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Determination of the Association and Dissociation Rate Constants of Muscarinic Antagonists on Rat Pancreas: Rank Order of Potency Varies with Time

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SUMMARY

Antagonist binding to rat pancreatic muscarinic receptors was relatively slow at 25° (tracer dissociation half-life, 50 to 60 min). We, therefore, chose this system to investigate the errors induced by nonequilibrium incubations on the estimates of receptor capacity and selectivity, in binding studies. We took advantage of the fact that muscarinic antagonists recognize only one receptor subtype in rat pancreatic homogenates and that association and dissociation kinetics conform to the law of mass action to analyze quantitatively the binding kinetics of [^{3}H]*N*-methylscopolamine and of several unlabeled progressive to these receptors. We observed no correlation between the affinities of drugs for muscarinic receptors and their dissociation rate constants. As a result, the apparent receptor specificity (based on relative affinities for different antagonists) varied markedly with the incubation period. We, therefore, strongly recommend that in general competition curves established for receptor classification should be compared at different incubation periods to ensure that equilibrium is attained. The association rate constants of muscarinic antagonists for rat pancreas receptors were remarkably low, when compared with other ligand-receptor systems. This suggests that the antagonist-pancreatic muscarinic receptor association reaction included a rate-limiting conformational change of the drug-receptor complex. This isomerization step was not directly detectable in our kinetic studies, due to the very low affinity and rapid dissociation rate of the initial nonisomerized complex.

The mathematics of competitive inhibition were first described by Aránvi (1). This author, followed by others (2–4), showed that the IC_{50} of an unlabeled drug may vary with time as binding equilibrium is approached. The variation of IC_{50} with time can be explained intuitively as follows. First, the unlabeled drug binds progressively to the receptor so that the IC_{50} value decreases as equilibrium comes near. This decrease of IC_{50} is counteracted, however, by increasing tracer binding, leading to increasing competitive inhibition of the binding of unlabeled drug. As clearly demonstrated by Motulsky and Mahan (3), this may result in complex variations of IC_{50} with time.

It is generally assumed that k_{off} and K_i values are directly correlated, low affinity drugs having a greater dissociation rate than high affinity drugs. As a result, the time necessary to allow equilibration of the tracer should correspond to the time necessary for equilibration of all competition curves, provided that the tracer is one of the drugs with highest affinity (3). As demonstrated by Motulsky and Mahan (3), for systems behaving according to the law of mass action, incubation intervals of about 5 $t_{1/2}$ (dissociation half-lives) are necessary to allow the tracer to reach equilibrium.

Some systems, like cardiac muscarinic receptors, equilibrate rapidly ([^{3}H]NMS has a $t_{1/2}$ of 1.5 min at 25°) (5). Tracers with very slow dissociation rates (necessitating very long incubations) are by no means exceptional. Another muscarinic antagonist, [^{3}H]quinuclidinyl benzilate, has a $t_{1/2}$ of the order of 10 hr in rat brain preparations (6). $t_{1/2}$ values ranging between 6 min and 2 hr are observed for various β-adrenergic antagonists at 10° (7), whereas Bürgisser *et al.* (8) found $t_{1/2}$ of 5.5 and 4.5 hr, respectively, for two other β-adrenergic antagonists (iodohydroxypindolol and carazolol) at 25°. [^{3}H]Etorphine dissociates from one subtype of opiate receptors with a $t_{1/2}$ of 70 min at 37° (9). Dissociation of ^{125}I-insulin (at 15°) and ^{125}I-human growth hormone (at 30°) from their respective receptors on IM-9 cells is biphasic, with a $t_{1/2}$ just over 30 min (10). ^{125}I-vasoactive intestinal peptide dissociation from pancreatic acinar cells is temperature dependent, with a $t_{1/2}$ of about 40 min at 37° (11).

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ABBREVIATIONS: [^{3}H]NMS, 1-[*N-methyl*-^{3}H]scopolamine methyl chloride; IC_{50}, concentration of unlabeled drug necessary to inhibit 50% of tracer binding; K_D, concentration necessary for 50% receptor occupancy; k_{on}, association rate constant; k_{off}, dissociation rate constant; AF-DX 116, 11-{[2-[(diethylamino)methyl]-1-piperidinyl}acetyl)-5-11-dihydro-6*H*-pyrido(2,3-b)(1,4)benzodiazepin-6-on; K_i, unlabeled drug dissociation constant; SOS, sum of squares.

Because it is not always possible to allow prolonged incubation periods (particularly when the system investigated is susceptible to degradation), we thought that it would be generally interesting to study the effect of incubation time on the apparent receptor specificity, in a slowly equilibrating system. We chose rat pancreatic muscarinic receptors for this study, because preliminary experiments (5) demonstrated that 1) rat pancreatic receptors are homogeneous, 2) tracer association and dissociation kinetics are in full agreement with the law of mass action, 3) tracer and muscarinic receptors are stable under our incubation conditions, and 4) the time scale of [^{3}H]NMS binding makes competition curves at, as well as far from, equilibrium equally easy to investigate.

