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Deoxamuscaroneoxime derivatives as useful muscarinic agonists to explore the muscarinic subsite: Demox, a modulator of orthosteric and allosteric sites at cardiac muscarinic M_2 receptors

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Abstract

A series of muscarinic agonists, straight chained, branched, cyclic alkyl and aromatic derivatives of the oxime **1** (demox) was designed with the aim of investigating their activity on muscarinic receptor subtypes. Effects on M_1 receptor were assessed functionally by a microphysiometer apparatus, while M_2, M_3, and M_4 receptor potency and affinity were studied on isolated preparations of guinea pig heart, ileum, and lung, respectively. The results suggest that the substitution of a hydrogen with a long side-chain or bulky group generally induces a decrease in potency at M_1 and M_3 subtypes, while a general increase in this parameter is obtained at M_2 subtype. Among the agonists **2**–**18**, compound **4** behaves as a full agonist with a preference for M_3 subtype. Moreover, compound **12** is inactive at M_1 and M_4 receptors while it displays a full agonist activity at M_2 and M_3 subtypes. Since demox displays a variable response on cardiac M_2 receptors regulating heart force, an in-depth inquiry of the functional behaviour of this compound was carried out at M_2 receptors. In presence of 10^{-11} and 10^{-10} M demox, the binding of [^{3}H]-NMS was increased by $\approx$ 30% as a consequence of an increase of the association of [^{3}H]-NMS to membranes; this effect was not observed in presence of a higher concentration of [^{3}H]-NMS. Higher concentrations of demox decreased the binding of [^{3}H]-NMS to heart atrial membranes but significantly retarded the dissociation of this radioligand. Our results suggest that demox

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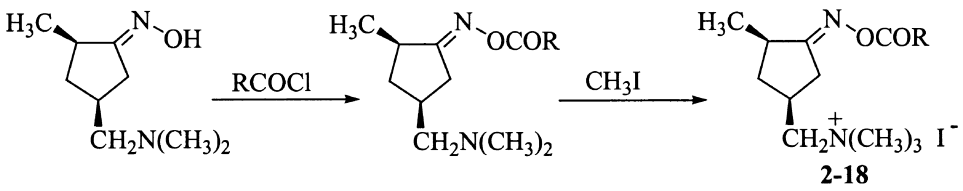
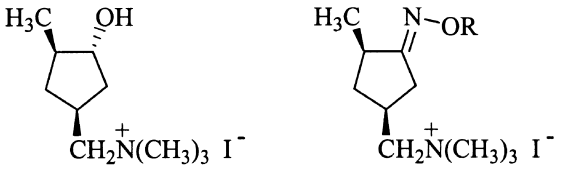
may interact with orthosteric and allosteric sites of atrial M_2 muscarinic receptor.

Keywords: Muscarinic subsite; Deoxamuscaroneoxime derivatives; Orthosteric and allosteric sites; Cardiac M_2 receptors

Introduction

Five different muscarinic receptor subtypes (m1–m5) from a variety of tissues and species have been cloned [1], while the pharmacological classification of these receptors distinguishes among M_1, M_2, M_3, and M_4 subtypes. The fifth subtype predicted by molecular cloning studies is still waiting for its physiological role to be defined [1–3]. Although the best results in the characterization of muscarinic receptor subtypes have been obtained with the discovery of selective antagonists [4], a great deal of effort has been made to design selective muscarinic agonists which could selectively modulate a distinct receptor subtype [5]; nevertheless, at this moment no agonist is known to discriminate among muscarinic receptor subtypes, according to its affinity, by more than one order of magnitude [6]. For many years our group has been involved in the study of the nature of the interaction involving the binding of the hydroxy group of muscarine with the complementary receptor subsite [7–10]. Many pentatomic cyclic muscarinic agonists have been synthesized and studied for this purpose, in order to determine the pharmacological parameters, such as affinity, relative efficacy and enantioselectivity, that define this subsite. On the other hand, oxime derivatives have been suggested as muscarinic ligands with a potential M_1 selective profile [11–13]. Moreover, the oxime moiety is present in arecoline and muscarone related agonists [12,14] and in allosteric muscarinic receptor modulators [15–21]. For all these reasons, we decided to design and study a series of oxime derivatives, deoxamuscarine analogues (Fig. 1), as useful tools to explore the requirements of the muscarinic subsite. This paper reports the results of the synthesis and the pharmacological investigation of these muscarinic agonists. Moreover, when testing demox, the reference agonist of the oxime derivatives **2**–**18**, our attention was attracted by its behaviour at the M_2 subtype. The peculiarity of this compound, in fact, is evident at cardiac M_2 receptors regulating heart force where, differently from what we reported (Piergentili *et al.*, submitted for publication), it displayed a large interval of potency (pD_2) and affinity values ($-\log K_D$), with these two parameters ranging from 5.83 to 8.07 and from 3.70 to 5.81, respectively (Table 3). This fact induced us to investigate this compound at cardiac M_2 receptors regulating heart rate as well. Figure 2 B shows the same trend of data already observed for heart force. Since monophasic and biphasic dose-response curves were obtained for this agonist at M_2 subtype (force and rate), the possibility of the involvement of two sites, at low and high activity (Fig. 2) was postulated. The implication of other receptors such as muscarinic M_1, adenosinic A_1 and β_1-adrenergic was also investigated, but our results proved that these receptors were not involved, supporting the specificity of the binding between the oxime demox and the M_2 muscarinic subtype(s) expressed in the cardiac tissue. Various compounds are known to allosterically modulate the binding of ligands to muscarinic receptors [22]. Interestingly, demox (3×10^{-5} M) as well as the allosteric agent W 84 [23] delayed the dissociation of [^{3}H]-N-methylscopolamine from rat atrial membranes, whereas lower con-





Deoxamuscarine

1. R = H (Demox)
2. R = $COCH_3$
3. R = $CO(CH_2)_2CH_3$
4. R = $CO(CH_2)_4CH_3$
5. R = $CO(CH_2)_6CH_3$
6. R = $CO(CH_2)_8CH_3$
7. R = $CO(CH_2)_{10}CH_3$
8. R = CO-*i*-Pr
9. R = CO-*c*-C_6H_{11}
10. R = CO-1-Adamantyl
11. R = $COCH{=}CH_2$
12. R = COC_6H_5
13. R = $COCH_2C_6H_5$
14. R = $COCH_2CH_2C_6H_5$
15. R = CO-1-Naphthoyl
16. R = CO-2-Naphthoyl
17. R = CO-2-Furoyl
18. R = CO-2-Thiophenyl

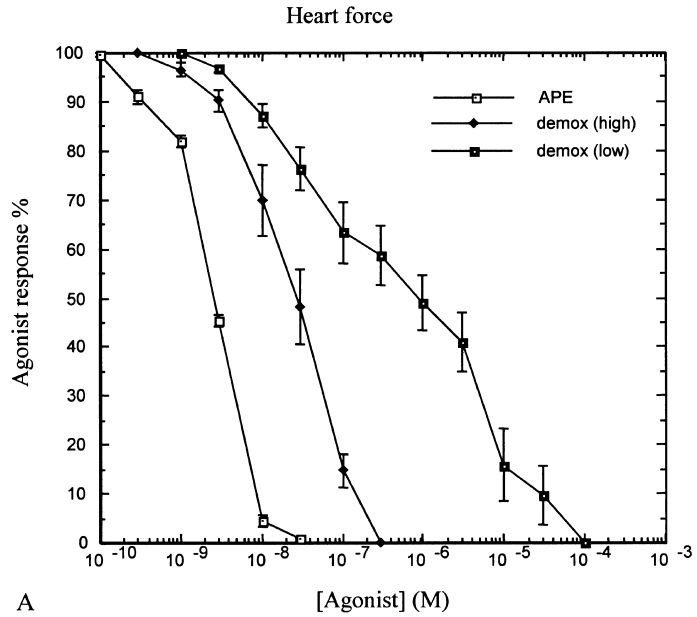
Fig. 1. Synthetic pathways and structural formulae of the muscarinic ligands discussed.

centrations (10^{-11} and 10^{-10} M) elevated the affinity of this radioligand to M_2 muscarinic receptors.

