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Parameters of drug antagonism: re-examination of two modes of functional competitive drug antagonism on intraocular muscles

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Abstract

There are two distinct kinetic functional pharmacological procedures by which the equilibrium affinity constant, K_B, of a competitive reversible blocker is obtained. The classical method on an organ system requires the study of the parallel displacement of the agonist concentration–response curve in the presence of the blocker. In the second method, the agonist-evoked functional mechanical response is reduced to half by the blocker IC50 (the concentration required for 50% inhibition). In relation to these parameters the role of the ionization constant pK_a and liposolubility log P_c or log D of blockers was examined. On the ciliary muscle from human eye, IC50/K_B ratios for (±)-atropine, its quaternary analogue (±)-methylatropine, (–)-scopolamine, (±)-cyclopentolate, (–)-tropicamide, (±)-oxybutynin and pirenzepine were 15, 23, 4.4, 2.6, 1.66, 1.46 and 1.71, respectively. The ratios on the iris sphincter were comparable with those of ciliary muscle. When compared with large proportions of ionized molecules with water soluble properties of (±)-atropine and (±)-methylatropine, relatively high amounts of un-ionized and/or with greater partitioning of all other blockers in the lipoid barrier co-related well to low IC50/K_B ratios, as predicted by the classical theory of competitive drug antagonism. It was hypothesized that due to receptor biophase access, the reduction of the mechanical response of the agonist by the highly ionized water-soluble antagonist at IC50 represented time-distorted ''pseudo-equilibrium'' estimation, where a higher concentration of the blocker was needed. On the other cholinergic effectors, like that of rat anococcygeus muscle or frog rectus abdominus muscle, IC50/K_B ratios of respective blockers atropine or (+)-tubocurarine and hexamethonium were close to 1. Thus physicochemical properties, which affect the distribution coefficient log D and the tissue morphology (where asymmetric distribution of receptors may occur), appeared to be a critical factor in the analysis of the affinity parameters of the competitive reversible blocker. On the intraocular muscles, two functional pharmacological procedures for obtaining K_B and IC50 values were not kinetically equivalent.

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Introduction

The utility of enzyme substrate kinetics as a model system for estimating affinity of drugs to the membrane receptors may be misleading. This is because enzymes in solution split the substrate whereas agonists activate receptor-mediated unidirectional cascade of intracellular events leading to the mechanical effect (Bowman & Rand 1980). Although both the product of enzyme substrate interaction and the mechanical effects mediated by the agonist-activated receptor in the organ system can be accurately measured, these events based on K_m and EC50 (the molar concentration required to produce 50% of the maximum possible response for the agonist) are not kinetically equivalent. For the highly potent agonist due to the presence of spare receptors, total saturation of the receptors in a tissue may not be required for production of maximum effect. Under these conditions, the EC50 parameter does not accurately represent the affinity of the agonist for the receptor (Colquhoun 1998).

The kinetic model for calculation of the affinity of a competitive reversible blocker (K_B) in both systems, however, exhibits remarkable similarity, because the "classical competitive reversible blocker" does not participate in other receptor-activated events except the interaction at the active site. A substrate or an agonist follows the laws of

mass action for the competition with the blocker at the enzyme or the receptor (Gaddum 1937, 1957; Schild 1947a, b; Ariëns 1954; Furchgott 1964, 1967, 1972).

The potency of a competitive blocker in organs can be calculated by two empirical procedures. In the first procedure the concentration–response curve of an agonist is obtained in the presence of blocker (or antagonist) and from the parallel linear shifts in the curve, the equilibrium affinity constant can be calculated (Furchgott 1967). The agonist presumably competes with the blocker at the site and the agonist-activated intracellular kinetics of events are not influenced by the procedure. The second pharmacological procedure includes reduction of the mechanical response generated by a concentration of an agonist to a half value, IC50, which is close to the K_B value obtained by the other procedure. In IC50 determinations on the organ system the blocker reverses or antagonizes the mechanical effect of the agonist. The steady-state kinetics of the agonist:antagonist interaction at the membrane receptor presumably translates the collective equilibrium of various intracellular processes "instantaneously" for the mechanical effect. If there is no receptor saturation by the agonist in estimation of IC50 of the competitive blocker, the ratio of $IC50/K_B$ approaches 1.

In light of this information, nearly equal K_B values of atropine (~1 nM) have been observed on the human iris sphincter at 37.5 and 17.5°C, by the parallel displacement of the agonist response in the presence of the blocker, but the IC50 values of 20 and 67 nM of the blocker were significantly greater at the two temperatures (Patil & Mauger 1992; Patil 1999, 2001). The agonist concentration used for the blocker IC50 determination was less than 70% of the maximum contraction of the iris sphincter. Transformation of the IC50 of atropine to the K_B of atropine as per the Cheng–Prusoff equation (Cheng & Prusoff 1973) did not change the value corresponding to the affinity constant of ~1 nM, as obtained by the other procedure in which the agonist mainly competed with the blocker at the receptor.

