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AGONIST MEDIATED CONFORMATIONAL CHANGES OF SOLUBILIZED CALF FOREBRAIN MUSCARINIC ACETYLCHOLINE RECEPTORS

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Abstract—Muscarinic receptors in calf forebrain membranes can be identified by the specific binding of the radiolabelled antagonist [^{3}H]dexetimide. These receptors (2.8 pM/mg protein) comprise two non-interconvertible subpopulations with respectively high and low agonist affinity but with the same antagonist affinity. For all the agonists tested the low affinity sites represent 85 ± 5% of the total receptor population. 0.5% Digitonin solubilized extracts contain 0.8 pM muscarinic receptor/mg protein. In contrast with the membranes, these extracts contain only sites with low agonist affinity.

The alkylating reagent *N*-ethylmaleimide causes an increase of the acetylcholine affinity for the low affinity sites in membranes as well as for the solubilized sites. This effect is time dependent until a maximal 3-fold increase in affinity is attained. The rate of *N*-ethylmaleimide action is enhanced by the concomitant presence of agonists. In contrast, *N*-ethylmaleimide does not affect antagonist binding. This suggests that agonists mediate a conformational change of both the membrane bound low affinity muscarinic sites and of the solubilized sites, resulting in their increased susceptibility towards NEM alkylation.

Radioligand binding studies have revealed that muscarinic receptors are cell plasma membrane proteins which recognize both muscarinic agonist and antagonist molecules [1, 2]. These studies have also revealed that, in different tissues, the muscarinic receptors are composed of discrete subpopulations with different affinities for agonists but with the same affinity for antagonists [2]. As an example, low agonist affinity sites (R_L)* represent 80% of the total amount of sites in rat forebrain membranes, one of the most investigated tissues [3, 4]. Using the alkylating reagent *N*-ethylmaleimide as a structural probe, we have recently demonstrated that only these R_L sites undergo agonist mediated conformational changes [3]. These sites are converted, by treatment with NEM, into an alkylated form which has a higher affinity towards agonists but the same affinity for antagonists. The agonist mediated conformational change is evidenced by their ability to enhance NEM action.

A similar heterogeneity towards agonist binding [5] is observed for β-adrenergic receptors in different tissues [6–9]. This β-receptor heterogeneity is linked to the ability of only part of the total receptors to undergo functional coupling to the adenylate cyclase regulatory unit [5, 7]. For the muscarinic receptors, there is only indirect evidence that receptor heterogeneity might be related to functional coupling to membrane effector components. Agonist mediated contractile responses in smooth muscle [10] and guanylate cyclase activation in mouse NG108-15 [11] and N1E-115 [12] neuroblastoma cells suggest that only the low affinity sites are physiologically active and hence, that they are probably coupled to an effector component. On the other hand, it has been reported on heart cell membranes that agonist mediated adenylate cyclase inhibition might encompass high affinity sites [13]. Finally, studies on agonist stimulated phosphatidylinositol breakdown in smooth muscle [14, 15] suggested that both high and low affinity sites are physiologically active.

In this study we have used solubilization as a tool for receptor uncoupling from putative effector components in membranes. We show that digitonin treatment of calf forebrain membranes results in the solubilization of low agonist affinity muscarinic sites. These sites retain their ability to undergo agonist mediated conformational changes suggesting that this characteristic does not require muscarinic coupling to a membrane effector component.

*Abbreviations: NEM, *N*-ethylmaleimide; K_D, equilibrium dissociation constant; R_H, muscarinic sites with high affinity for agonists; R_L, $R_{L(NEM)}$, muscarinic sites with low affinity for agonists in the native and the alkylated form respectively; $K_{D(H)}$, $K_{D(L)}$ and $K_{D(L,NEM)}$, agonist equilibrium dissociation constants for binding to R_H, R_L, and $R_{L(NEM)}$ respectively.

MATERIALS AND METHODS

Materials. Acetylcholine bromide, carbamylcholine chloride, atropin sulfate monohydrate, dexetimide hydrochloride and oxotremorine sequifumarate were from Aldrich Europe (Beerse, Belgium). *N*-Ethylmaleimide (NEM), cysteine, neostigmine bromide and carbamyl-β-methylcholine

were from Sigma Chemical Company (St. Louis, U.S.A.). Digitonin was purchased from Merck (Darmstadt, Germany). Benzylcholine Mustard was from NEN (Dreieich, W. Germany). [^{3}H]Dexetimide (14 Ci/mM) was supplied by IRE (Fleurus, Belgium).