Materials and methods

Chemistry. General methods

Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. ^{1}H-NMR spectra were recorded on a Varian VXR-300. Chemical shifts are reported as δ-values (parts per million) relative to Me_4Si as an internal standard. The microanalyses were performed by the microanalytical laboratory, and the elemental compositions of the compounds agreed to within ± 0.4 % of the calculated value. Chromatographic separations were performed on silica gel columns (kiesegel 4.0 0.040–0.063, Merck) by flash chromatography. Reactions were monitored by thin-layer chromatography (TLC) with Fluka plates with fluorescent indicator. Spots were detected by ultraviolet light (254 nm) and iodine vapour.



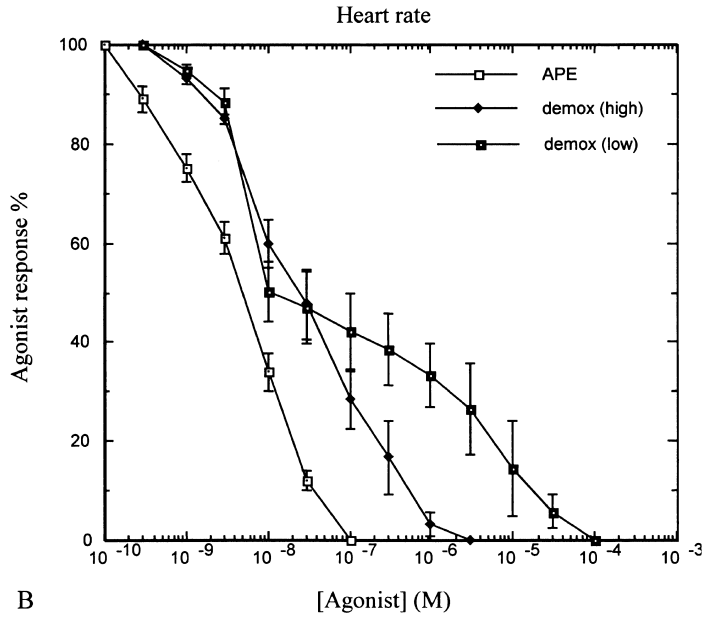


Fig. 2. Panel A. Experimental dose-response curves of APE (control) and demox at high ($pD_2 = 8.07$; $pK_D = 5.81$; correlation coefficient = 0.954) and low ($pD_2 = 5.83$; $pK_D = 3.70$; correlation coefficient = 0.855, # 1) activity and affinity respectively, at cardiac M_2 receptors regulating heart force. An average 50% of monophasic and 50% of biphasic curves was obtained. Each point is the mean ± SEM of 20–30 observations. Panel B. Experimental dose-response curves of APE (control) and demox at high ($pD_2 = 7.71$; $pK_D = 4.68$; correlation coefficient = 0.951) and low ($pD_2 = 6.46$; $pK_D = 2.50$; correlation coefficient = 0.847, # 1) activity and affinity respectively, at cardiac M_2 receptors regulating heart rate. An average 50% of monophasic and 50% of biphasic curves was obtained. Each point is the mean ± SEM of 20–30 observations.

General procedure for the preparation of compounds 2–18

The appropriate acyl chloride (3.0 mmol) (Fig. 1) was added to a solution of oxime (0.5 g, 2.9 mmol) and triethylamine (0.45 mL) in dry $CHCl_3$ (20 mL). After stirring at r.t. for 3 h under a nitrogen stream, the solution was washed with 2N NaOH. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by column chromatography. Eluting with $CHCl_3$-CH_3OH-(33%) NH_4OH (9-1-0.1) gave the acyl oxime derivatives. By treating the free bases with an excess of CH_3I, the corresponding methiodides were obtained. The yields of products were 50%–80% (Table 1).

In vitro tests. General considerations

Male guinea pigs (200–300 g) were killed by cervical dislocation. The organs required were set up rapidly under 1 g of tension in 20 mL organ baths containing physiological salt solution (PSS) maintained at an appropriate temperature (see below) and aerated with 5% CO_2–95% O_2. Two dose-response curves were constructed by cumulative addition of the reference agonist. The concentration of agonist in the organ bath was increased approximately 3-fold at each step, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady. Following 30 min of washing, a new dose-response curve to the agonist under study was obtained. Responses were expressed as a percentage of the maximal response obtained in the control curve. The results are expressed in terms of pD_2, which is the $-\log ED_{50}$, the concentration of agonist required to produce 50% of the maximum effect. Contractions were recorded by means of a force displacement transducer connected to the MacLab system PowerLab/800 and to a two-channel

Table 1
Physicochemical properties of compounds **2–18**

Compd.	R	m p (°C)	Yield	Recrytn. solvent
2	CH_3	150-151	50%	*i*-PrOH/Ethyl Ether
3	$(CH_2)_2CH_3$	134-136	80%	*i*-PrOH/ Ethyl Ether
4	$(CH_2)_4CH_3$	116-117	80%	EtOH/ Ethyl Ether
5	$(CH_2)_6CH_3$	102-103	80%	*i*-PrOH/ Ethyl Ether
6	$(CH_2)_8CH_3$	113-114	73%	i-PrOH
7	$(CH_2)_{10}CH_3$	115-116	53%	*i*-PrOH/ Ethyl Ether
8	*i*-Pr	75-76	80%	*i*-PrOH/ Ethyl Ether
9	*c*-C_6H_{11}	145-146	74%	i-PrOH
10	1-Adamantyl	142-143	79%	i-PrOH
11	$CH{=}CH_2$	133-134	23%	EtOH/ Ethyl Ether
12	C_6H_5	184	85%	EtOH
13	$CH_2C_6H_5$	131-133	24%	*i*-PrOH/ Ethyl Ether
14	$CH_2CH_2C_6H_5$	91-92	59%	*i*-PrOH/ Ethyl Ether
15	1-Naphthoyl	184-185	58%	EtOH
16	2-Naphthoyl	185-186	61%	EtOH
17	2-Furoyl	168-169	75%	EtOH
18	2-Thiophenyl	187-188	57%	*i*-PrOH

Gemini polygraph (U. Basile). In all cases, parallel experiments in which tissues received only the reference agonist were run in order to check any variation in sensitivity.

All animal testing was carried out according to the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Determination of dissociation constants

Dissociation constants (K_D) were determined as previously described according to the method of Furchgott and Bursztyn for full agonists ($\alpha = 1$) [24] by means of a software computation process supplied by Dr. Roberto Gagliardi [Piergentili *et al.*, submitted for publication]. When demox behaved as a partial agonist ($\alpha = 0.9$), K_p was determined according to Waud [25]. For partial agonism with $\alpha << 1$ ($\alpha = 0.4$), the affinity constant (K_B) was calculated from the equation $\log (DR - 1) = \log$ [partial agonist] $- \log K_B$, where DR (dose ratio) is the ratio of ED_{50} values of the reference agonist after and before incubation (1×10^{-4} M) with partial agonist [26]. $K_{D/}$ ED_{50} values, which are indicators of agonist efficacy, are reported.

