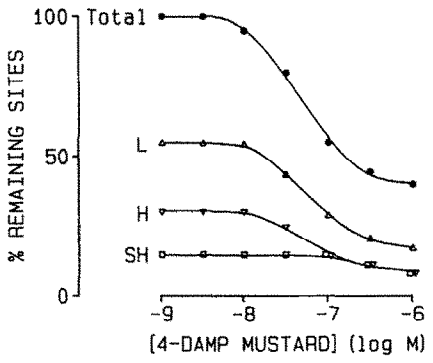

Fig. 5. The total concentration of receptors (●) after pretreatment with the indicated 4-DAMP mustard concentrations was calculated from saturation curves, like those shown in Fig. 2. The absolute concentrations of SH (□), H (▽) and L (△) sites were then calculated using the proportion of each site found in carbamylcholine competition curves (Fig. 4). The results are all expressed as a percentage of the total receptor concentration observed under control conditions.

Agonist binding

[^{3}H]NMS shows a $M_4 > M_1$, $M_3 > M_2$ preference [3, 16]. At the saturating (1.0 nM) concentration used for the pirenzepine and AF-DX 116 competition curves, IC_{50} values correspond to 21 K_i at M_4 receptors, 9 K_i at M_1 and M_3 receptors and only 3 K_i at M_2 receptors [3, 16]. Carbamylcholine has a similar affinity (K_i) for each L (or H or SH) receptor state of M_1 (previously called A), M_2 (C) and M_3 + M_4 (B) receptors [19]: its IC_{50} values will be very different from subtype to subtype if a large [^{3}H]-NMS concentration is used. We therefore decided to use a low [^{3}H]NMS concentration to analyse the carbamylcholine competition curves, even though the M_2 subtype was poorly labeled in these conditions.

The carbamylcholine IC_{50} value was decreased 2-fold following 4-DAMP mustard pretreatment (Fig. 4, bottom panel), but only if this treatment was performed at neutral pH (7.0) rather than at pH 9.0 and at a low temperature (25°), rather than 37° (not shown). Analysis of the competition curves, assuming that carbamylcholine recognized three receptor states [19] with the same affinities in control and treated membranes, suggested that this shift of the competition curve reflected a relative resistance of receptors in the super high (SH) affinity state as compared to those in the high (H) or low (L) affinity states (Figs 4 and 5).

The competition curves shown in Fig. 4 (bottom panel) were fitted assuming that carbamylcholine inhibited [^{3}H]NMS binding to SH receptors with an IC_{50} value of 0.1 μM, binding to H receptors with an IC_{50} of 5.0 μM, and binding to L receptors with an IC_{50} value of 300.0 μM. The proportions of SH:H:L receptors were 15:30:55 in control membranes, and changed to 25:25:50 after treatment with 0.1, 0.3 or 1.0 μM preactivated 4-DAMP mustard.

DISCUSSION

Slow binding of 4-DAMP mustard

Preactivated 4-DAMP mustard was able to interact with the muscarinic binding sites of cerebral cortex (Fig. 1, top panel). We observed a decrease of the muscarinic receptor concentration after pre-incubation with (preactivated) 4-DAMP mustard, followed by extensive washing of cortex membranes (Fig. 2). Furthermore, reappearance of muscarinic receptors in NB-OK1 cells pretreated with pre-activated 4-DAMP mustard was absolutely dependent on protein synthesis (unpublished results). These data suggest that activated 4-DAMP mustard interacted reversibly with muscarinic receptors (Fig. 1), then formed a stable covalent bond with receptors (Fig. 2), as described by the following model:

$$M + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MR \xrightarrow{k_3} M\text{–}R'$$

(where M is the mustard; R, the muscarinic receptor; MR, the reversibly bound mustard; M–R', the covalent mustard–receptor complex; and k_1, k_2 and k_3, respectively, the association, dissociation and inactivation rate constants) (see also Ref. 15).

As shown in Fig. 1, we did not observe any decrease of the [^{3}H]NMS receptor concentration when cortex membranes were incubated in the simultaneous presence of tracer and preactivated 4-DAMP mustard. On the other hand, 4-DAMP mustard was able to inactivate the muscarinic receptors when used in the absence of [^{3}H]NMS (Fig. 2). This suggests that the tracer and the aziridinium ion of 4-DAMP mustard had comparable binding kinetics: [^{3}H]NMS, at high concentrations, was able to occupy the muscarinic receptors first, and thereby prevent receptor recognition and alkylation by the mustard. If the 4-DAMP mustard had been able to recognize muscarinic receptors faster than [^{3}H]NMS, we would have found either a mixed, or non-competitive inhibition of [^{3}H]NMS binding in the experiment shown in Fig. 1 (bottom panel).