This allowed us to use the equations described by Motulsky and Mahan (3) to estimate the kinetic constants of various muscarinic antagonists and to test the hypothesis that their association rate constants (k_{on}) were identical. We will see that this was not the case.

Materials and Methods

Chemicals

[^{3}H]NMS (72 Ci/mmol) was obtained from Amersham International (Bucks, England). Atropine, scopolamine, trihexyphenidyl, bacitracin, and polyethylenimine were obtained from Sigma Chemical Co. (St. Louis, MO). The following drugs were gifts: pirenzepine from Dr. R. Hammer (Institute di Angeli, Milano, Italy), dicyclomine from Merrell-Dow (Brussels, Belgium), dexetimide from Janssen Pharmaceutica (Beerse, Belgium), and AF-DX 116 from Dr. K. Thomae GmbH (Biberach, FRG). Trasylol (apronitin) was from Bayer (Brussels, Belgium).

Methods

Male Wistar albino rats (200–250 g) were killed by decapitation and the pancreas was immediately removed.

The pancreases were minced with scissors and homogenized in 8 ml/pancreas of 300 mM sucrose that contained 0.2 mg/ml bacitracin and 500 kallikrein inhibitor units/ml Trasylol, using a glass-Teflon homogenizer (seven up and down strokes). The resulting homogenate was filtered through two layers of medical gauze and immediately diluted 11-fold with the incubation buffer. One milliliter of the diluted homogenate (800 to 1000 μg of protein/assay) was added to 200 μl of [^{3}H]NMS and unlabeled drug (in water) to obtain final concentrations of 50 mM sodium phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 1% bovine serum albumin, 0.2 mg/ml bacitracin, and 500 kallikrein inhibitor units/ml Trasylol.

The [^{3}H]NMS-binding capacity of the pancreas homogenate prepared as described above was unchanged after a 4-hr preincubation at 25°. The total receptor concentration in the assay was 30 to 40 pM (=50 fmol/mg of protein).

[^{3}H]NMS binding in the presence of 1 μM atropine was defined as nonspecific and was subtracted from all other determinations.

To terminate the incubation, we added 2 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.4) to each sample, followed by immediate filtration on GF/C glass fiber filters (Whatman, Maidstone, England) that were presoaked overnight in 0.05% polyethylenimine. The filters were rinsed three times with the same phosphate buffer and dried and the radioactivity was counted by liquid scintillation.

[^{3}H]NMS association kinetics. The [^{3}H]NMS association was measured at seven tracer concentrations (from 0.025 to 1.6 nM), after incubation periods of 5, 10, 20, 40, 60, 120, or 240 min.

Competition kinetics. [^{3}H]NMS (1.6 nM) and the indicated unlabeled drug concentrations were added simultaneously to rat pancreas homogenates, and [^{3}H]NMS specific binding was measured after a 5-min, 10-min, 20-min, 40-min, 1-hr, 2-hr, or 4-hr incubation period at 25°. We used three drug concentrations per log (concentration), so that each competition curve was defined by seven or eight points.

All competition curves were repeated at least three times, in duplicate. The standard deviation of repeated percentage of [^{3}H]NMS bound measurements was typically ±5%, corresponding to a ±26% variation of the IC_{50} values.

Analysis. We assumed throughout this work that binding of [^{3}H]NMS and of unlabeled drugs to pancreas muscarinic receptors is a reversible, one-step reaction and that [^{3}H]NMS and drug binding is mutually exclusive:

$$L + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} RL$$

$$I + R \underset{k_4}{\overset{k_3}{\rightleftharpoons}} RI$$

so that

$$\frac{d(RL)}{dt} = k_{on}R\cdot L - k_{off}(RL) \quad (1)$$

$$\frac{d(RI)}{dt} = k_3R\cdot I - k_4(RI) \quad (2)$$

$$R_o = R + (RL) + (RI) \quad (3)$$

For [^{3}H]NMS association kinetics experiments, up to 25% of the added [^{3}H]NMS was bound to muscarinic receptors at equilibrium, at the lowest tracer concentration used. We, therefore, chose to use Eq. 4 to calculate the k_{on} values:

$$\ln\left[\frac{B_e[L - (B\cdot B_e/R_o)]}{L[B_e - B]}\right] = \left[k_{on}t\left(\frac{L\cdot R_o}{B_e} - B_e\right)\right] \quad (4)$$

with L = total tracer concentration; B_e = tracer binding at equilibrium; B = tracer binding at time t; t = incubation period; R_o = total receptor concentration; and k_{on} = association rate constant.

The [^{3}H]NMS dissociation rate constant was calculated from independent experiments, using Eq. 6.