The functional assays on guinea-pig ileum (M_3), bladder (M_3), stimulated left atria (M_2), right atria (M_2) and lung strips (M_4) were conducted as previously described [10, 27].

Measurement of extracellular acidification rates

CHO cells, stably expressing human mAChRs M_1, were supplied by Professor Roberto Maggio (University of Pisa, Italy). Cells were maintained in α-minimum essential medium containing 10% foetal bovine serum at 37 °C under 5% CO_2–95% O_2. Cells were grown to confluence and harvested by scraping in fresh medium. Changes in extracellular acidification were determined using a Cytosensor microphysiometer (Molecular Devices). Cells were seeded into cytosensor cell capsules 20 h prior to experiments at a density of 3×10^5 cell per well. The cell capsules, mounted in sensor chambers, were placed on the instrument, and the cells were perfused with media *via* a peristaltic pump, during which the pH of the microenvironment surrounding the sensor was kept constant. The removal of acid from the cells by the perfusate was periodically halted (pump turned off), allowing a build up of acid metabolites and, therefore, a change in chamber pH (acidification rate). This on-off cycle was repeated throughout the experiment and the effect of compounds was determined by adding the compound to the chamber. The extracellular acidification rates were measured in each 120-s pump cycle; flow on at 100 μl min^{-1} for 90 s with a 20-s exposure to the test compound, flow off for 30 s. The acidification rate was determined between 98 s and 118 s, using the cytosoft program (Molecular Devices). Concentration-effect curves were obtained by exposing the cells sequentially to increasing concentrations of agonist at intervals of 21 min.

In vivo tests. Pithed rat

This preparation was set up as previously described [10].

Binding studies of demox to the five cloned human muscarinic receptor subtypes

These studies were performed as previously described [28]. [^{35}S]-GTPγS binding assay was performed according to Lazareno and Birdsall [29]. Membranes were suspended in a buffer containing 20 mM HEPES, 100 mM NaCl and 10 mM $MgCl_2$ pH 7.4, at a protein con-

centration of 35 μg/ml. One milliliter of membrane suspension was incubated with 1 μM GDP and the agonist (acetylcholine and demox) at 30 °C for 20 min and then transferred to ice for 15 min. [^{35}S]-GTPγS was added to a final concentration of 100 pM and the samples were incubated for 30 min at 30 °C. The samples were filtered over glass fibre filters (Whatman GF/C) using a Brandel cell harvester and washed thee times with 4 ml water. The filter discs were extracted overnight in 4 ml scintillant and counted in a β-counter.

Binding assays on rat heart atria homogenates

Male Sprague-Dawley rats (220–250 g) were killed by decapitation, the hearts were quickly removed and processed as described [30]. Protein concentration was determined using the method of Lowry *et al.* [31]. Homogenates (500 μg of protein) were incubated for 2 h at 22 °C in 1 mL of assay buffer in presence of 300 pM or 2 nM [^{3}H]-N-methyl-scopolamine (NMS; DuPont NEN, specific activity: 3034 GBq/mmol; K_d= 420 ± 38 pM; B_{max}= 180 ± 20 fmol/mg of protein; n = 3) and the indicated concentration of demox. In saturation binding assays, the homogenates were incubated as indicated above, in the presence of [^{3}H]-NMS (25 – 3200 pM) and the indicated concentrations of demox. Non-specific binding was defined as binding in the presence of 1 μM atropine. Each sample was filtered on GF/C glass fiber filters as described [30]. Radioactivity was counted by liquid scintillation.

Association and dissociation kinetics of [^{3}H]-NMS to atrial membranes

The conditions used in measurements of [^{3}H]-NMS association and dissociation to rat heart atrial homogenates were similar to the conditions described for binding assays. In association experiments, the compounds under investigations were incubated with the atrial membranes (500 μg of protein) for 30 min at 22 °C before [^{3}H]-NMS (300 pM) was added for various periods of time up to 120 min. In dissociation experiments, selected compounds were incubated with the membranes for 30 min and then with [^{3}H]-NMS (300 pM) for 120 min; than atropine (1 μM) was added for a further 120 min. Membranes were separated by rapid filtration as previously described. Data were analyzed by computer-aided, non-linear regression analysis using Prim (GraphPAD Software, San Diego, CA). Curve fitting to the dissociation data was based on a monoexponential decay equation; a biexponential decay equation did not yield significantly better fits (partial F-test, $P>0.05$).

Statistical analysis

The results are expressed as means ± S.E.M. of *n* experiments. Student's t-test was used to assess the statistical significance of the difference between two means.

Results and discussion

Activity of oxime derivatives at muscarinic receptors M_1 – M_4

The steric tolerance of M_1, M_2, M_3, and M_4 muscarinic receptor subtypes was systematically examined by synthesizing a series of straight chained, branched, cyclic alkyl, and aromatic derivatives of oxime **1** (demox). M_1 receptor potency was assessed from functional studies at human cloned muscarinic receptor using a microphysiometer apparatus. M_2, M_3,

and M_4 receptor potency and affinity of the oxime derivatives were assessed on isolated preparations of guinea pig heart, ileum and lung, respectively. These parameters are shown in Table 2 for the various muscarinic receptor subtypes. When testing demox, the reference agonist of the oxime derivatives, at cardiac M_2 receptors regulating heart force, in order to confirm previous data [Piergentili *et al.*, submitted for publication], we sometimes noted ED_{50} values significantly different from those already acquired. In fact, in some experiments carried out at guinea-pig stimulated left atria (M_2 subtype), a pD_2 value of 8.07 was obtained against the earlier value of 5.83 [Piergentili *et al.*, submitted for publication], while no differences were noted at the other muscarinic subtypes. None of the new compounds in Table 2 behaves as a full agonist in all the four muscarinic subtypes. Compounds **4** and **7** are full agonists in three subtypes ($\alpha \geq 0.90$), compounds **2** and **12** in two. Potency values range from 3.32 (compound **9**) to 5.55 (demox) at the M_1 subtype, from 5.69 (compound **3**) to 8.59 (compound **9**) at the M_2, from 4.50 (compound **16**) to 6.01 (compound **6**) at the M_3, and from 3.89 (compound **7**) to 6.31 (compound **15**) at the M_4 subtype. The agonists **2–18** display pD_2 values which are lower than that of demox at M_1 receptors, where only compounds **2**, **4**, **8**, and **14** show good intrinsic activity ($\alpha \geq 0.80$). The trend is different at the M_2 receptors where all the agonists show high pD_2 values and compounds **2**, **4**, **7**, and **12** display good intrinsic activities at this subtype. At the M_3 receptors, demox shows the best pD_2 value, with the exception of compound **6**, and all

Table 2
Intrinsic activity, potency, affinity, and efficacy of compounds **1–18** at M_1, M_2, M_3, and M_4 muscarinic receptors