Membrane preparations. Calf brains were obtained at a slaughterhouse and kept in an ice-cold 145 mM NaCl solution (pH 7.4) at 4°. Further manipulations were performed within less than one hour. Cerebellum and medulla oblongata were discarded. The remaining forebrains were homogenized in 10 vol of ice-cold sucrose buffer (0.25 M sucrose, 5 mM Tris–HCl, pH 7.4) with a polytron mixer (15 sec) and then with a Potter homogenizer (3 strokes at maximum speed). The homogenate was centrifuged at 700 *g* for 15 min and the supernatant further centrifuged at 10,000 *g* for 15 min. The resulting supernatant was then centrifuged at 29,000 *g* for 20 min and the pellet suspended in a 12% glycerol solution in water. The suspension (10 mg protein/ml) was stored at −20° for up to two months without loss of muscarinic receptor number nor agonist and antagonist binding characteristics. Protein determinations were performed by the method of Lowry *et al.* [16] using bovine serum albumin as the standard.

Membrane solubilization. Membranes (final concentration 5 mg/ml) were solubilized by mixing in a 0.5% (w/v) digitonin suspension in Tris–buffer (75 mM Tris–HCl, pH 7.4) for 25 min at 4°. The suspension was then centrifuged at 29,000 *g* for 30 min at 4°. 55 ± 10% of the original amount of membrane protein was recovered in the supernatant. Centrifugation of the solubilisate at 105,000 *g* for 1 hr caused no appreciable further precipitation of [^{3}H]-dexetimide binding sites: i.e. B_{max} = 2.1 pmoles/ml after 29,000 *g* centrifugation and 1.9 pmoles/ml after 105,000 *g* centrifugation. The affinity for the radioligand was also similar: i.e. 4.7 and 5.8 nM respectively. Cholinesterase activity, measured by the method of Knedel *et al.* [17], was below the limit of detection in the membrane as well as in the solubilized preparations.

[^{3}H] *Dexetimide binding to muscarinic receptors in calf forebrain membranes.* Calf forebrain membranes (0.2 mg/ml) were incubated with [^{3}H]dexetimide in the presence of other ligand when indicated, for 20 min at 37° in Tris buffer in a total volume of 2 ml. Three micromolar neostigmine bromide was included to block acetylcholine esterase activity. At the end of the incubation 3 ml of ice-cold Tris buffer was added and the samples filtered under suction through Whatman GF/F glass fiber filters (2.5 cm ϕ). The filters were washed rapidly twice with 4 ml of ice-cold buffer and placed in 20 ml polyethylene scintillation vials. After addition of 1 ml NaOH (0.1 M) and 9 ml aqualuma (Lumac), they were counted for 10 min in a Packard liquid scintillation spectrometer. Non-specific binding of [^{3}H]dexetimide was defined by binding of the tracer in the presence of 1 μM unlabelled atropin, and was subtracted from total binding to yield 'specific binding'. Non-specific binding did not exceed 10% of the total [^{3}H]dexetimide binding. Curves shown in Fig. 1 were fitted according to the Scatchard analysis of [^{3}H]dexetimide saturation binding. Curves in Figs. 2 and 3(B) were fitted according to the Hofstee plot analysis (1 site model) and curves in Fig. 3(A) according to the parameters obtained from Minneman's two-site analysis [22].

[^{3}H] *Dexetimide binding to solubilized receptors.* Incubation of solubilized membranes (1.2 mg protein/ml) with [^{3}H] dexetimide was performed as described above. Free and bound [^{3}H] dexetimide were then separated from each other by the Sephadex G50 gelfiltration method as described [18]. Radioactivity was counted in a liquid scintillation spectrometer after addition of 10 ml aqualuma (Lumac). Radioligand binding to solubilized receptors was routinely carried out on the supernatant obtained after a 30 min centrifugation at 29,000 *g*. Further centrifugation (1 hr, 100,000 *g*) did not decrease the concentration of [^{3}H] dexetimide binding sites in the supernatant.