To analyze the competition kinetics of unlabeled drugs, we chose a large (1.6 nM) tracer concentration. Under these conditions, tracer and drug binding was negligible as compared with the added concentrations. We, therefore, used Eq. 5 to describe [^{3}H]NMS binding in the absence of unlabeled drug and Eq. 6 to describe [^{3}H]NMS binding in the presence of unlabeled drug (from Ref. 3).

$$B = \frac{R_o k_{on} L}{K_A}(1 - e^{-K_A t}) \quad (5)$$

$$B = \frac{R_o k_{on} L}{K_F - K_S}\left[\frac{k_4(K_F - K_S)}{K_F K_S} + \frac{(k_4 - K_F)}{K_F}e^{-K_F t} - \frac{(k_4 - K_S)}{K_S}e^{-K_S t}\right] \quad (6)$$

using the following new variables: k_3 = unlabeled drug association rate constant; k_4 = unlabeled drug dissociation rate constant; I = unlabeled drug concentration; $K_A = k_{on}L + k_{off}$; $K_B = k_3I + k_4$;

$$K_F = \tfrac{1}{2}[K_A + K_B + \sqrt{[(K_A - K_B)^2 + 4k_{on}k_3L\cdot I]}],$$

and

$$K_S = \tfrac{1}{2}[K_A + K_B - \sqrt{[(K_A - K_B)^2 + 4k_{on}k_3L\cdot I]}]$$

Statistics. The kinetic constants of the labeled drug ([^{3}H]NMS) used for curve fitting were determined in independent experiments (see above).

To determine the kinetic constants of the unlabeled drugs, we used a spreadsheet to calculate simultaneously the seven competition curves expected for a given set of k_3 and k_4 values, at the same drug concentrations and seven incubation intervals, as in the experiment. The sum of squared differences between the "theoretical" (calculated) and "experimental" (average of three determinations) values (hereafter called SOS) was used as an index for the quality of the curve fitting.

We first calculated the expected competition curves with k_3 = 0.12 $nM^{-1}min^{-1}$ (as for [^{3}H]NMS) and $k_4 = K_i\cdot k_3$ [K_i was determined with

the Cheng and Prusoff equation (12), after a 4-hr incubation, as shown in Table 1]. We then modified, stepwise, k_3 and k_4 in order to decrease the SOS, while maintaining a constant $K_i = k_4/k_3$. When a minimum SOS was obtained by this procedure, we attempted to further decrease it by alternatively modifying k_4 and k_3 (allowing K_i variations).

Independent fitting by two people gave very similar k_3 and k_4 values for the "slowest" drugs. The best fit constants obtained for trihexyphenidyl and AF-DX 116 were far less reproducible. This was due to the fact that 3- to 10-fold modifications of the assumed k_3 and k_4 values affected very little the theoretical competition curves when k_4 was large.

To obtain a quantitative estimate of the precision of the curve fitting, we increased or decreased the k_3 and k_4 values (maintaining K_i constant) in order to obtain a SOS (at one drug concentration and seven incubation intervals) equal to 18 times the experimental standard error at the same drug concentration (the drug concentration chosen corresponded approximately to the IC_{50} at 5 min). This SOS corresponds to an F ratio of 3, indicating that, given these "true" k_3 and k_4 values, the probability of observing our set of experimental curves was only 5%. We also checked that the 95% confidence intervals of k_3 and k_4 were indeed smaller if K_i variations were allowed.

Results

Effect of time on tracer binding. [^{3}H]NMS binding was concentration and time dependent. At the highest concentration used in this work (1.6 nM), apparent equilibrium was reached within 20 min of incubation; progressively greater incubation periods were necessary at lower tracer concentrations (Fig. 1, *upper*). Because we had measured [^{3}H]NMS (at each time) over a large range of tracer concentrations, we were able to analyze the variation of [^{3}H]NMS saturation curves with time. As shown in Fig. 1 (*lower*), the results were compatible with linear Scatchard plots at all time points. The total concentration of receptors was overestimated by about 30% at 5 min, a minimum of 20 min of incubation being necessary to obtain a correct estimate of the receptor concentration (Fig. 1, *lower*). The apparent K_D value of [^{3}H]NMS decreased with time, as shown in Table 1 and Fig. 1 (*lower*).

Analysis of saturation curves at different time points allowed us to calculate the following kinetic constants for [^{3}H]NMS: $k_{on} = 0.12$ nM^{-1}min^{-1}; $k_{off} = 1.210^{-2}$min^{-1}; and $K_D = 100$ pM.