		M_1		M_2				M_3				M_4			
	R	α^a	$pD_2{}^b$	α^a	$pD_2{}^b$	$pK_D{}^c$	$\frac{K_D{}^d}{ED_{50}}$	α^a	$pD_2{}^b$	$pK_D{}^c$	$\frac{K_D{}^d}{ED_{50}}$	α^a	$pD_2{}^b$	$pK_D{}^c$	$\frac{K_D{}^d}{ED_{50}}$
1	H (demox)	1.00	5.55	[e]	[e]	[e]	[e]	1.00	5.97	4.86	13	0.37	4.87	<5	
2	$COCH_3$	0.85	5.14	0.94	6.27	3.80	295	0.94	5.38	3.79	39	0.57	4.46	4.43	1
3	$CO(CH_2)_2CH_3$	0.53	3.96	0.60	5.69	4.30	24	1.00	5.27	4.46	7	0		<5	
4	$CO(CH_2)_4CH_3$	1.01	4.73	1.00	6.15	3.52	427	0.96	5.39	4.92	3	0.23	5.51	<5	
5	$CO(CH_2)_6CH_3$	0.50	4.11	0.74	6.30	4.73	37	0.92	4.97	5.01	1	0		<5	
6	$CO(CH_2)_8CH_3$	0.21	4.70	0.48	7.51	5.91	40	0.92	6.01	4.91	13	0		<5	
7	$CO(CH_2)_{10}CH_3$	0.41	5.30	0.94	6.07	4.15	83	1.00	5.63	4.32	20	1.00	3.89	3.89	1
8	CO-i-Pr	0.85	4.23	0.75	7.06	5.42	43	0.97	5.26	4.62	4	0.25	5.03	<5	
9	CO-c-C_6H_{11}	0.16	3.32	0.34	8.59	4.44	14125	0.91	4.80	4.75	1	0		<5	
10	COAdamantyl	0.12		0.15		5.66		0.84	4.96	4.52	3	0.27	5.17	<5	
11	$COCH{=}CH_2$			0.70	6.30	4.35	89	0.88	5.31	4.46	7	0.34	4.51	4.50	1
12	COC_6H_5	0.10		0.90	7.69	5.90	62	1.00	5.93	4.31	42	0		<5	
13	$COCH_2C_6H_5$			0.77	7.16	6.05	13	0.92	5.37	5.38	1	0.27	5.71	4.17	35
14	$COCH_2CH_2C_6H_5$	0.89	4.68	0.65	6.54	4.55	98	0.90	5.06	5.01	1	0.10		<5	
15	CO-1-Naphthoyl			0.36	7.15	4.54	407	0.88	4.56	4.82	1	0.20	6.31	<5	
16	CO-2-Naphthoyl	0.10		0.34	7.83	4.17	4571	0.77	4.50	3.83	5	0.15		<5	
17	CO-2-Furoyl	0.23	4.34	0.45	6.73	4.31	263	1.00	5.14	4.29	7	0.12		<5	
18	CO-2-Thiophenyl	0.27	3.57	0.46	6.36	3.56	631	0.95	5.02	4.31	5	0		<5	

[a] Intrinsic activity, measured by the ratio between the maximum response of the agonist and the maximum response of 4-Cl-McN-A-343 at M_1 and arecaidine propargyl ester (APE) at M_2, M_3, and M_4 subtypes. [b] $-\log ED_{50}$. Number of observations varies between 4 and 6. The standard error of the estimate was always less than 10%. [c] $-\log K_D$. Number of observations varies between 4 and 6. The standard error of the estimate was always less than 10%. [d] This ratio indicates the efficacy of the agonist. [e] This compound actually displays a large interval of potency and affinity at cardiac M_2 receptors (see Table 3).

the agonists display high values of intrinsic activity (compound **16** is the only partial agonist with α slightly lower than 0.80). Compound **7** is the only full agonist at the M_4 receptors. As far as agonist affinity is concerned, this parameter (Table 2) was determined at M_2, M_3, and M_4 receptor subtypes according to the method of Furchgott and Bursztyn [24], since the only comparison of agonist potencies on different tissues is not sufficient to speculate on the heterogeneity of muscarinic receptors. For this reason, in Table 2 we also report the K_D/ED_{50} ratios of the agonists, which give a rough indication of their efficacy. Affinity values range from 3.52 to 6.05 at M_2 receptors while they are in the range 3.79–5.38 at M_3, and 3.89–4.50 at M_4 receptors. Efficacy variations (Table 2) are quite large at M_2 receptors (a 1087-fold ratio between compounds **9** and **13**) while they are modest (42- and 35-fold) at M_3 and M_4 receptors, respectively. These results suggest that a similar trend in the behaviour of potency exists between M_1 and M_3 subtypes where the substitution of hydrogen with a long side-chain or bulky group generally induces a decrease in potency. On the contrary, a general increase in potency is obtained with the same substitution at the M_2 subtype, this fact suggesting different steric requirements between subtypes M_1 and M_3, on the one hand, and M_2 on the other. No sound relations are possible in the case of the M_4 subtype. An analysis of the affinity and efficacy values of the agonists at the four muscarinic receptors can give useful information about the recognition site (affinity) and the activation site (efficacy) of the receptor subtypes. Compounds **6**, **12**, and **13** show the highest affinities at M_2 subtype, the affinity of compound **13** is again the highest at M_3 subtype, while no useful indication is evident at the M_4 one. As far as efficacy is concerned, compounds **9** and **16** at M_2 subtype, compounds **2** and **12** at M_3, subtype and compound **13** at the M_4 one, are the most efficacious muscarinic agonists. Moreover, this parameter is much higher at M_2 subytpe on average. These data seem to suggest the the oxime derivatives in general maintain a good fit for the recognition site of the M_2, M_3, and M_4 subtypes, with a slight preference for the first one. This preference is definitely more pronounced for M_2 receptors when efficacy is considered. In fact, our agonists display more modest efficacy at M_3 receptors, while some ligands even behave as antagonists (no efficacy) at M_4 subtype. As a consequence, the activation site of M_2 receptors can be easily fitted by this series of agonists, among which the cyclic derivative **9** and the bulky aromatic derivative **16** occupy a prominent position. When tissue selectivity is considered, differences in potency on different tissues are not sufficient to differentiate among receptors because of the presence of spare receptors and/or possible differences in receptor-effector coupling in the various tissues. In this respect, Furchgott suggested that differences in pK_D of at least 0.5 may be indicative of receptor heterogeneity [32]. A horizontal analysis of the data in Table 2 evidences few compounds with significative differences in affinity and efficacy values. Among them compound **4** shows a 25-fold higher affinity and a 142-fold lower efficacy when moving from M_2 to M_3 receptors. This is the only full agonist of this study with a preference for the receptor recognition site of the M_3 subtype, while, at M_4 receptors, it behaves as a partial agonist ($\alpha = 0.23$). Compound **12** shows the most interesting behaviour since this agonist is lacking in activity at M_1 and M_4 receptors while it displays a full agonist activity at M_2 and M_3 ones. A 39-fold higher affinity at M_2 receptors in comparison with the M_3 ones makes this ligand an M_2 selective muscarinic agonist which also shows a 58-fold M_2/M_3 potency ratio. These results evidence once again the particular role of the phenyl ring in receptor activation processes and the importance of the muscarinic subsite in subtype discrimination.