RESULTS

Binding characteristics of muscarinic receptors in calf forebrain membranes. The antagonist [^{3}H] dexetimide has earlier been reported to specifically

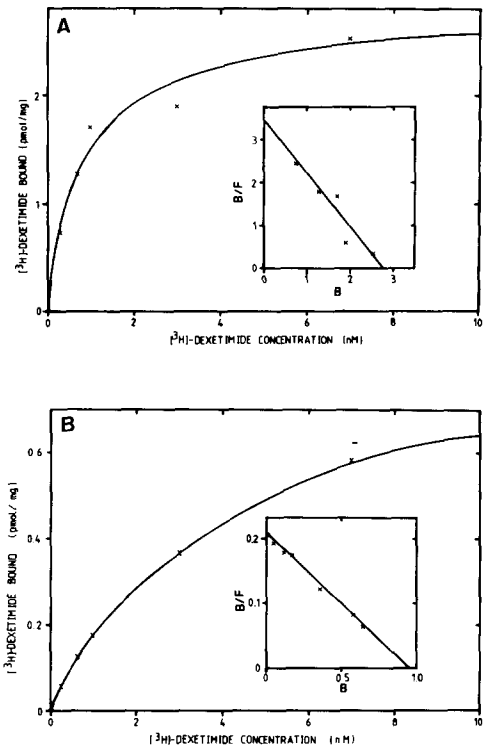

Fig. 1. [^{3}H]Dexetimide saturation binding to calf forebrain membranes and to 0.5% digitonin solubilized preparation. Calf brain membranes (A) or solubilisate (B) are incubated with increasing concentrations of [^{3}H]dexetimide for 30 min at 37°, and filtered as described in Materials and Methods. Specific binding of [^{3}H]dexetimide is presented as a function of its free concentration. Values shown are means of two experiments.

Inserts: Scatchard plot of the saturation binding data. The slope is determined by linear regression analysis. The total amount of binding sites (B_{max}) is 2.8 pM/mg protein for the membranes and 0.8 pM/mg protein for the solubilisate. The equilibrium dissociation constants are 1.6 and 4.5 nM, respectively.

Table 1. Antagonist binding to calf forebrain membranes: effect of NEM

Antagonist	NEM (1 mM)	K_D (nM)	$K_D/K_{D(NEM)}$
Atropine	−	3.1	
	+	3.2	0.98
Dexetimide	−	0.5	
	+	0.4	1.30
Levitimide	−	2100	
	+	2900	0.73

Competition binding studies with the listed antagonists are performed as described in Fig. 2A. The Hofstee plots are rectilinear. The slopes are determined by linear regression analysis to yield IC_{50} values. The corresponding K_D values are calculated according to the method of Cheng and Prusoff [23] and Chou [24]. Listed K_D values refer to the same membrane preparation. $K_D/K_{D(NEM)}$ refers to the ratio of K_D values obtained in absence and presence of NEM.

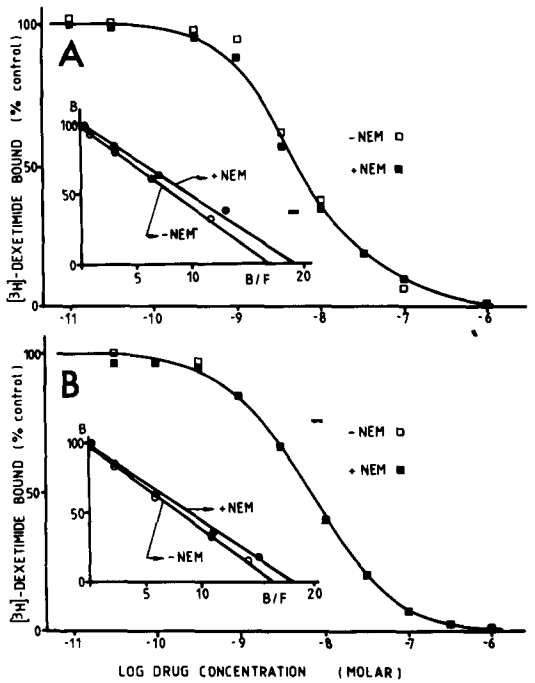

Fig. 2. Atropine/[^{3}H]dexetimide competition binding to membranes and solubilisate: effect of NEM. Membranes (A) or solubilisate (B) are incubated with 2 nM [^{3}H] dexetimide and increasing concentrations of atropine in the absence or presence of NEM (1 mM for A and 0.1 mM for B) for 20 min at 37°. [^{3}H]Dexetimide binding is presented as a function of the concentration of competitor. Control binding (100%) is measured in the presence of buffer only. Values are means of two experiments.