Various methods in homogenates and on organ systems are used for characterizing receptors by studying affinity constants of competitive blockers (Arunlakshana & Schild 1959; Kenakin 1993; Lazareno & Birdsall 1993; Patil 1996). In a given organ, the affinity constant is influenced by the physicochemical properties of the blocker and drug equilibrium in the receptor biophase, the cellular distribution of receptors in the organ, the type of the contraction coupling processes, intracellular calcium homeostasis, pH and temperature. (Furchgott 1964, 1972; Salazar et al 1976; Waud et al 1978; Jarv et al 1980; Barlow & Chan 1982; Motulsky & Mahan 1984; El Tayar et al 1988; Itoh & Kuriyama 1994; Barlow 1995). In a given system, if equilibrium of the agonist:antagonist interaction is instantaneously reflected with the equilibrium of the mechanical response, then the $IC50/K_B$ ratio will be close to 1. Such an idealized, simplistic concept at a single concentration of antagonistic drugs is illustrated in Figure 1. The so-called steady state of the mechanical response, however, may not instantaneously reflect the "invisible" equilibrium of the antagonistic drugs at the membrane receptor. In this situation the IC50 will be greater than K_B, because more intracellular-activated processes will have to be terminated at different rates and higher concentration of the blocker may be needed. Slope changes of the inhibition parameter will occur.

(±)-Atropine, (±)-methylatropine (quaternary atropine), (−)-scopolamine, (±)-cyclopentolate, (−)-tropicamide, (±)-oxybutynin and pirenzepine provide a collection of antimuscarinic drugs with varying ionization constants, pK_a values and liposolubility, log P_c, properties (Barlow & Chan 1982; Hansch 1990; Foye et al 1995) to test the hypothesis that drug distribution in the receptor biophase plays a vital role in estimation of affinity of blockers by two procedures. The study of atropine antagonism was carried out against both exogenous agonist and endogenous acetylcholine, the latter preserved by physostigmine. Human intraocular muscles and a thin and robust smooth muscle preparation, rat anococcygeus tissue, sensitive to muscarinic drugs were used. Frog rectus abdominus skeletal muscle, containing cholinergic nicotinic ion channel receptors, was also used to explain the $IC50/K_B$ ratio paradox of anticholinergic drugs in

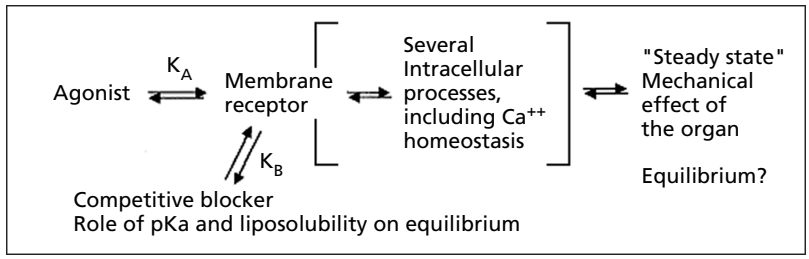

Figure 1 The classical simplistic view of the agonist:antagonist interaction at the membrane receptor, leading to the mechanical functional effect is indicated. The antagonist is evaluated by two procedures. In one, the agonist competes in the presence of the blocker (K_B). In the other, the agonist-induced response is terminated by the blocker (IC50). The latter parameter for water-soluble antagonist may or may not be converted to K_B value. Receptor biophase drug distribution related to the physicochemical properties, such as ionization constant and liposolubility of blockers, appears to be critical in establishing the equilibrium at various sites. However, when IC50 concentration is far greater than that of the K_B of the competitive blockers, the steady state of the mechanical response by two methods of study becomes a critical problem. Drug interaction equilibrium at the receptors may not be expressed linearly or proportionately with the steady state of the mechanical effect. (Refer to the text for additional details.)

different organs. Some observations were communicated at the XIV[th] World Congress of Pharmacology, July 2002, San Francisco, CA (Patil 2002).

Materials and Methods

Isolated tissues were suspended in oxygenated (95% $O_2 + 5\%$ CO_2) physiological salt solution of pH 7.4, maintained at 37.5°C. The solution was of the following composition: (mM) NaCl, 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 0.57, $NaHCO_3$ 25, NaH_2PO_4 1.0, dextrose 11.0, dissolved in demineralized distilled water. The tissue-optimum tension was maintained throughout the experiment. The tissue equilibrium time for the needed sensitivity to the agonist varied between 1 to 3 h. Tension changes were recorded via a pre-calibrated Grass force displacement transducer (FT03) on slowly advancing paper of a polygraph ink recorder. Two types of experiments were planned.

K_B determinations

Cumulative concentration–response relationship of the stable, charged molecule carbachol was obtained (van Rossum & Van den Brink 1963). The tissue was thoroughly washed and then incubated with the competitive blocker for 1 h, thus the cumulative agonist-mediated response in the presence of the blocker was established. Two concentration–response curves of carbachol without the blocker were reproducible (Patil 1992). Only one cumulative concentration–response curve was obtained from each tissue and the maximum response of the indirectly acting drug was normalized to the percent maximum of the carbachol obtained immediately after the maximum response of physostigmine. The dissociation constant (K_B) of the blocker from the parallel-displaced curve of the agonist was calculated as described by Furchgott (1967).