Effect of demox on responses mediated by $M_2 - M_4$ muscarinic receptors

The peculiar profile displayed by demox prompted us to further explore its pharmacological effects. Demox was tested *in vitro* on guinea pig heart (force and rate) (M_2), guinea pig ileum and bladder (M_3), and guinea pig lung (M_4). Muscarinic potency and affinity [24–26] (Table 3) were evaluated for this agonist at the three receptor subtypes M_2–M_4: it behaves as a full agonist at M_2, and M_3 (ileum), and as a partial agonist at M_3 (bladder, $\alpha = 0.9$) and M_4 ($\alpha = 0.4$) subtypes. Two different sets of data for the oxime demox at cardiac M_2 subtype(s) regulating heart force and rate are reported; Figure 2 clearly shows both mono- and biphasic dose-response curves (correlation coefficients > 0.9 and # 1, respectively) at the M_2 subtype(s) regulating heart force (Fig. 2 A) and heart rate (Fig. 2 B). The two sets of potency and affinity values recorded *in vitro* for demox at cardiac M_2 receptors regulating heart force first, and then at those regulating heart rate, were surprising and stimulating at the same time. The measurement of these two parameters were repeated many times, carefully keeping the experimental conditions, e.g. pH and temperature of the physiological solution, constant. On average, 50% monophasic and 50% biphasic curves were obtained. The incidence of the mono- and the biphasic curves was evenly distributed during the experimental period with no variation ascribable to the animal stock under study or to housing conditions. On the other hand, a complete ^{1}H-NMR analysis of demox carried out in the space of eight days at temperatures ranging from − 50 to + 50 °C, in solvents such as D_2O, DMSO, and CD_3OD, showed no isomerization and confirmed the presence only of the E stereochemical configuration of the oxime demox [Piergentili *et al.*, submitted for publication]. The hypothesis that this agonist could recognize two sites at cardiac M_2 receptors at high and low activity and af-

Table 3
Potency[a], affinity[b] and efficacy[c] of compound **1** at M_2, M_3 (guinea pig ileum and bladder) and M_4 muscarinic receptors

	tissue											
	heart force M_2	ileum M_3	bladder M_3	lung M_4	heart force M_2	ileum M_3	bladder M_3	lung M_4	heart force M_2	ileum M_3	bladder M_3	lung M_4
	pD_2[a]	pD_2[a]	pD_2[a]	pD_2[a]	pK_D[b]	pK_D[b]	pK_D[b]	pK_D[b]	$\frac{K_D}{ED_{50}}$[c]	$\frac{K_D}{ED_{50}}$[c]	$\frac{K_D}{ED_{50}}$[c]	$\frac{K_D}{ED_{50}}$[c]
	8.07 (α = 1)[d,e]				5.81[f]				182			
	(7.85-8.29)				(5.61-6.01)							
1		5.97 (α = 1)[d]	4.21 (α = 0.9)[d]	4.87 (α = 0.4)[d]		4.86	3.87[g]	< 5[h]		12.9	2.2	-
		(5.73-6.21)	(3.99-4.43)	(4.51-5.23)		(4.66-5.06)	(3.69-4.05)					
	5.83 (α = 1)[d,i]				3.70[l]				135			
	(5.61-6.05)				(3.42-3.98)							

Mean values and 95% confidence limits are given. Number of observations varies between 6 and 10 (20 and 30 for guinea pig heart). [a] $-\log ED_{50}$. [b] $-\log K_D$. [c] This ratio indicates the efficacy of the agonist. [d] Intrinsic activity, measured by the ratio between the maximum response of **1** and the maximum response of arecaidine propargyl ester (APE) at M_2, M_3, and M_4 subtypes. [e] High activity site. [f] High affinity site. [g] $-\log K_p$. [h] $-\log K_b$. [i] Low activity site. [l] Low affinity site.

finity was a convincing one, particularly when we verified that muscarinic M_1, adenosinic A_1 and β_1-adrenergic receptors were not involved. In fact, 1×10^{-5} M of the A_1 antagonist ANR 106, 3×10^{-7} M of the M_1 antagonist pirenzepine, and 1×10^{-6} M of the β_1-adrenergic antagonist propanolol did not influence the corresponding dose-response curves to demox. The behaviour of this compound which, in the same experimental conditions, is able to display sometimes a monophasic and sometimes a biphasic curve, could depend on individual differences among animals or on the physiological conditions of the individual animal used. Stress due to handling [33] or to social behaviour [34–39] of the animals inside the cages could result in the imbalance of sympathetic and parasympathetic nervous system activation. This could result in a difference in the relative distribution of the M_2 receptors between the plasma membrane and the internal compartment of the cells, or in sensitivity to M_2 stimulation itself [40]. Demox was also tested *in vivo* on pithed rat, and its muscarinic activity at ganglionic M_1 receptors mediating tachycardia and cardiac M_2 receptors mediating bradycardia was evaluated (Fig. 3), according to the method reported by Angeli *et al.* [41]. Analogously to other pentatomic cyclic muscarinic agonists such as muscarine, *cis*-oxathiolane [41], and *cis*- and *trans*-oxathiolane sulfoxide [42], the same dose of demox produces biphasic heart rate responses in this preparation: an initial bradycardia mediated by cardiac M_2 receptors followed by an increase in heart rate due to the stimulation of M_1 receptors. Pirenzepine and tripitramine, selectively blocking M_1 and M_2 receptors, respectively, allow determination of the "pure" M_2 and M_1 responses [9]. An accurate analysis of Fig. 3 clearly shows how the M_2 response is prevalent over the M_1 one: in fact, only after blocking the M_2 receptors with tripitramine is it possible to detect a significant increase in heart rate (M_1 effect) (Fig. 3 A), while blocking the M_1 receptors with pirenzepine has no effect on the already relevant decrease in heart rate (M_2 effect) (Fig. 3 B).

Binding affinity of demox to cloned human muscarinic receptors

Affinity constants at cloned muscarinic receptors were also estimated for demox which displayed a narrow difference in affinity values among the five human muscarinic receptor subtypes, $-\log K_i$ ranging from 4.71 to 5.23. The comparison among these values and the $-\log K_D$ of Table 3 evidences no significant differences in the affinities calculated in the two preparations. Moreover the activity of demox was evaluated on the stimulation of [^{35}S]-GTPγS binding to CHO-K_1 cell membranes containing Hm2 muscarinic receptor (Fig. 4). The results confirm that demox behaves as a full agonist ligand at this subtype when compared with acetylcholine.

Orthosteric and allosteric interaction of demox with heart atrial muscarinic receptors

Binding studies carried out on rat heart atrial homogenates (expressing M_2 muscarinic receptors) indicated that demox has a positive allosteric effect on the binding of [^{3}H]-NMS to cardiac muscarinic receptors in a concentration range of 10^{-11} – 10^{-10} M (Fig. 5). The increase in [^{3}H]-NMS binding (which was present at a subsaturating concentration) induced by 10^{-10} M and 10^{-11} M demox showed variability among the experiments. Additional experiments, performed to make sure that these results were not due to any artefacts, confirmed these data (the experiments were repeated at least 10 times; Fig. 5). When heart atria