Inserts: Hofstee plots of the competition binding data. B represents percentage displacement of [^{3}H]dexetimide binding by atropine and F the free atropine concentration (in nM). The slopes are determined by linear regression analysis and yield IC_{50} values for atropine in the absence and presence of NEM: respectively 6.8 ± 0.5 nM and 6.9 ± 0.7 nM for the membranes and 5.1 ± 0.5 nM and 5.2 ± 0.6 nM for the solubilisate. The corresponding K_D values, calculated according the method of Cheng and Prusoff [23] and Chou [24] are presented in Table 1.

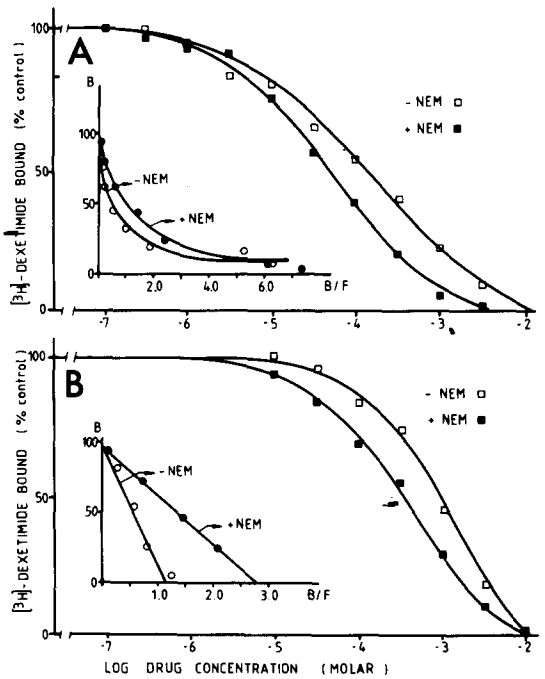

Fig. 3. Acetylcholine/[^{3}H]dexetimide competition binding to membranes and solubilisate: effect of NEM. Membranes (A) or solubilisate (B) are incubated with 2 nM [^{3}H] dexetimide and increasing concentrations of acetylcholine (in the presence of 5 μM neostygmine bromide) in the absence or presence of NEM (1 mM for A and 0.1 mM for B). [^{3}H]dexetimide binding is presented as described for Fig. 2.

Inserts: Hofstee plots of the competition binding data. B represents the percentage displacement of [^{3}H]dexetimide binding and F the free acetylcholine concentration in μM. The non-linear Hofstee plots for membranes (A) are analysed according to a two-site model by the iterative method of Minneman *et al.* [22] and the linear plots for the solubilisate are analysed by linear regression. The percentage of each affinity population (for A) and the K_D values for acetylcholine are given in Table 2.

label muscarinic receptors in membranes from different tissues [19, 20]. Specific binding of this radioligand to calf forebrain membranes is a saturable process (Fig. 1A). The Hill coefficient equals unity ($n_h = 1.0$) and the Scatchard plot is linear ($r = 0.99$) which argues for the absence of cooperative interactions. The total number of binding sites determined by the Scatchard plot equals 2.8 pM/mg membrane protein and the equilibrium dissociation constant (K_d) for dexetimide binding amounts to 1.6 ± 0.4 nM.

Binding of the other antagonists, listed in Table 1, follow also the simple law of mass action. As an example the Hofstee plot of an atropine/[^{3}H] dexetimide competition binding is rectilinear ($r = 0.98$) and the Hill coefficient is close to unity ($n_h = 0.98$) (Fig. 2). In agreement with muscarinic receptors in other tissues, levitimide has a considerable lower affinity (i.e. 3800-fold) than its dexorotary isomer dexetimide (Table 1).

In contrast, the agonist/[^{3}H]dexetimide competition curves are shallow. The Hofstee plot for

acetylcholine is curvilinear (Fig. 3) and the Hill coefficient equals 0.44. This can be explained either by negative cooperativity or by the existence of two or more receptor subpopulations with different agonist affinities. We have retained the second possibility on the basis of the ability of the irreversible antagonist PrBCM to block a considerable fraction of the receptors (83% under the conditions defined in Table 3) without altering the agonist competition binding curves for the remaining sites. A similar conclusion has been drawn by Birdsall *et al.* [21] for rat forebrain membranes and is based on the fact that random inactivation of a large receptor proportion should diminish the agonist cooperativity (if present) whereas there should be no effect on the agonist binding pattern in the case of two receptor subclasses with different agonist affinity.