IC50 of the antagonist or the blocker

The IC50 value is the concentration of antagonist that will reduce the response of carbachol or physostigmine to half. The concentration of the agonist that would produce the response between 20% and 80% of its maxima, obtained on the same or contra-lateral tissue, was selected. Depending on the sensitivity of the preparation, the concentration of carbachol for the response varied between 0.3–3 μM. When the response reached the plateau, two to five cumulative concentrations of the antagonist were added until the agonist response was fully reversed. IC50 of the antagonist was calculated from the graphic plot. The agonist response, without the antagonist, served as the control baseline plateau during the experiments. Schematics for the determinations of K_B and IC50 of the blocker are presented in Figure 2. A single concentration of the competitive reversible antagonist was used to obtain the K_B value.

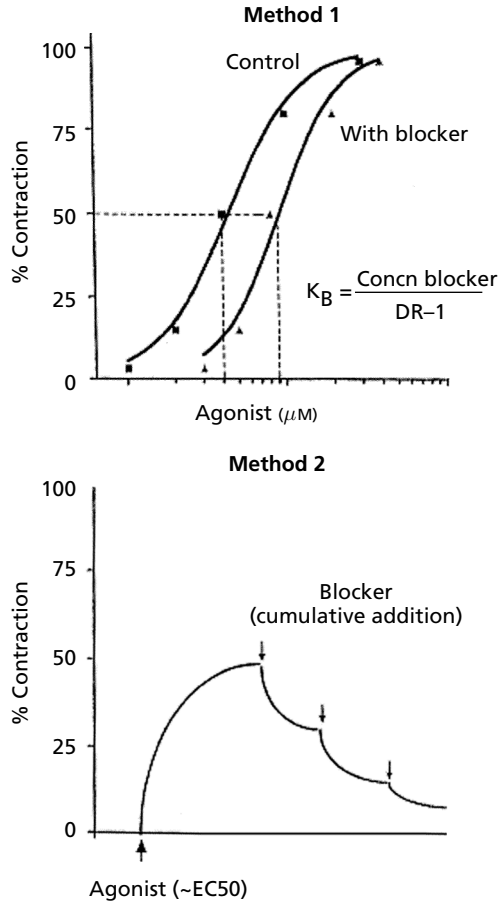

Figure 2 Methods for determination of K_B and IC50 concentration of the anticholinergic drugs (Gaddum 1937, 1957; Schild 1947a, b; Furchgott 1967). Dose ratio (DR) = EC50 with blocker/EC50 of control.

Cholinergic effectors

Human iris-sphincter and ciliary muscles

Ocular globes were obtained from various US eye banks. The essential donor responsibility as well as the shipment of organs to the laboratory was coordinated by the National Disease Research Interchange (Philadelphia, PA). Within 48 h of the death of the donor, either iris-sphincter and/or circular ciliary muscle of the eye were dissected. The investigation was carried out as described by Patil (1992). Data were collected over approximately 24-h experimentation in-vitro.

Anococcygeus muscle

Rats (300–450 g) were anaesthetized with pentobarbital (~50 mg kg^{-1}, i.p.) and both contra-lateral tissues were dissected as described by Gillespie (1972). In-vitro tension changes were recorded at a resting tissue load of 1 g. An agonist:antagonist study was conducted for 6 h.

Frog rectus abdominus

Xenopus laevis (24–106 g) were pithed, and both abdominal skeletal muscles were dissected as described in the

laboratory manual (The Staff 1968). Tissues were suspended in oxygenated Clark–Ringer solution, maintained at room temperature, which varied between 18 and 21°C. The composition of the Frog–Ringer solution was: (mM) NaCl 111.23, KCl 1.88, $CaCl_2$ 1.08, NaH_2PO_4 0.08, $NaHCO_3$ 6.6, glucose 11.01. A resting tissue tension of 1 g was maintained throughout the experiments. Since nicotine is known to produce desensitization, only one dose–response curve of the agonist was obtained on each tissue. K_B and IC50 values of nicotinic blocker were obtained.

Drugs

(±)-Atropine sulfate (Sigma Chemical Co., St Louis, MO); carbachol chloride (Aldrich Chemical Co., Milwaukee, WI); (±)-cyclophentolate HCl and (−)-tropicamide HCl were a gift from Alcon, Fort Worth, TX; hexamethonium chloride (General Biochemicals, Chagrin Falls, OH); (±)-methylatropine (Boehringer Ingelheim, KG, Germany); nicotine hydrogen bitartrate (Sigma Chemical Co., St Louis, MO); (±)-oxybutynin chloride (Sigma-Aldrich Company, St Louis, MO); pirenzepine, 2 HCl (Dr Karl Thomae GmbH, Bibernach order Riss, Germany); physostigmine salicylate (Mallinckrodt, St Louis, MO); (−)-scopolamine HBr (ICN Biochemicals Inc., Aurora, OH). All drugs were freshly prepared in normal saline; physostigmine was prepared in saline containing EDTA.

Data analysis

Each concentration–response curve was converted to the percent of its own maxima or percent of a reference agonist, which produced the maximum response of the tissue. EC50 values, before and after the blockers, were obtained from graphic plots. K_B values and IC50 values of the antagonist were averaged for the geometric mean with s.e. and 95% confidence interval (CI). Student's *t*-test was used to compare two means and the difference at $P < 0.05$ was considered to be statistically significant.