Effect of time on competition curves. Competition curves using the six unlabeled muscarinic antagonists indicated in Table 1 were obtained after different incubation intervals, at a high tracer concentration (1.6 nM). As shown in Fig. 2, the IC_{50} value of all antagonists tested varied with time. The Hill coefficients (data not shown) of competition curves varied between 1.25 and 1.0 at short time points and were equal to 1.0 after 4 hr of incubation (except for dexetimide: $n_H = 1.15$). The SD of n_H was typically 0.05 and was always below 0.1. We calculated the K_i values according to the method of Cheng and Prusoff (12) (i.e., assuming competitive binding) using the IC_{50} values of the unlabeled drugs and the [^{3}H]NMS K_D values obtained at each time. As shown in Table 1, the values varied markedly with time.

All competition curves could be adequately described by the Motulsky and Mahan equations (Eqs. 5 and 6) (3), suggesting that unlabeled drug binding, like tracer binding, could be described by the law of mass action (Eqs. 1 to 3). This is illustrated in Fig. 3 by comparison of experimental data points and fitted curves for dexetimide and trihexyphenidyl (respectively the slowest and fastest drugs for which kinetic constants could be defined). The estimated k_3 and k_4 values and their 95% confidence intervals are shown in Table 2.

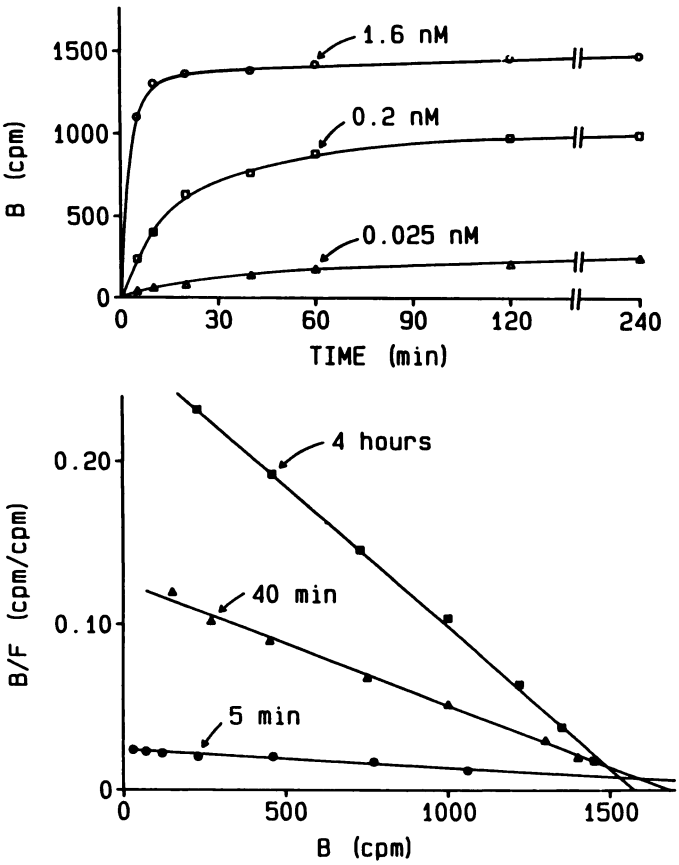

Fig. 1. *Upper*, Specific [^{3}H]NMS to rat pancreatic homogenates was measured as a function of time at three tracer concentrations, 0.025 nM ($K_D/4$) (△), 0.2 nM (2 K_D) (□), and 1.6 nM (16 K_D) (○). *Lower*, Specific [^{3}H]NMS binding to rat pancreatic homogenates was measured as a function of time at seven tracer concentrations (between 0.025 and 1.6 nM). The results obtained after 5 min (●), 40 min (▲), and 4 hr (■) of incubation are represented according to Scatchard. The data are representative of three experiments performed in duplicate.

Effect of time on apparent receptor selectivity. The apparent receptor selectivity observed after 5 min of incubation at 25° was very different from that observed after 4 hr (Table 1 and Fig. 4). Pancreatic receptors clearly had low affinities for AF-DX 116 and pirenzepine at all times (confirming previous data; Ref. 5); however, the apparent affinity of drugs like [^{3}H]NMS, scopolamine, dexetimide, etc., increased by 10- to 100-fold with prolonged incubation periods (Table 1).

Discussion

Analysis of tracer binding after nonequilibrium incubations. As shown in Fig. 1 (*lower*), the determination of the total binding capacity of rat pancreatic homogenates was not markedly affected by incubation time. In contrast, the tracer affinity was markedly underestimated after short incubation periods; incubations of at least 2 hr (over two $t_{1/2}$) were required to approach the equilibrium K_D value.

As shown in Fig. 1 (*upper*), association kinetics should be used with caution to evaluate the equilibrium incubation time, because the time needed to approach steady state binding varies with tracer concentration.

Motulsky and Mahan (3) estimated that, assuming monoexponential dissociation rates, incubations of at least five tracer half-lives should be performed to approach equilibrium. This

TABLE 1

Apparent [^{3}H]NMS K_D values obtained by Scatchard analysis of saturation curves obtained at various incubation periods with rat pancreatic homogenates. Apparent K_i values of unlabeled drugs were obtained by applying the Cheng and Prusoff equation to competition curves obtained with 1.6 nM [^{3}H]NMS, using the [^{3}H]NMS K_D values obtained after the same incubation periods.