Receptor inactivation by the bound 4-DAMP mustard (k_3) was also slow: after a 1 hr incubation with 1 μM preactivated 4-DAMP mustard (a saturating concentration: see Fig. 1), 40% of the receptors were still intact (Figs 2 and 5) and inactivation progressed exponentially with time over at least 3 hr (not shown).

Lack of muscarinic receptor selectivity

4-DAMP mustard produces a markedly greater (reversible + irreversible) inhibition on ileum (M_3 receptors) as compared to atria (M_2 receptors) [15]. We obtained qualitatively similar results in this work: 4-DAMP mustard inhibited [^{3}H]NMS binding to cerebral cortex (mainly M_1 + M_4 sites) at slightly lower concentrations than binding to cardiac homogenates (M_2 receptors) (Fig. 1, top panel). We therefore hoped that 4-DAMP mustard would inactivate preferentially the M_1 and M_4 receptors rather than the M_2 receptors present in cerebral cortex.

This assumption was however based on the (wrong) hypothesis that "equilibrium binding" was achieved very quickly and that the inactivation rate was proportional to k_3 and to receptor occupancy (as in Ref. 20).

As shown in Fig. 3, we did not observe any modification of the proportion of M_1 or M_2 receptors after pretreatment for 1 hr with different 4-DAMP mustard concentrations. This can be explained if we assume that k_3 was comparable to k_2, so that the receptor occupancy increased according to the sum of two exponentials, both exponents being complex functions of k_1 M, k_2 and k_3 (see Ref. 21). Under these conditions, receptor inactivation is *not* directly proportional to receptor occupancy (compare Fig. 1 and Fig. 5!).

Slow dissociation rates are by no means exceptional for muscarinic antagonists: the tracers used in binding studies typically have dissociation half-lives of a few minutes to several hours (see *inter alia* Refs 3, 5, 10 and 19).

The activated mustard can diffuse through vesiculated membranes and alkylates receptors that are usually inaccessible

As indicated in the introduction, [^{3}H]QNB recognizes in rat brain a muscarinic receptor population which is not recognized by quaternary ammonium derivatives like [^{3}H]NMS. These inaccessible receptors belong to the M_1 and to non-M_1 subclasses [22].

It is interesting to observe that 4-DAMP mustard was able to alkylate to the same extent all [^{3}H]QNB binding sites, including those which were not accessible to quarternary amines. This can be explained if one remembers that the "preactivated 4-DAMP mustard" is in fact a mixture of three compounds: 4-diphenylacetoxy-1-(2-chloroethyl) piperidine (4-DAMP mustard itself), the aziridinium ion and 4-diphenyl-acetoxy-1-(2-ethanol) piperidine formed by reaction of the aziridinium ion with water. Titration with sodium thiosulfate indicates that at 25° at most 65% of the mustard is converted to the aziridinium ion [15]. Our results suggest that the remaining 4-diphenylacetoxy-1-(2-chloroethyl) piperidine, being very hydrophobic, penetrated into the closed vesicles, formed the aziridinium ion, and inactivated the "inaccessible" receptors. By contrast, [^{3}H]*N*-methylscopolamine and *N*-methylatropine, being permanently charged quarternary drugs, were not able to penetrate inside these vesicles, and were therefore unable to recognize this subset of receptors.

This is an important result, since it supports the hypothesis that the barrier between the quarternary antagonists and the binding site is formed by the membrane of closed vesicles and that the conformation of the receptor itself did *not* change to a state unable to recognize quaternary compounds.

Relative resistance of receptors in the super high affinity state for the agonist carbamylcholine

Another interesting result is the observation that the SH state of muscarinic receptors was somewhat more resistant than the H or L states to 4-DAMP mustard alkylation. This result suggests that some of the effector proteins (e.g. G proteins) interacting with the receptors to form the SH state, were concentration limitant (relative to muscarinic receptors) and free to associate with other molecules of receptors. These mobile effector proteins were probably relatively unstable, since we did not observe any effect of 4-DAMP mustard pretreatment on agonist binding properties when the 1 hr pretreatment was performed either at a high pH (9.0) or a higher temperature (37°), i.e. under relatively long incubation conditions that facilitate protein denaturation.

In conclusion, 4-DAMP mustard behaved as a non-selective, non-competitive antagonist for brain cortex M_1, M_2 and M_4 muscarinic receptors and inactivated with an equal potency muscarinic receptors that were accessible and those which were not accessible to quaternary ammonium derivatives. The mustard was less efficient on receptors in the super high affinity state.

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