Assuming the presence of two major receptor subpopulations agonist/[^{3}H]dexetimide competition curves can be analysed by the computerized iterative method of Minneman *et al.* [22] to yield the proportion of the sites with high (R_H) and low (R_L) agonist affinity and their respective K_D's. As depicted in Table 2 the proportion of the two sites is approximately the same for the different agonists tested, i.e. $R_H = 15 \pm 5\%$ and $R_L = 85 \pm 5\%$.

Using the alkylating reagent NEM as a structural probe, we have earlier demonstrated the ability of muscarinic agonists to cause conformational changes of the muscarinic R_L sites in rat forebrain membranes [3]. The same experimental picture is observed in calf.

The presence of 1 mM NEM has no effect on [^{3}H] dexetimide saturation binding, competition binding of other antagonists (Table 1), the R_H/R_L-ratio and agonist binding to R_H (Table 2). However the reagent causes a leftward shift for agonist competition binding to the R_L sites (Fig. 3). This phenomenon can be explained either by an agonist/NEM-mediated receptor inactivation [7] or by a NEM-mediated increase in agonist affinity [3]. The first interpretation can, however, be ruled out on the basis of the fact that [^{3}H]dexetimide saturation binding experiments yield the same number of receptor sites for membranes preincubated with buffer only, with NEM or with a combination of agonist and NEM (Table 4). The NEM-mediated increase in agonist affinity for R_L ($K_{D(L)}/K_{D(L,NEM)}$) decreases in the following order: acetylcholine (2.9) > carbachol (2.4) > oxotremorine (2.0) > carbamyl-β-methylcholine (1.2) (Table 2).

Preincubation of the membranes with NEM causes a time dependent increase in apparent agonist affinity (i.e. decrease in [^{3}H]dexetimide binding in presence of a submaximal concentration of agonist) until a limit value is reached, corresponding to agonist binding to the fully alkylated R_L sites (Fig. 5A). The concomitant presence of agonist (at a concentration

Table 2. Agonist binding to membranes: effect of NEM

Agonist	NEM (1 mM)	R_H (%)	$K_{D(H)}$ (nM)	$K_{D(L)}$ (μM)	$K_{D(L)}/K_{D(L,NEM)}$
Oxotremorine	−	10	1.1	2.6	
	+	11	2.3	1.2	2.0
Acetylcholine	−	19	390	52	
	+	17	350	18	2.9
Carbachol	−	11	36	300	
	+	17	91	124	2.4
Carbamyl-β-methylcholine	−	10	390	746	
	+	12	850	620	1.2

Membranes are incubated with the listed agonists in the absence and presence of 1 mM NEM as described in Fig. 3(A). Iterative analysis of the Hofstee plots is performed according to the two-site model by the computerized method of Minneman *et al.* [22]. The agonist K_D values for R_H and R_L sites (i.e. $K_{D(H)}$ and $K_{D(L)}$) are calculated from the computer determined IC_{50} values by the method of Cheng and Prusoff [23] and Chou [24]. In this method we also use the K_D of the radioligand on the same membrane preparation. $K_{D(L)}/K_{D(L,NEM)}$ refers to the ratio of $K_{D(L)}$ values obtained on absence and presence of NEM.

Table 3. Antagonist and agonist binding to membranes: effect of PrBCM pretreatment

	[^{3}H]Dexetimide binding characteristics			Acetylcholine binding characteristics			
Pretreatment	n_H	K_D (nM)	B_{max} (pM/mg)	n_H	R_H (%)	$K_{D(H)}$ (μM)	$K_{D(L)}$ (μM)
none	0.94	2.18	2.02	0.39	23	0.74	69.5
PrBCM (30 nM)	1.10	1.98	0.35	0.46	22	0.56	74.0

Membranes are preincubated during 30 min at 30° with 30 nM PrBCM (cyclized to the aziridinium ion as described earlier [29]). The membranes are then washed by centrifugation (29,000 *g* for 30 min), and resuspended in ice-cold buffer. The K_D and B_{max} for [^{3}H]dexetimide is determined from Scatchard analysis of saturation binding data. The acetylcholine competition binding parameters are determined as described in Table 1.