Results

Time course of response of intraocular muscles to agonists and its antagonism by atropine

A time range of 6–16 min was required for the iris sphincter or ciliary muscle to reach a steady-state contraction due to carbachol before atropine was introduced to antagonize the agonist response. The rate of relaxation of the iris sphincter at each concentration of atropine, from 1 to 100 nM, was very slow. As much as 1 h was required for the completion of the concentration response protocol on the tissue. The liposoluble physostigmine produced a slowly developing contraction of the tissue. On average, 28 min was required to obtain a stable contraction of the sphincter muscle. Carbachol 1 μM (n = 8) at 17.5°C produced a contraction that was 68% of its own maximum response. In three of the eight sphincter preparations, two phases of contraction to carbachol were observed. The average time difference in plateau phase was 7 min. As compared with that at 37.5°C, the total onset for the maximum contraction was slow at 17.5°C, but the difference was not significant. These time parameters are summarized in Table 1. Due to inter-tissue variations, such a time course of interaction of other antimuscarinic drugs was not pursued.

K_B and IC50 values of atropine

Table 2 details the essentials of previously published data on the human iris sphincter at 37.5 and 17.5°C, and the data on K_B and IC50 values of atropine on ciliary muscle. The sensitivity of the tissue to agonists varied from one preparation to another. The single, lowest concentration of carbachol (100 nM) produced 58% of its maximum response on the tissue, whereas a relatively high concentration of the agonist (1 μM) produced only 28% of the response on the other tissue. IC50 values of atropine in these two preparations were very close. Therefore, carbachol responses from all seven preparations used in concentrations ranging from 0.1 to 1 μM were averaged to 68% of the maximum contraction. In each preparation the value was far below the maximum response of the

Table 1 Time course of cholinergic response by carbachol (0.1–3 μM) and physostigmine (3–30 μM) with its inhibition by atropine (10–100 nM)

Human intraocular muscle	Carbachol or physostigmine peak time (min)[a] ± s.e.m.	Atropine-mediated inhibition (min)[b] ± s.e.m.	Total time for inhibition by atropine (min)[c] ± s.e.m.
Iris sphincter n = 7 37.5°C	9.17 (C) ± 1.35	10.28 ± 0.47	49.30 ± 3.3
n = 8 17.5°C	12.60 (C) ± 1.8	12.80 ± 1.3	43.40 ± 6.0
Ciliary muscle n = 7 37.5°C	10.82 (C) ± 1.98	12.80 ± 1.92	50.71 ± 4.17
Iris sphincter n = 4 37.5°C	28.80 (P) ± 4.86	14.30 ± 2.73	57.20 ± 4.9
Ciliary muscle n = 6 37.5°C	25.83 (P) ± 4.72	19.00 ± 1.86	57.46 ± 3.81

[a]Time of carbachol (C) or physostigmine (P) response from addition to the plateau. [b]Time of relaxation of the carbachol pre-contracted muscle to half inhibition by a single or cumulative addition of atropine. [c]Total time from the beginning to the complete inhibition of carbachol by cumulative addition of atropine.

Table 2 K_B and IC50 values of atropine for cholinergic antagonism

Organ	Atropine K_B nM (95% CI)	Atropine IC50 nM (95% CI)	IC50/K_B
Human iris sphincter:carbachol 17.5°C	1 (0.4–2.0) n = 5	67[a] (27–170) n = 8	67
37.5°C	2 (1.0–5.0) n = 7	20[b] (10–39) n = 7	10
Iris sphincter:physostigmine 37.5°C	(Reduction in max.)	17[c] (6–44) n = 4	9[f]
Ciliary muscle:carbachol 37.5°C	1.6 (1–2) n = 11	24[d] (12–43) n = 7	15
Ciliary muscle:physostigmine 37.5°C	(Reduction in max.)	27[e] (12–55) n = 6	17[g]

Average contraction produced by carbachol at a, b and d determinations were 68% ± 6, 68% ± 8 and 64% ± 7 of its maxima, respectively. Average contraction produced by physostigmine at c and e were 60% ± 8 and 64% ± 4 of carbachol maxima, respectively. (The ratios f and g represent physostigmine:atropine IC50 to the carbachol:atropine K_B at 37.5°C.) Data from Patil (1992, 1999, 2001).

agonist. Similarly, contraction produced by physostigmine, 3–30 μM, was averaged to 61% of the carbachol maxima.

Analysis of K_B value data on ciliary muscles was similar to that for the iris-sphincter muscle (Patil 1992, 1999). The K_B and IC50 values of atropine obtained with carbachol are summarized in Table 2. In the presence of 30 nM atropine the maximum effect of physostigmine was significantly reduced. Therefore, K_B from non-parallel displacement of physostigmine response was not calculated. IC50 of atropine in physostigmine pre-contracted tissue was equal to that observed from carbachol-contracted tissue. Summary data, including IC50/K_B ratios, are presented in Table 2.

Ionization constants (pK_a), partition coefficient (P_c), K_B and IC50 of antimuscarinic drugs

Antagonists (±)-atropine, (±)-methylatropine, (−)-scopolamine, (±)-cyclopentolate, (−)-tropicamide, (±)-oxybutynin and pirenzepine were selected for the study on the basis of ionization characteristics and liposolubility. Based on pK_a values of weak bases the ratio of ionized (charged) and un-ionized (neutral) species for each antagonist at pH 7.4 was calculated from the Henderson–Hasselbalch equation $pK_a = pH + \log(R\text{-}NH^+_3)/(R\text{-}NH_2)$. Water-soluble methylatropine, a quaternary salt, will be 100% ionized. Atropine with a pK_a of 9.8 will have the proportion of 251 (ionized):1 (un-ionized) molecules at physiological pH 7.4. The ratio is reversed for (−)-tropicamide with pK_a of 5.25, i.e. the proportion changes to 1 (ionized):99 (un-ionized) molecules of the drug. The liposolubility index log P_c of the antimuscarinic drugs as reported before varied between 1.1 for (−)-tropicamide to 2.90 for (±)-oxybutynin.