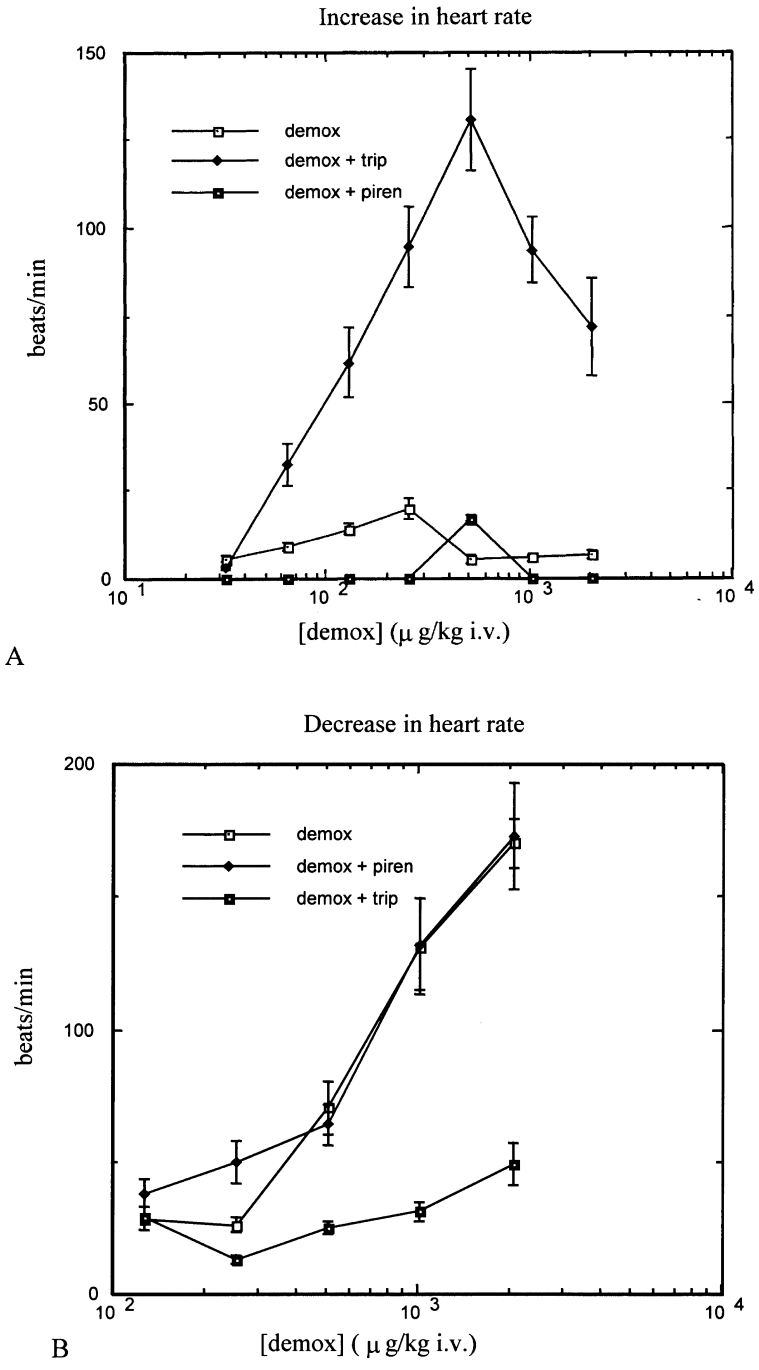


Fig. 3. Panel A. Increase in heart rate caused by demox alone, after pretreatment with tripitramine (30 μg/kg), and pirenzepine (50 μg/kg). Data are presented as means ± SEM (n = 9 for agonist curve; n = 5 for antagonist curves). Panel B. Decrease in heart rate caused by demox alone, after pretreatment with pirenzepine (50 μg/kg), and tripitramine (30 μg/kg). Data are presented as means ± SEM (n = 9 for agonist curve; n = 5 for antagonist curves).

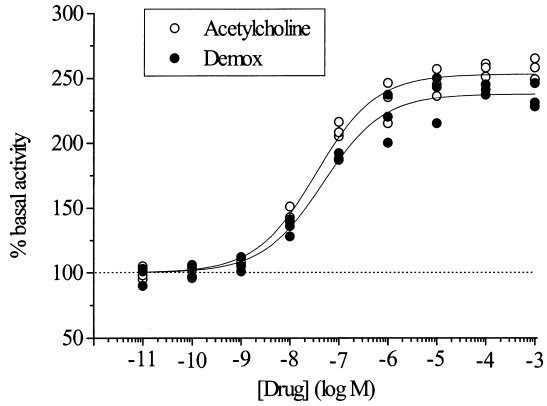


Fig. 4. Stimulation of [^{35}S]-GTPγS binding to CHO-K1 cell membranes containing Hm2 muscarinic receptor by acetylcholine and demox. The data of three experiments are plotted as percentage of basal activity. The points represent the mean of triplicate observations in each experiment. The dotted line shows the basal level of stimulation. The calculated parameters are as follow: acetylcholine $pEC_{50} = 7.47 \pm 0.07$, $E_{max} = 252.9 \pm 3.13$ %; demox $pEC_{50} = 7.33 \pm 0.12$, $E_{max} = 237.6 \pm 1.97$ %.

homogenates were incubated in presence of 2 nM [^{3}H]-NMS (a concentration which saturated M_2 receptors) and increasing concentrations of demox, the positive effect of low concentrations of demox on binding this radioligand was not observed; on the contrary, the amount of [^{3}H]-NMS specifically bound to the membranes was progressively diminished by higher concentrations of demox ($> 3 \times 10^{-7}$ M) (Fig. 5). The K_i value for demox in presence of 300 pM and 2 nM [^{3}H]-NMS was 3.2×10^{-6} M and 5.7×10^{-6} M (mean of at least five different experiments), respectively. Hill slopes were in the range of 0.66 – 0.74, clearly lower than unity (data not shown). Saturation experiments were carried out on heart atria

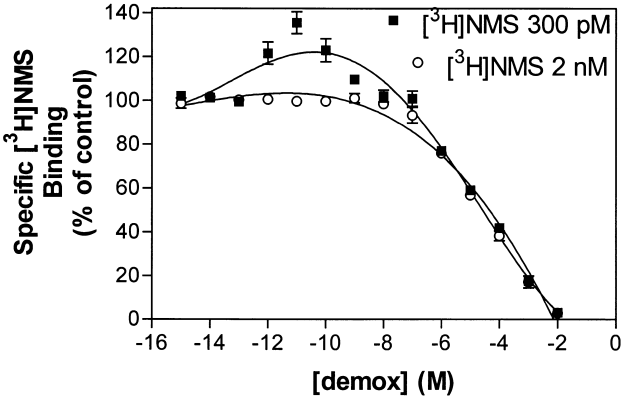


Fig. 5. Effect of demox on the binding of [^{3}H]-NMS in rat heart homogenates. The concentration of [^{3}H]-NMS was 300 pM or 2 nM and the incubation was carried out at 22 °C for 2 h. Values are means ± standard error of ten experiments carried out in duplicate. * $P<0.05$ *vs.* control (Student's t test).