Muscarinic antagonist	K_D						
	5 min	10 min	20 min	40 min	60 min	120 min	240 min
				nM			
[^{3}H]NMS	1.4	0.62	0.33	0.20	0.15	0.13	0.10
Scopolamine	2.5	1.0	0.60	0.40	0.30	0.27	0.24
Dexetimide	20	6	4	2	1.1	0.7	0.4
Dicyclomine	15	11	10	9	9	10	8
Trihexyphenidyl	13	10	10	13	15	20	18
Pirenzepine	350	190	180	200	190	180	190
AF-DX 116	1300	1000	1200	1600	1800	1900	2100

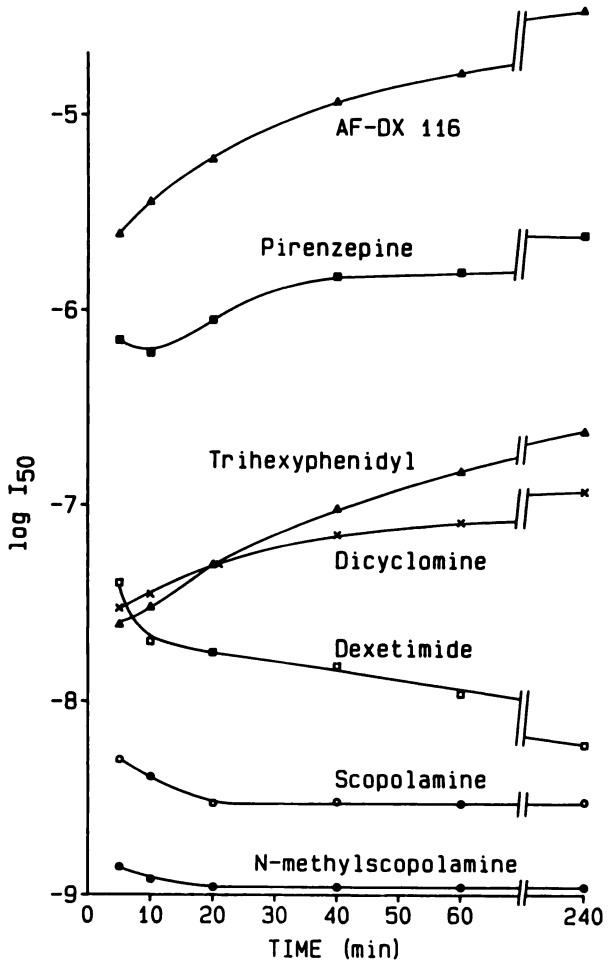

Fig. 2. [^{3}H]NMS binding was measured as a function of time, in the absence or presence of various unlabeled drug concentrations (as shown in Fig. 3). The concentration of unlabeled antagonist inhibiting 1.6 nM [^{3}H]NMS binding by 50% (IC_{50}) was represented, on a log scale, as a function of incubation time, using N-methylscopolamine (unlabeled) (●), scopolamine (○), dexetimide (□), dicyclomine (×), trihexyphenidyl (▲), pirenzepine (■), and AF-DX 116 (△). The data are representative of three to five experiments performed in duplicate.