Except for (−)-scopolamine, either K_B or IC50 values of blockers were obtained from at least three different ocular pairs. On several preparations, K_B and IC50 values were tested on the paired tissues, so that inter-donor variability was reduced. For (±)-oxybutynin and pirenzepine, the K_B and IC50 evaluation on iris sphincter was not pursued. The percent tone of ciliary muscle on iris sphincter, induced by carbachol for the IC50 study of antagonists, varied between 41% for pirazepine to 75% for (±)-cyclopentolate. Data are summarized in Table 3.

Other cholinergic effectors and antagonists

Repeated exposure to carbachol on the rat anococcygeus muscle exhibited small desensitization (1.87-fold); hence, dose ratios for the calculation of K_B of atropine were corrected. IC50 of atropine on the tissue was obtained against 4 μM of the carbachol.

Nicotine was used as an agonist on the rectus abdominus muscle of *Xenopus laevis* to obtain K_B and IC50 values of two competitive antagonists, (+)-tubocurarine and hexamethonium. There was a parallel shift of the nicotine concentration–response curve by both antagonists. A single concentration of nicotine (3 μM) produced an average 69% of the maximum contraction of the tissue. IC50 values of both antagonists were then obtained. Nicotine 1 μM produced an average 25% of the contraction and IC50 of (+)-tubocurarine was slightly lower than that obtained at the higher concentration of nicotine. IC50/K_B ratios of all antagonists on cholinergic effectors were less than 3. Data are summarized in Table 4.

Discussion

In an organ system containing multiple types or subtypes of receptors, the chemical structure and associated physicochemical properties dictate the affinity of the competitive reversible antagonist for the receptor. The method of investigation of the affinity of a blocker at equilibrium conditions of the antagonistic drugs in the organ is of crucial importance for classification and utility of drugs. The initial observation was that K_B of atropine on the isolated iris sphincter, either at 37.5 or 17.5°C, was ~1 nM. The value was inconsistent with the 10–67 nM IC50 values of the antagonist that reduced the agonist response by half. According to the law of mass action, the ratio of IC50/K_B should approach 1. These paradoxical observations triggered the present inquiry into the elucidation of factors which govern the estimates of affinity of series of antimuscarinic drugs.

Table 3 IC50/K_B ratios in relation to the physicochemical properties of the muscarinic antagonists studied on human intraocular muscles

Blocker	Ionized to un-ionized	log Pc	Iris sphincter			Ciliary muscle		
			K_B nM	IC50 nM	IC50/K_B	K_B nM	IC50 nM	IC50/K_B
(±)-Atropine[a] pK_a 9.8	251:1	1.30	2 (1–5) n = 7	20 (10–39) n = 7	10	1.6 (1–2) n = 11	24 (12–43) n = 7	15
(±)-Methyl-atropine	All ionized	Water soluble	0.29 (0.15–0.55) n = 4	3.17[c] (1–9) n = 4	11	0.59 (0.3–1.1) n = 7	13.40[g] (10–17) n = 4	23
(−)-Scopolamine pK_a 7.70	2:1	1.2	—	10[d] n = 1		1.99 (1.91–2.0) n = 3	8.83[h] (6–14) n = 5	4.4
(±)-Cyclopentolate pK_a 7.93	3:1	2.51	8.3 (4–16) n = 4	30[e] (13–71) n = 5	3.6	16 (4–58) n = 5	42[i] (20–88) n = 6	2.6
(−)-Tropicamide pK_a 5.25	1:99	1.1	15 (10–22) n = 4	116[f] (78–172) n = 5	7.7	24 (15–38) n = 7	40[j] (19–83) n = 9	1.66
(±)-Oxybutynin pK_a 8.04	4:1	2.90	—	—	—	69 (41–115) n = 9	101[k] (54–191) n = 8	1.46
Pirenzepine[b] pK_a 8.2 (2.9)	6:1	1.2	120	—	(5.44)	380 (116–1242) n = 3	653[L] (403–1059) n = 4	1.71

[a]Atropine data from Table 2, (±)-cyclopentolate and (−)-tropicamide K_B data from Ishikawa et al (1998), K_B and IC50 values with 95% CI. [b]Ionized to un-ionized ratios for pirenzepine is based on pK_a 8.2 of the drug. [c, d, e, f, g, h, i, j, k] and [L]Average contraction of Carbachol was 61%, 67%, 63%, 75%, 57%, ~45%, 61%, 73%, 60%, and 41% of the maximum respectively.