Table 4. Antagonist and agonist binding to solubilized membranes: effect of NEM

	n_H	K_D (without NEM) (μM)	K_D (with NEM) (μM)	$K_D/K_{D(NEM)}$
agonist				
oxotremorine	0.88	0.36 ± 0.01	0.32 ± 0.01	
acetylcholine	0.85	42 ± 4	17 ± 2	2.5
carbachol	0.90	339 ± 7	68 ± 4	4.9
carbamyl-β-methylcholine	1.2	494 ± 25	372 ± 18	1.3
antagonists				
atropine	0.90	0.0039 ± 0.0002	0.0033 ± 0.0001	1.2
dexetimide	1.02	0.0045 ± 0.0001	0.0044 ± 0.0002	1.0
levitimide	0.89	14.2 ± 0.02	13.1 ± 0.07	1.1

Antagonist as well as agonist competition binding curves yield rectilinear Hofstee plots. The K_D values of the listed antagonists and agonists are calculated as described in Fig. 2.

corresponding to the IC_{50} value) in the NEM preincubation medium causes an increase in the rate of NEM alkylation whereas the presence of an equipotent concentration of antagonist has no effect (Fig. 4A).

Binding characteristics of solubilized muscarinic receptors. Muscarinic receptors can be solubilized with a yield of 16 ± 5% by treatment of the membranes with a 0.5% digitonin suspension. Specific binding of [^{3}H]dexetimide to the soluble sites is a saturable process (Fig. 1B) and the K_D equals 4.5 ± 1.1 nM. The total number of binding sites equals 0.8 ± 0.2 pM/mg protein. The Hill coefficient (n_H = 0.95) and the linear Scatchard plot (r = 0.99) argue for the absence of cooperative interactions. Binding of the other antagonists tested, still follow the law of mass action and their affinities, including the stereospecificity towards the benzetimide analogs (i.e. dexetimide and levitimide), are unaffected upon solubilization (Table 4).

In contrast to the situation on membranes, the agonist/[^{3}H]dexetimide competition binding curves

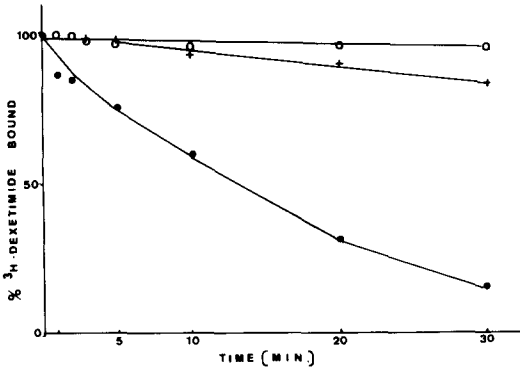

Fig. 4. Time dependent inactivation of [^{3}H]dexetimide binding sites by NEM. Membranes are preincubated with 1 mM NEM (○—○) and solubilisate with 0.1 mM (+ — +) and 1 mM NEM (●—●) for increasing periods of time (abscissa). The reagent is inactivated by addition of cysteine (10 mM for membranes 4 mM for solubilisate) after which binding of 2 nM [^{3}H]dexetimide is measured. Cysteine has no effect on the total number of binding sites on membranes (i.e. B_{max}/B_{max}(cys) = 0.96), the affinity for the muscarinic antagonists (i.e. the K_D/K_D(cys) = 1.04 for dexetimide and 1.1 for atropine) or the agonist acetylcholine (i.e. K_D/K_D(cys) ≈ 1.2 for R_H and 0.9 for R_L) nor on the proportion of R_H and R_L sites (i.e. 17 and 83%). Cysteine alone has also no effect on total amount of binding sites and the binding characteristics of the different ligands (data not shown).