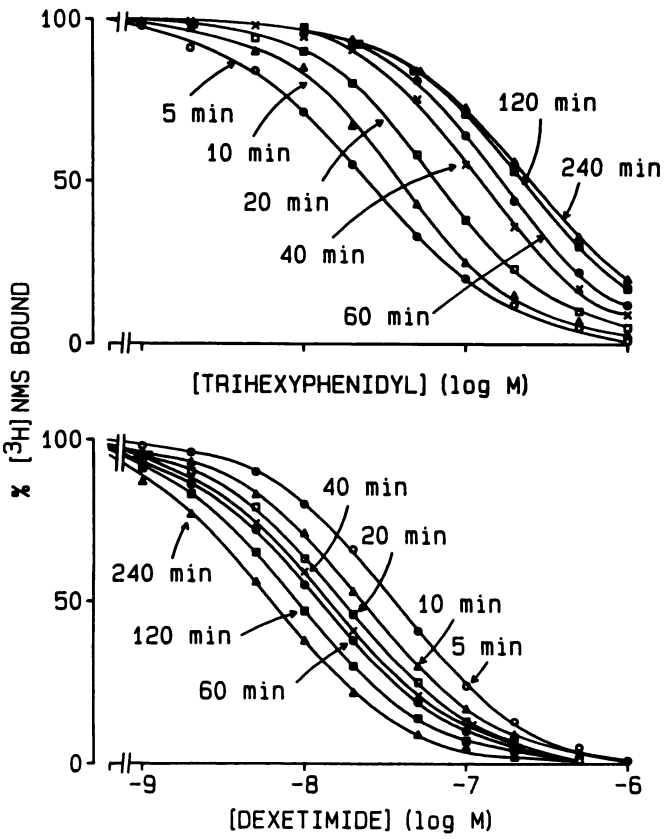

Fig. 3. [^{3}H]NMS was measured in the absence or presence of the indicated concentrations of trihexyphenidyl (*upper*) or dexetimide (*lower*) after 5 (○), 10 (△), 20 (□), 40 (×), 60 (●), 120 (■), or 240 min (▲) of incubation. The experimental results are compared with fitted curves (——), using the equations published by Motulsky and Mahan (3) and the corresponding rate constants are indicated in Table 2. Averages of three to five experiments performed in duplicate are shown.

can be extended to any drug-receptor interaction as follows. To ensure that the system is as close as possible to true equilibrium, tracer binding should be measured at a very low drug concentration. Because, by definition, the dissociation kinetics measure (re)equilibration of tracer binding at a zero tracer or receptor concentration, the time interval necessary to obtain for example 97% of tracer dissociation is equal to the time interval necessary to reach 97% of equilibrium binding, at an infinitely low tracer concentration. At finite tracer concentrations, binding will be ≥97% of true equilibrium.

Analysis of unlabeled drug binding after nonequilibrium incubations. The unlabeled drug apparent affinities demonstrated variable time dependence (see Fig. 2). The "receptor selectivity" observed after 5-min and 4-hr incubation (Fig. 4) was in fact so different that, under different circumstances, these results might be taken as supporting the existence of different receptor subtypes.

TABLE 2

Association (k_3) and dissociation (k_4) rate constants and equilibrium dissociation constant (K_i) of seven muscarinic antagonists estimated by analysis of three competition curves obtained after different incubation periods of rat pancreatic homogenates

The 95% confidence intervals of the kinetic constants are indicated in parentheses under each best fit value.

Muscarinic antagonist	k_3	k_4	K_i
	$M^{-1}\ sec^{-1}$	sec^{-1}	nM
NMS	3.3×10^8 (3.1×10^8–4.8×10^8)	2.0×10^{-4} (1.9×10^{-4}–2.9×10^{-4})	0.06
Scopolamine	1.25×10^8 (0.8×10^8–1.9×10^8)	2.5×10^{-4} (1.7×10^{-4}–3.7×10^{-4})	0.20
Dexetimide	1.83×10^5 (1.4×10^5–2.3×10^5)	4.2×10^{-5} (3.2×10^{-5}–5.2×10^{-5})	0.23
Dicyclomine	2.5×10^5 (1.0×10^5–1.0×10^6)	2.0×10^{-3} (8×10^{-4}–8×10^{-3})	8.00
Trihexyphenidyl	2.5×10^6 (2.5×10^5–large)*	3.83×10^{-2} (3.8×10^{-3}–large)	15.00
Pirenzepine	1.0×10^4 (0.7×10^4–2.0×10^4)	1.4×10^{-3} (1.0×10^{-3}–2.9×10^{-3})	140
AF-DX 116	$\gg 1.0 \times 10^5$ (9.3×10^3–large)	$\gg 0.17$ (1.7×10^{-2}–large)	1800

* Large, when k_4 is equal to or above 0.17 sec^{-1}, the expected competition curves after incubation intervals of 5 min or greater are almost independent of the k_3 and k_4 values chosen. It is, therefore, not possible to determine an upper limit for the 95% confidence intervals of k_3 and k_4.