homogenates incubated with increasing concentrations of [^{3}H]-NMS without or with 10^{-11} M demox and showed that B_{max} was not modified (control: 180 ± 20 fmol/mg protein; n=3; demox-treated samples: 166 ± 32 fmol/mg protein; n=3) whereas the K_d of [^{3}H]-NMS was slightly, but not significantly, reduced (control: 420 ± 38 pM; n=3; demox-treated samples: 360 ± 15 pM; n=3; P>0.05, Student's t test). In absence of demox, the binding of 300 pM [^{3}H]-NMS to M_2 receptors proceeded monophasically and reached equilibrium within 90 min (Fig. 6 A). Demox (10^{-11} M) increased the amount of [^{3}H]-NMS bound and reduced the association rate of this radioligand to muscarinic receptors ($T_{1/2}$ = 26.84 min *vs.* 30.30 min mean of at least five different experiments) (Fig. 6 A). When demox was present at higher concentration (3×10^{-5} M), the amount of [^{3}H]-NMS bound to atrial homogenates was reduced by ≈ 50%, (Fig. 6 A). W 84 (3×10^{-5} M), a prototype allosteric modulator of cardiac muscarinic receptors [23], reduced by ≈ 50% the binding of [^{3}H]-NMS to atrial homogenates (Fig. 6 A). The time course of [^{3}H]-NMS dissociation by atrial homogenates in presence of 1 μM atropine was monophasic whether in control conditions or in presence of test compounds. Under control conditions, dissociation of [^{3}H]-NMS was rapid and half-life was $T_{1/2}$ = 6.096 min (mean of three different experiments) (Fig. 6 B). Demox (10^{-11} M) did not modify the [^{3}H]-NMS dissociation rate (Fig. 6 B). On the contrary, in presence of 3×10^{-5} M demox ($T_{1/2}$ = 114.60 min; mean of three experiments), as well as 3×10^{-5} M W 84 ($T_{1/2}$ = 110.60 min; mean of three experiments), [^{3}H]-NMS dissociation was retarded (Fig. 6 B). Demox showed a complex interaction with muscarinic receptors occurring in atrial homogenates. At low concentrations (10^{-11} M and 10^{-10} M), it had a positive effect on the binding of a subsaturating concentration of [^{3}H]-NMS, whereas higher concentrations (300 pM and 2 nM) reduced the binding of this radioligand to muscarinic atrial receptors and slowed down

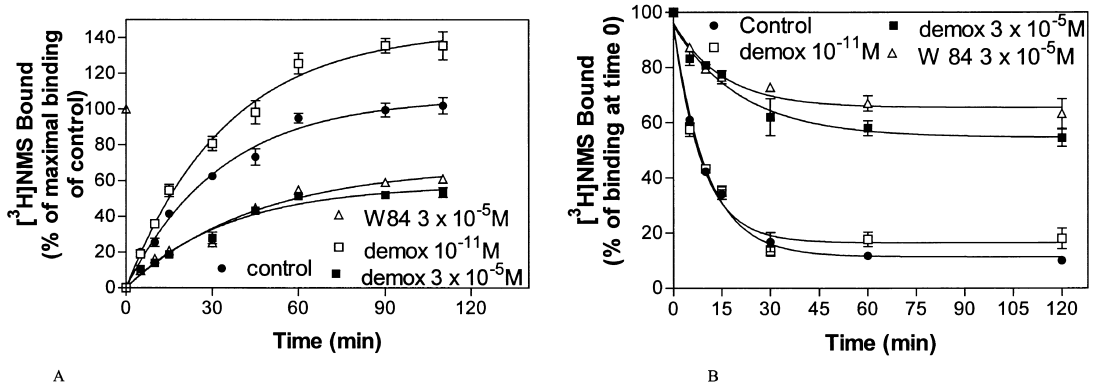


Fig. 6. Panel A. Association of [^{3}H]-NMS (300 pM) at rat atrial membranes under control conditions and in the presence of demox and W 84 (3×10^{-5} M) added to the membranes for 30 min before [^{3}H]-NMS. The data are expressed as percentage of maximal [^{3}H]-NMS binding reached under control conditions and are the mean ± standard error of three experiments performed in duplicate. Panel B. Dissociation of [^{3}H]-NMS (300 pM) from atrial membranes. Membranes were incubated for 30 min in the presence of demox and W 84 (3×10^{-5} M) and for 120 min with the radioligand; then atropine (1 μM) was added (here indicated as time 0). The data are expressed as percentage of [^{3}H]-NMS binding before atropine addition and are the mean ± standard error of three experiments performed in duplicate. See Materials and methods.

the dissociation of [^{3}H]-NMS. Therefore, these data could be explained by assuming that its effect is both competitive and allosteric. The positive effect displayed by low concentrations of demox cannot be clearly explained; however, it could be due to any modulator effect of this compound on muscarinic M_2 receptor conformation, and thus to an increase in the binding of subsaturating amounts of [^{3}H]-NMS. On the contrary, higher concentrations of demox could better stabilize the [^{3}H]-NMS-receptor complex since it is present in an amount sufficient to modulate it allosterically. Consequently, demox binds to free receptors at lower concentrations than it does to receptors occupied by [^{3}H]-NMS, and it displays an apparent biphasic allosteric effect. The positive allosteric effect on muscarinic receptors has been described for alcuronium [22,43] whereas gallamine and W 84 have a negative allosteric effect [23]. In order to clarify the functional behaviour shown by demox at cardiac M_2 receptors and the nature of the sites at high and low activity and affinity, we performed further *in vitro* experiments using the M_2 allosteric antagonist gallamine and the M_2 selective antagonist methoctramine. In fact, in our opinion, the possibility that demox could interact with the allosteric site present at cardiac M_2 receptors, in addition to the classic muscarinic agonist site recognized by acetylcholine, should not be discarded. On the other hand, the ability of some bispyridinium oximes to promote an allosteric action at M_2-cholinoceptors is well known [15–21], as is the possibility for some ligands and small molecules to interact with muscarinic receptors both competitively and allosterically [44,45]. Muscarinic agonists behaving as noncompetitive ligands in the myocardium [46–49], and cardiac tissues having different subpopulations of muscarinic receptors [50–53], have been postulated lately. Moreover, according to recent theories [54,55], allosteric phenomena on G protein-coupled receptors are explained by different receptor-active states promoted by agonist-receptor-G protein interactions which, in turn, can be affected by allosteric modulators. Hearts displaying biphasic dose-response curves were chosen in order to study the shape of the curve and the amount of the shift caused by a 1×10^{-4} M concentration of gallamine. Figure 7, in fact, shows a biphasic curve with a correlation coefficient of 0.862, which undergoes a right-forward shift after treatment with gallamine. This second curve displays a correlation coefficient > 0.9 (0.942) with a shape which is typical of a monophasic dose-response curve. A possible explanation of this behaviour is the ability of demox to bind both to the orthosteric and allosteric sites of the receptor: the occupancy of the allosteric site by gallamine causes demox to bind only to the orthosteric site, thus resulting in a monophasic curve. In our experiments, the value of $-\log K_B$ obtained for gallamine was 7.41, which is significantly higher than the values usually reported (~ 6) in *in vitro* studies when measured with classical muscarinic agonists such as carbachol [56], or in binding studies [57]. Methoctramine was included in this study because of its mixed behaviour; in fact, the binding domain of polymethylene tetraamines, which are long divalent molecules, most probably encompasses the orthosteric and allosteric sites of the receptor [58,59]. With a procedure similar to that used with gallamine, a 3×10^{-8} M concentration of methoctramine was used to shift the biphasic dose-response curve of demox (Fig. 8). After treatment with the antagonist, a new biphasic curve with an upper plateau was obtained. The ability of methoctramine, and possibly of demox, to interact with the orthosteric and allosteric sites of the receptor accounts for the new biphasic curve. Again, as we observed with gallamine, the $-\log K_B$ of methoctramine (9.67) was different from that calculated with classical muscarinic agonists (~ 8.00) [56,58].