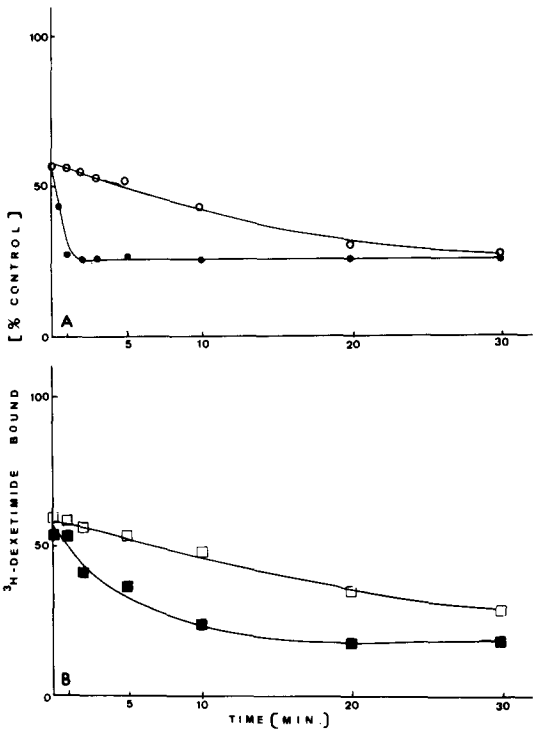

Fig. 5. Time dependent increase in apparent agonist affinity by NEM for membranes and solubilisate. Membranes (A) and solubilisate (B) are preincubated for increasing periods of time (abscissa) with NEM (1 mM for A and 0.1 mM for B). Then, the reagent is inactivated by addition of an excess of cysteine (10 mM for A and 4 mM for B) and binding of 2 nM [^{3}H]dexetimide is measured. Values shown represent binding in the presence of 0.1 mM acetylcholine, either added at the start of the [^{3}H]dexetimide incubation phase (○, □) or already present during the preincubation phase (●, ■). Control binding (100%) is measured in the presence of NEM only.

are steep (Fig. 3B). The Hofstee plot for acetylcholine is rectilinear ($r = 0.97$) and the Hill coefficient is close to unity ($n_H = 0.85$) indicating that only one population of agonist binding sites is present in the digitonin solubilized membranes. The K_D for acetylcholine, calculated from its IC_{50} value amounts to 42 ± 4 μM (Table 4). Other agonists, listed in Table 4, compete with [^{3}H]dexetimide with the same order of affinity as in membranes, i.e. oxotremorine > acetylcholine > carbachol = carbamyl-β-methylcholine. All agonist K_D values are very close to the $K_{D(L)}$ values in membranes (Table 4), suggesting that the solubilized sites are in the R_L form.

Whereas 1 mM NEM has no effect on membranes, it readily inactivates solubilized binding sites (Fig. 5B). To reduce the rate of this effect the NEM concentration was lowered to 0.1 mM. At this concentration the reagent causes only slow inactivation (Fig. 5B), but is still able to cause a leftward shift of the agonist competition binding curve. As for the membranes, this effect does not correspond to an agonist/NEM-mediated receptor inactivation (Table 4) but to an increase in the agonist affinity for the total amount of binding sites (Fig. 3B). As shown in Table 4, the acetylcholine affinities amounted to 42 ± 4 μM and 17 ± 2 μM in absence and presence of NEM respectively. The degree of the NEM shift ($K_D/K_{D(NEM)} = 2.5$) remains close to the value observed for the R_L sites in membranes (Table 4). NEM has no effect on antagonist affinity for the solubilized receptor sites (Table 4).

As for the membranes, the NEM-mediated increase in the apparent agonist affinity is time dependent (Fig. 4B) and the concomitant presence of agonist but not of antagonist increases the rate of NEM alkylation.

DISCUSSION

In this study we have characterized the muscarinic receptors in calf forebrain membranes by the specific binding of the antagonist [^{3}H]dexetimide (Fig. 1A and Table 1). As expected, these receptors behave as a homogeneous population of non-cooperative sites with regard to antagonist binding [4] (Figs. 1A and 2A). On the other hand, agonist/[^{3}H]dexetimide competition binding curves are shallow. The possibility of negative cooperativity being excluded (Table 3), these curves evidence the presence of two or more receptor subpopulations with different agonist affinity, but with the same antagonist affinity. In this context, Birdsall *et al.* [21] have shown that rat forebrain membranes might contain three receptor subpopulations with respectively superhigh, high and low agonist affinity. The superhigh affinity sites, representing no more than 3–5% of the total receptor population, can only be detected by direct [^{3}H] agonist binding and remain undetected in radiolabeled antagonist competition binding experiments. Accordingly we analysed the agonist/[^{3}H] dexetimide competition binding curves on calf forebrain membranes in terms of a two-site model (i.e. R_H and R_L with respectively high and low agonist affinity). For all the agonists tested, R_H represented 15 ± 5% of the sites. This is very close to the R_H value obtained in rat forebrain membranes (i.e. $R_H = 20$% [3]). Moreover the order of the agonist IC_{50} and $K_{D(L)}$ values is also the same for rat and calf forebrain membranes (i.e. oxotremorine > acetylcholine > carbamylcholine > carbamyl-β-methylcholine).