Table 4 K_B and IC50 values of antagonists for cholinergic antagonism on other muscarinic and nicotinic effectors

Organ agonist:antagonist	K_B (CI)	IC50 (CI)	IC50/K_B
Rat, anococcygeus muscle carbachol:atropine 10 nM 37.5°C	0.17 nM (0.08–0.4) n = 5	0.52[a] nM (0.3–0.9) n = 8	3
Frog rectus abdominus			
Nicotine:(+)-tubocurarine 30 μM (18–21°C)	0.7 μM (0.6–0.8) n = 5	2.36[b] μM (2.0–2.8) n = 9	3
	—	1.5[c] (1.2–1.9) n = 6	2
Nicotine:hexamethonium 300 μM	65 μM (48–88) n = 4	111[d] μM (87–140) n = 9	2

[a]Carbachol (4 μM) on the average produced 50% of the maximum contraction. [b] and [d]Nicotine (3 μM) on the average produced 69% of the maximum contraction. [c]Nicotine (1 μM) on the average produced 25% of the maximum contraction.

On both ocular muscles, carbachol and physostigmine produced slowly developing contractions of the muscle. The rate of onset of the response by the liposoluble acetylcholinesterase inhibitor physostigmine (pK_{a1} 2.0; pK_{a2} 8.2) relative to that by the quaternary water-soluble carbachol was much slower. The average magnitude of contraction of the tissue by both agents in various series of experiments was remarkably close, between 60% and 68%. The receptor saturating concentration or supra-maximum functional response was avoided. Under these conditions, at 37.5°C, the IC50 of atropine varied between 17 and 27 nM. The values were statistically equal. Thus, effects of exogenous muscarinic agonists or that of endogenous acetylcholine, presumably preserved by physostigmine, on the antagonistic potency of atropine were equal. This was expected. Furthermore, graphic analysis of atropine inhibition of the physostigmine concentration–response curve was non-parallel with reduced maxima because in-vitro endogenous acetylcholine at the highest concentration of the indirect acting drug could be limiting. K_B values cannot be obtained by this procedure. The kinetics of inhibition related to the distribution of physostigmine response by atropine was somewhat slower than that of the carbachol. It simply reflected the biophase drug distribution patterns of the antagonistic drugs in the organ. When these IC50 values of atropine were corrected as per Cheng–Prusoff equation for K_B, the resulting values varied 9–12 nM. The values deviated significantly from the K_B of ~1 nM obtained by parallel displacement of carbachol in the presence of the blocker. These deviations were even greater at 17.5°C. This indicated that IC50 and K_B determination procedures under so-called equilibrium conditions are not equivalent.

In one procedure, presumably the blocker competed with the agonist, and the subsequent agonist-activated cascade was not affected by the inhibitor. In the second IC50 procedure, the agonist-activated receptor related and non-related cascade of events at steady state, the mechanical response was "competitively" antagonized by the blocker. Even though in both procedures the blocker presumably competes with the receptor, in the second procedure primary receptor-mediated events, as well as secondary amplified signals will be antagonized by the blocker, but with difficulty. Therefore, IC50 appears greater than K_B of atropine. Intracellular "off" mechanisms were spatially separated from the initial "on" mechanism providing delay in the measured mechanical effects (Figure 1). The higher IC50 of the blocker will be needed for the latter procedure than that in the former K_B determination. Several investigators have thoroughly discussed the IC50 and K_B paradox of the antimuscarinic drugs in the biophase (Jarv et al 1980; Motulsky & Mahan 1984; Eglen & Whiting 1989; Lazareno & Birdsall 1993; Kenakin 2002; Ostrum 2002). I partly agree with their explanations, but the issue of physicochemical properties of antimuscarinic drugs in relation to IC50 was not investigated or discussed. In addition to the chemical structure, which dictates the affinity of the blocker for the receptor, the drug penetration-distribution related to pK_a and P_c remain vital in estimates of affinity in the organ system (Ritchie et al 1965).

Liposolubility and un-ionized molecules in relation to IC50/K_B ratios

The important aspect of drug diffusion onto the receptor biophase of the competitive blocker in estimates of receptor affinities was presented by Furchgott (1964). Seven antimuscarinic drugs with significant differences in pK_a and P_c were selected for the study. Completely ionized, high-affinity water-soluble quaternary analogue of atropine provided a large IC50/K_B ratio (up to 23) comparable with that of atropine on both iris sphincter and ciliary muscle. This was expected. Based on the high liposolubility of (±)-cyclopentolate and oxybutynin, we expected low IC50/K_B ratios, <2. Note the overlapping confidence intervals of two parameters of the blocker in Table 3. (−)-Tropicamide with a pK_a of 5.25, at pH 7.4 would remain 99% un-ionized. The latter molecular species can diffuse freely into the receptor compartment, providing low IC50/K_B ratios when compared with methylatropine. The ratio on the iris sphincter was somewhat higher compared with the ciliary muscle. The organ preparations in the test series provided more variations for IC50 evaluations. Scopolamine, with relatively low liposolubility index but with a pK_a of 7.7, had a relatively low IC50/K_B ratio. Thus a series of competitive blockers with differing physicochemical properties clearly established the importance of the liposolubility and ionization

characteristics of the molecule in estimates of IC50 and K_B values. In this context, a report by Barlow & Chan (1982) on pirenzepine indicated that the antimuscarinic potency of the blocker increased with increasing proportions of the protonated form in the medium. Those investigators reported an equal affinity constant K_B of the competitive blocker at 37.5 and 30°C. These observations were consistent with nearly equal dissociation constants K_B of atropine at 37.5 and 17.5°C.