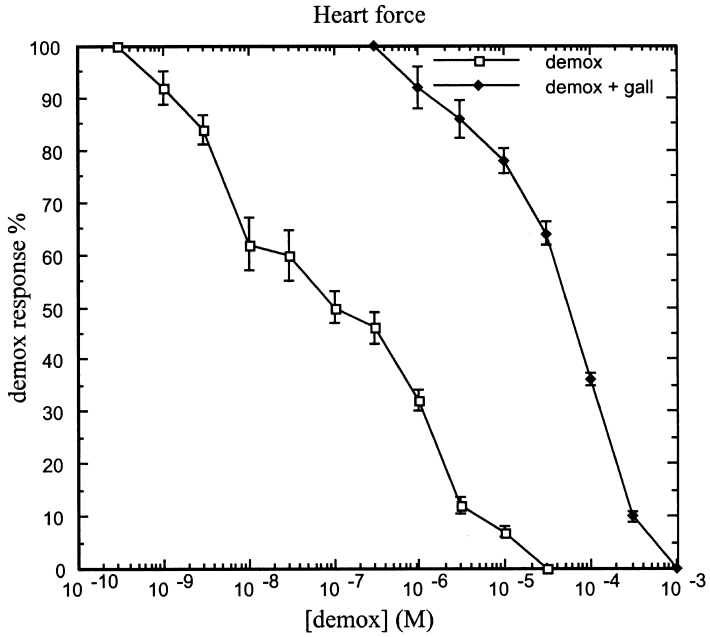


Fig. 7. Experimental dose-response curves of demox in absence (biphasic curve) and in presence (monophasic curve) of 100 μM gallamine. Each point is the mean ± SEM of 4 to 6 observations.

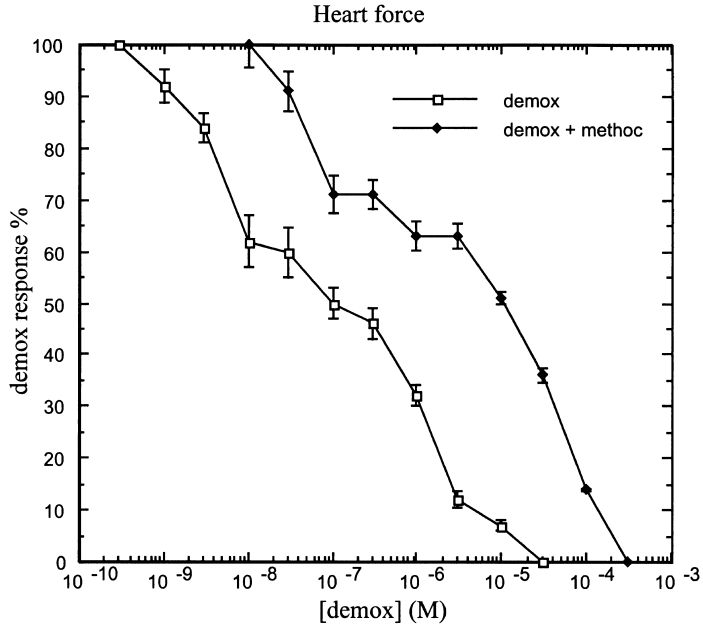


Fig. 8. Experimental dose-response curves of demox in absence and in presence of 0.03 μM methoctramine. Each point is the mean ± SEM of 4 to 6 observations.

Conclusion

Taken together, all these observations seem to indicate that the oxime demox could bind both to the classical agonist site and to the orthosteric and allosteric sites, both present at cardiac muscarinic M_2 receptors. Our working hypothesis seems to be supported by more recent studies with allosteric modulators [60] which suggest the picture of an allosteric binding site close to the orthosteric one, located at a more extracellular level, at the entrance of the ligand binding pocket of the M_2 receptor. Bivalent ligands possessing two positive charges separated by six or seven methylene groups in a distance of 10 Å, and two aromatic skeletons in lateral positions (Fig. 9), seem to be the ideal candidates with the right structural requirements for the best approach to, and binding of, the allosteric site by means of electrostatic and hydrophobic interactions [61]. Nevertheless, the structural similarities between the oxime demox and allosteric modulators such as obidoxime and TMB 4 (Fig. 9), and the presence of only one charge on this agonist, lead us to assume that demox possibly binds only one lateral lipophilic pocket of the allosteric sites present at the cardiac M_2 receptor. The synthesis and the biological and molecular modelling study of the divalent oxime demox, with two inner

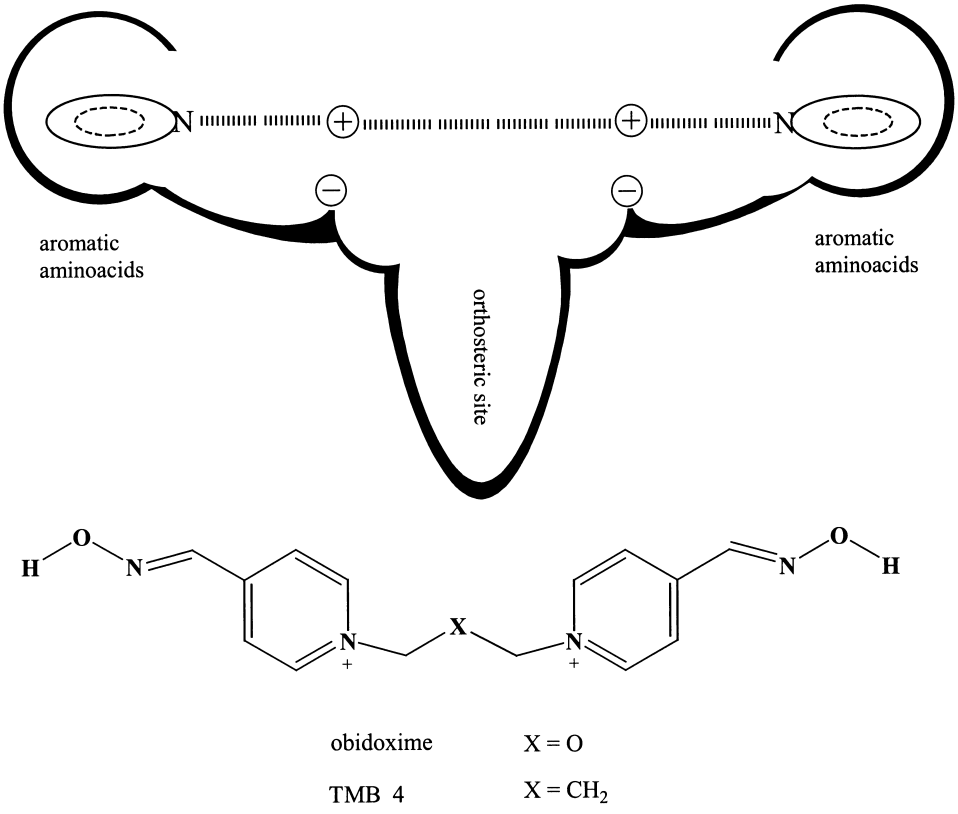


Fig. 9. Schematic representation of the binding area including the orthosteric and the allosteric sites of the recepotor protein.

charged nitrogens separated by three methylene groups, could add decisive evidence to our working hypothesis.

Finally, the present investigation shows that the oxime derivatives of demox are an interesting series of agonists which are able to discriminate among the M_1–M_4 pharmacologically defined subtypes of the muscarinic receptor. In particular, compounds **4** and **12** evidence the importance of the muscarinic subsite and stand out as useful tools in studying subtype discrimination.

Acknowledgments

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