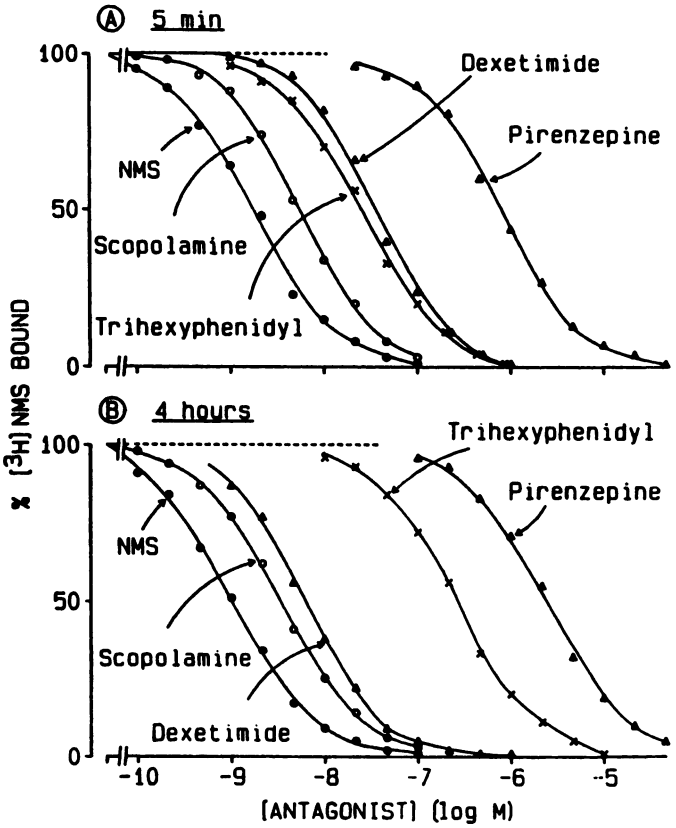

Fig. 4. Binding of 1.6 nM [^{3}H]NMS was measured after 5 min (A) or 4 hr (B) of incubation, in the absence or presence of the indicated concentrations of unlabeled *N*-methylscopolamine (*NMS*) (●), scopolamine (○), trihexyphenidyl (×), dexetimide (▲), and pirenzepine (△). Averages of three to five experiments performed in duplicate are shown.

We attempted in Table 1 to take into account the competitive inhibition of unlabeled drug binding by [^{3}H]NMS, by using the Cheng and Prusoff equation (12). This did not give satisfactory results; the K_i values of most drugs were under- or overestimated after short incubations. Furthermore, we found no correlation between the equilibrium K_i value and the error made by analysis of nonequilibrium binding.

Fitting of the competition curves obtained at all time points with the Motulsky and Mahan equations was very satisfactory (see Figs. 3 and 4 for a few examples). This suggested that rat pancreatic muscarinic receptors bind the antagonist reversibly, with kinetics in agreement with the law of mass action. Evaluation of the unlabeled drug dissociation rate constants obtained with this model allowed us to understand the variations of K_i values with time observed in pancreatic homogenates (Table 1).

1) Trihexyphenidyl and AF-DX 116 had $t_{1/2}$ values of, respectively, 20 and $\ll$1 sec. They equilibrated very rapidly with muscarinic receptors, thereby preventing [^{3}H]NMS from recognizing these sites. The Cheng and Prusoff correction (which assumes equilibrium binding) was, therefore, overevaluated at early time points ($\leq$40 min), and their affinities were overestimated.

2) Dicyclomine and pirenzepine had $t_{1/2}$ values of approximately 6 and 8 min, respectively. They were unable to equilibrate in 5 min (the earliest time point studied) and their affinity was underestimated at this time point. The K_i values obtained after 10- and 20 min-incubations, respectively, were almost correct.

3) Longer incubations were necessary to obtain acceptable evaluations of the K_i values for scopolamine ($t_{1/2}$ of 46 min), NMS ($t_{1/2}$ of 58 min), and dexetimide ($t_{1/2}$ of 280 min).

The time necessary to obtain acceptable K_i estimates was, thus, related to the unlabeled drug dissociation rates, with the fastest and slowest drugs necessitating the longest incubations. The unlabeled drug k_{off} values were, however, *not* correlated with their K_i values (Fig. 5). The variation of the k_{on} values (shown in Fig. 5) explained the remarkable difference between the drug binding patterns observed after 5-min and equilibrium incubations.

The calculated k_{on} values obtained in this work for muscarinic antagonists binding to rat pancreatic receptors were far lower than expected for a diffusion-controlled bimolecular reaction. This is probably not explained by diffusion barriers, such as sealed vesicles containing the muscarinic binding site, because 1) the hydrophilic, quaternary ammonium, drug NMS had a greater k_{on} value than the hydrophobic, tertiary amino, drug dexetimide and 2) it is generally admitted that [^{3}H]NMS labels only the "accessible" muscarinic receptors as opposed to receptors in an hydrophobic environment (13). We, therefore, suspect that the muscarinic association reaction in pancreas was far more complex than just collision of drug and receptor and the immediate formation of a stable complex.

Higher concentrations of allosteric drugs like gallamine or *d*-tubocurarine are necessary to inhibit tracer dissociation, as compared with tracer association, in pancreas[1] as in heart and brain (14, 15). This suggests that the conformation of the allosteric site changes after drug binding (14), i.e., that antagonists (like agonists) change the receptor conformation upon

[1] M. Waelbroeck, J. Camus, M. Tastenoy, and J. Cristophe. Unpublished data.