The distribution coefficient (log D, which represents the concentration of ionized form in octanol, divided by the total concentration in aqueous phase) was calculated (Hansch & Leo 1995). The values obtained were atropine −1.1, methylatropine approximately −1.5, scopolamine 0.72, cyclopentolate 1.87, tropicamide 1.1, oxybutynin 2.17, and pirenzepine 0.34. Although a perfect linear correlation of the $IC50/K_B$ ratios of seven antimuscarinic drugs with that of the distribution coefficients was lacking, the lower and higher log D values provided a much better explanation of the role of physicochemical properties for the blocking activity.

Other cholinergic effectors

$IC50/K_B$ ratios of blockers on two other cholinergic effectors were examined to establish the versatility of the observations made on the cholinergically-innervated human intraocular muscles. On rat anococcygeus muscle, IC50 and K_B values of atropine were not significantly different. An IC50 of 0.52 nM was easily correctable by the Cheng–Prusoff equation to 0.26 nM K_B, giving an $IC50/K_B$ ratio of 1.5. At the nicotinic cholinergic junction of rectus abdominus of *Xenopus laevis*, IC50 values of (+)-tubocurarine at two concentrations of nicotine were 2–3-times higher than that of K_B. IC50 values for (+)-tubocurarine and for hexamethonium were corrected according to the Cheng–Prusoff equation, leading to the ratio 1. The law of mass action for competitive blockers on two cholinergically innervated organs was expected. At the mammalian nicotinic cholinergic junction, however, K_B for (+)-tubocurarine by the conventional method was 0.33 μM and the IC50, against the cholinergic phrenic nerve-mediated contractions, was 4.45 μM, leading to an $IC50/K_B$ ratio of 13.5 (Barlow 1995). Again the explanation for such a paradox in relation to the receptor biophase drug distribution, either in the skeletal muscle or in the heart, remains to be elucidated (Waud et al 1978).

This series of experiments on IC50 and K_B determinations of competitive blockers in various organs has raised some important issues concerning kinetic equilibrium and steady-state evalution in functional receptor pharmacology. Physicochemical properties of the competitive reversible blocker play a profound role in the determination of IC50. The equilibrium dissociation constant K_B obtained by the incubation of the blocker with organ preparation is still a useful parameter to compare the potency of antagonists. However, it is equally important to establish IC50 values of the competitive blockers. The latter concentrations presumably antagonize half of the response mediated by the endogenous transmitter or the exogenous agonist. The K_B concentration of the blocker in plasma and/or in the biophase will reduce the response of the exogenous agonist to half. Therefore, both the IC50 and K_B values of competitive antagonists are useful in pharmacotherapeutics and drug toxicity.

Conclusion

The concentration of atropine or its quaternary analogue, methylatropine, to reduce the carbachol-activated response of iris sphincter or ciliary muscle to half (IC50) was 10–23-times greater than that of the K_B. The $IC50/K_B$ ratios of (−)-scopolamine, (±)-cyclopentolate, (−)-tropicamide, (±)-oxybutynin and pirenzepine were much smaller and closer to 1 as predicted by the law of mass action. Based on pK_a and log P_c or log D of these antimuscarinic drugs, the receptor biophase distribution of atropine and methylatropine appeared to be greatly distorted when the affinity constant was studied by the IC50 and/or K_B parameters. The equilibrium of the water-soluble atropine at the receptor might not be translated properly for the so-called equilibrium of the mechanical response, particularly when the blocker was studied using the IC50 parameter. The physicochemical properties played a profound role in the estimation of the receptor-related parameters of the competitive blockers by these two procedures.

References

Ariëns, E. J. (1954) Affinity and intrinsic activity in the theory of competitive inhibition Part I. Problems and theory. *Arch. Int. Pharmacodyn.* **99**: 32–49

Arunlakshana, O., Schild, H. O. (1959) Some quantitative uses of drug antagonists. *Br. J. Pharmacol.* **14**: 48–58

Barlow, R. B. (1995) Use of antagonist for estimating the degree of agonist stimulation during physiological release. *Trends Pharmacol. Sci.* **16**: 262–264

Barlow, R. B., Chan, M. (1982) The effects of pH on the affinity of pirenzepine for muscarinic receptors in the guinea pig ileum and rat fundus strip. *Br. J. Pharmacol.* **77**: 559–563

Bowman, W. C., Rand, M. J. (1980) *Textbook of pharmacology*. 2nd edn, Blackwell Scientific Publications, Oxford, pp. 39–41

Cheng, Y.-C., Prusoff, W. H. (1973) Relationship between the inhibition constant (K_i) and the concentration which causes 50 percent inhibition IC_{50} of an enzymatic reaction. *Biochem. Pharmacol.* **22**: 3099–3108

Colquhoun, D. (1998) Binding, gating, affinity and efficacy: the interpretation of structure activity relationships for agonists and effects of mutating receptors. *Br. J. Pharmacol.* **125**: 923–947

Eglen, R. M., Whiting, R. L. (1989) Problems associated with the application of the Cheng-Prusoff relationship to estimate atropine affinity constants using functional tissue responses. *Life Sci.* **44**: 81–94

El Tayar, N., Testa, B., Van de Waterbeemd, H. V., Carrupt, P.-A., Kaumann, A. J. (1988) Influence of liposolubility and chirality on the selectivity of ligands for β_1- and β_2-adrenoceptors. *J. Pharm. Pharmacol.* **40**: 609–612