Specific [^{3}H]dexetimide binding sites could be solubilized by treatment of calf brain membranes with 0.5% digitonin. The treatment preserved the high affinity of the sites for the tracer (Fig. 1B), the order of the agonist affinities (Table 2) and the stereoselectivity. This indicates the absence of gross conformational changes of the muscarinic receptor binding sites upon solubilization with digitonin. Interestingly, agonist/[^{3}H]dexetimide competition binding curves follow the simple law of mass action after solubilization (Fig. 3B). The agonist K_D's for the solubilized sites are very close to the $K_{D(L)}$'s for the membranes (Table 3). It appears thus that the solubilized membranes may only contain low agonist affinity sites. This is in agreement with the findings of Hurko *et al.* [30] who solubilized bovine brain homogenates with digitonin, but in contrast with the results of Carson *et al.* [31] who solubilized calf brain membranes with 2 M NaCl. This difference might be explained by the alternative method of solubilization.

The same experimental picture of NEM action is observed for the R_L sites in rat [3] and calf brain membranes and is moreover, preserved after solubilisation of the receptors:

(1) NEM alkylation results in a time-dependent increase in the apparent agonist affinity but not the antagonist affinity.

(2) The NEM effect is observed, even in the absence of ligand. However the rate of this effect is increased by the concomitant presence of agonist but not of antagonist.

These data on membrane-bound and solubilized receptors can both be explained by a model which is based on (a) the Monod–Wyman–Changeux "Plausible Model" [25] and (b) on the assumption that NEM "blocks" the sites in a state resembling the "active" conformation by alkylation of either the sites themselves or of groups in their vicinity i.e.

$$\begin{array}{ccc} H + R_{L(i)} & \rightleftharpoons & R_{L(a)} \\ K_i \uparrow\downarrow & & \uparrow\downarrow K_a \\ H.R_{L(i)} & & H.R_{L(a)} \end{array}$$

In this model R_L can exist in two forms: the active (NEM sensitive) form, $R_{L(a)}$, and the inactive (NEM resistant) form $R_{L(i)}$, to which the ligand H can bind with the respective microscopic equilibrium dissociation constants K_a and K_i. $R_{L(a)}$ and $R_{L(i)}$ are in equilibrium, even in the absence of ligand and ligands can act as agonists if $K_i > K_a$ or as antagonists if $K_i = K_a$.

Agonist molecules thus appear to mediate conformational changes of muscarinic R_L sites (in the membrane as well as solubilized), resulting in an increased accessibility of alkylable groups at their surface. Interestingly, β-adrenergic receptors also undergo agonist mediated conformational changes which can be monitored by use of NEM as a structural probe. Whereas NEM causes a moderate

increase in agonist affinity for the muscarinic R_L sites, the reagent causes the formation of a stable agonist β-adrenergic receptor complex (resulting in an apparent 'inactivation' of these receptors [6, 26]). For both the muscarinic and β-adrenergic receptors, only a subpopulation appears to undergo such conformational changes. A further similarity between both receptors resides in the fact that the NEM sensitive and insensitive receptor subpopulations have a different agonist affinity but the same antagonist affinity (i.e. NEM-sensitivity for the high affinity sites for β-adrenergic [6–9] and low affinity sites for muscarinic receptors [3]).

For the β-adrenergic receptors, it has been demonstrated that the agonist/NEM sensitivity requires the functional coupling to the adenylate cyclase regulatory component [5, 7]. As a result of the loss of receptor–regulator coupling digitonin solubilization of the β-adrenergic receptors abolishes the agonist/NEM sensitivity [27]. Solubilized muscarinic receptors appear also to be free from putative regulatory components, since SDS-PAGE analysis of the affinity chromatography purified receptors contains only one protein band of approximately 70 K Dalton [28]. There is however, a marked difference between β-adrenergic and muscarinic receptor behaviour since the solublilized muscarinic receptors still undergo agonist mediated conformational changes. Indeed, it is still possible to observe a NEM dependent increase in agonist affinity for the solubilized receptor sites. As for membranes, this NEM effect is clearly enhanced by the concomittant presence of agonist molecules. It can thus be concluded that agonist mediated conformational changes of muscarinic receptors do not require their coupling to a membrane effector component.

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