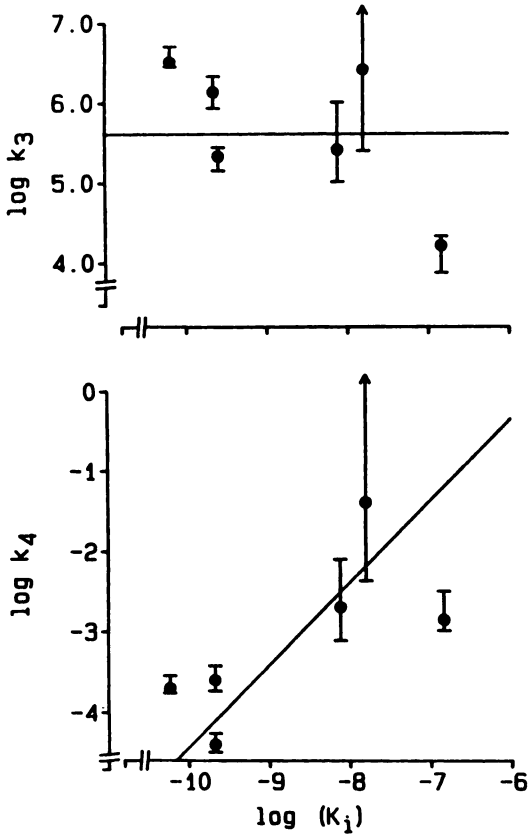

Fig. 5. *Upper*, The k_3 values shown in Table 2 and their confidence intervals were plotted as a function of K_i on a log/log scale. *Lower*, the k_4 values shown in Table 2 and their confidence intervals were plotted as a function of K_i on a log/log scale. AF-DX 116 was omitted from both graphs because we could not obtain an estimate of k_3 and k_4. The *lines* drawn in both panels correspond to the hypothesis that k_3 was the same for all drugs (average, 4.1×10^6 M^{-1} sec^{-1}), with k_4 being directly proportional to K_i.

binding. We, therefore, suspect that the muscarinic antagonist-receptor binding reaction was controlled by a rate-limiting step involving receptor isomerization.

It is sometimes possible to demonstrate experimentally that drug-receptor binding is a two-step reaction, described by:

$$L + R \underset{K_D}{\leftrightarrow} LR \overset{k_i}{\underset{k_{-i}}{\rightleftharpoons}} LR \tag{7}$$

1) Isomerization of the drug-receptor complex is often detected by biphasic dissociation kinetics, reflecting fast dissociation of LR and slow dissociation of LR' (16).

2) In some cases, the association reaction is also biphasic (16). In other systems, the initial binding reaction is too fast to be measurable, but formation of LR' is proportional to the concentration of LR, not of L. Deviations from the one-step model can then be observed at very high L concentrations (above K_D), because of progressive saturation of LR (see for example Ref. 17). The association kinetics are then described by Eq. 8:

$$\ln\left(\frac{B_{eq} - B_t}{B_{eq}}\right) = -\left[\left(\frac{k_i}{K_D + L}\right) \times L + k_{-i}\right]t \tag{8}$$

However, 1) if the affinity of the initial complex LR is very low ($K_D \gg L$), and 2) if $K_{iso} = k_{-i} = k_{-i}/k_i \ll 1$, the association rate equation becomes:

$$\ln\left(\frac{B_{eq} - B_t}{B_{eq}}\right) = -\left[\left(\frac{k_i}{K_D}\right) L + k_{-i}\right]t \tag{9}$$

The dissociation rate equation is:

$$B_t = B_{eq} e^{k_{-i}t} \tag{10}$$

The equilibrium dissociation constant $K = \frac{K_D K_{iso}}{K_{iso} + 1}$, with $K_{iso} = k_{-i}/k_i$, becomes equal to $K_D K_{iso}$.

Under these conditions, receptor isomerization cannot be detected by kinetic studies.

Experimentally, 1) we were unable to detect variations of the [^{3}H]NMS k_{on} with tracer concentrations in the concentration range investigated; 2) its dissociation kinetics were monoexponential; 3) the equilibrium dissociation constant K_D for [^{3}H]NMS was equal to its k_{off}/k_{on} ratio; and 4) the competition curves obtained with several muscarinic antagonists, after varying incubation periods, could be fitted with the Motulsky and Mahan equations (3) without difficulty (see Fig. 3). Taken together, these results are compatible with a one-step reaction.

However, the association rate constants were low. As discussed above, this indicates that the initial (collision) complex formed had a very low affinity and that the association (and dissociation) rates of the drugs were dominated by an isomerization reaction.

Conclusion. As previously demonstrated by others on theoretical grounds or practically on isolated organs or membranes (1–4), we confirmed that the apparent affinity of unlabeled drugs may be either over- or underestimated if nonequilibrium incubations are performed. The minimum time necessary to obtain acceptable estimates of the K_i values depends on the relative tracer and unlabeled drug dissociation rates and is greatest with very slowly and very rapidly dissociating drugs. In the case of rat pancreatic muscarinic receptors, there was a large variation of the association rate constants among muscarinic antagonists; the effect of nonequilibrium incubations on the apparent K_i value of each drug was, therefore, unpredictable.

In practice, to determine the incubation period necessary for equilibrium incubations, we strongly recommend that the tracer dissociation rather than association kinetics be investigated. We also suggest that the competition curves obtained with those drugs that are used for receptor classification should be compared at two incubation times, one equal to 4× the other, to ensure that equilibrium K_i values are obtained.

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