Foye, W. O., Lemke, T. L., Williams, D. A. (1995) *Principles of medicinal chemistry*. 4th edn, Williams and Wilkins, Baltimore

Furchgott, R. F. (1964) Receptor mechanisms. *Ann. Rev. Pharmacol.* **4**: 21–50

Furchgott, R. F. (1967) The pharmacological differentiation of adrenergic receptors. *Ann. NY Acad. Sci.* **139**: 553–570

Furchgott, R. F. (1972) The classification of adrenoceptors (adrenergic receptors): an evaluation from standpoint of receptor theory. In: Blaschko, H, Muscholl, E. (eds) *Catecholamines. Handbook of experimental pharmacology*, Vol XXXIII, Springer Verlag, New York, p. 283

Gaddum, J. H. (1937) The quantitative effects of antagonistic drugs. *J. Physiol.* (London): 7P–9P

Gaddum, J. H. (1957) Drug antagonism. *Pharmacol. Rev.* **9**: 211–217

Gillespie, J. S. (1972) The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. *Br. J. Pharmacol.* **45**: 404–416

Hansch, C. (Chair Ed. Board) (1990) *Comprehensive medicinal chemistry*. Vol 6, Pergamon Press, New York

Hansch, C., Leo, A. (1995) *Exploring QSAR*. American Chemical Society, Washington, DC, p. 97

Ishikawa, H., DeSantis, L., Patil, P. N. (1998) Selectivity of muscarinic agonists including (±)-aceclidine and antimuscarinics on the human intraocular muscles. *J. Ocular Pharmacol. Ther.* **14**: 363–372

Itoh, T., Kuriyama, H. (1994) Excitation-contraction coupling mechanisms in visceral smooth muscle cells. In: Szekeres, L., Papp, J. (eds) *Pharmacology of smooth muscle. Handbook of experimental pharmacology*, Vol 111. Springer Verlag, New York, p. 57

Jarv, J., Hedlund, B., Barfai, T. (1980) Kinetic studies on muscarinic antagonist-agonist competition. *J. Biol. Chem.* **255**: 2649–2651

Kenakin, T. P. (1993) *Pharmacologic analysis of drug-receptor interaction*. 2nd edn, Raven Press, New York

Kenakin, T. (2002) Drug efficacy at G protein-coupled receptors. *Annu. Rev. Pharmacol. Toxicol.* **42**: 349–379

Lazareno, S., Birdsall, N. J. M. (1993) Estimation of competitive antagonist affinity from inhibition curves using the Gaddum, Schild and Cheng-Prusoff equation. *Br. J. Pharmacol.* **109**: 1110–1119

Motulsky, H. J., Mahan, L. C. (1984) The kinetics of competitive radioligand binding predicted by the law of mass action. *Mol. Pharmacol.* **25**: 1–9

Ostrum, R. S. (2002) New determinants of receptor-effector coupling: trafficking and compartmentation in membrane microdomains. *Mol. Pharmacol.* **61**: 473–476

Patil, P. N. (1992) Reactivity of human iris sphincter to muscarinic drugs in vitro. *Naunyn Schmiedebergs Arch. Pharmacol.* **346**: 614–619

Patil, P. N. (1996) Pharmacologic quantitation. *Indian J. Exp. Biol.* **34**: 615–633

Patil, P. N. (1999) Enhanced sensitivity of the iris sphincter to the muscarinic agonist carbachol at lower temperature. *J. Ocular Pharmacol. Ther.* **15**: 65–72

Patil, P. N. (2001) Reversal of cholinergic response of intraocular muscles by atropine: drug antagonism re-examined. In: Gupta, S. K. (ed.) *Pharmacology and therapeutics in the new millennium*. Narosa Publishing House, New Delhi, p. 579

Patil, P. N. (2002) *Two processes of competitive drug antagonism*. XIV World Congress of Pharmacology Abstract, July 7–12, San Francisco, CA

Patil, P. N., Mauger, T. F. (1992) Cholinergic sensitivity of irides from donors with various pathological conditions and lens implants. *Naunyn Schmiedebergs Arch. Pharmacol.* **346**: 620–628

Ritchie, J. M., Ritchie, B., Greengard, P. (1965) The active structure of local anesthetics. *J. Pharmacol. Exp. Ther.* **150**: 152–159

Salazar, M., Shimada, K., Patil, P. N. (1976) Iris pigmentation and atropine mydriasis. *J. Pharmacol. Exp. Ther.* **197**: 79–88

Schild, H. O. (1947a) pA, a new scale for the measurement of drug antagonism. *Br. J. Pharmacol.* **2**: 189–206

Schild, H. O. (1947b) The use of drug antagonist for the identification and classification of drugs. *Br. J. Pharmacol.* **2**: 251–258

The Staff (1968) Department of Pharmacology, University of Edinburgh, *Pharmacological experiments on isolated preparations*. E & S Livingstone, Ltd, London

van Rossum, J. M., Van den Brink, F. G. (1963) Cumulative dose-response curves. I Introduction to the technique. *Arch. Int. Pharmacodyn. Ther.* **143**: 240–246

Waud, D. R., Leeson, S., Waud, B. E. (1978) Kinetic and empirical analysis of dose-response curves illustrated with a cardiac example. *Life Sci.* **22**: 1275–1286