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Schild's Equation and the Best Estimate of pA2 Value and Dissociation Constant of an Antagonist

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Calculation of the pA2 value and dissociation constants for an antagonist from the effects observed on isolated smooth muscles can be done in two ways: using Schild's plot procedure or Schild's equation. In our study we used the effects of muscarinic antagonists observed in experiments on isolated human and feline stomach and rat gastric fundus. Only the estimates of pA2 values and dissociation constants made using the Schild's equation on the basis of the lowest antagonist concentrations were not significantly different from the values calculated using the Schild's plot procedure. This suggest that, when it is impractical to perform the full Schild's plot procedure, the best estimate of pA2 values and dissociation constants can be done with the lowest antagonist concentration that significantly inhibits the effects of an agonist on an isolated smooth muscle preparation.

Key Words: acetylcholine; antimuscarinic agonists; atropine; bethanechol; carbachol; cholinergic agonists; gastric fundus; muscarinic agonists; parasympatholythics; receptors

In experiments on isolated organs and tissues, the calculation of dissociation constant (KB) and pA2 value (a negative logarithm of KB when the slope of the Schild's plot is exactly 1) of an antagonist is considered to be an indirect measure of antagonist's affinity for its receptors. A direct measure of the affinity is the reciprocal of dissociation constant (1). In order to obtain these values for an antagonist, one must first register the effects of increasing concentrations of the agonist on intact tissue, and then on the same tissue incubated previously with at least three different concentrations of the antagonist (2). If the agonist concentration producing half the maximal response on intact tissue is denoted EC50, then the agonist concentrations producing half the maximal response on tissue incubated with the lowest, medium, and the highest antagonist concentrations will be EC50', EC50'' and EC50''', respectively. Making linear regression of the series EC50'/EC50, EC50''/EC50 and EC50'''/EC50 (in the form of log10(EC50'/EC50-1), log10(EC50''/EC50-1) and log10(EC50'''/EC50-1) upon -log10(antagonist concentration) one is able to calculate pA2 value (intercept on the y-axis) and KB (antilog(-pA2) if the slope is exactly 1), provided that the slope of regression is within the limits of 0.8 to 1.2 (Fig. 1) (3).

Figure 1: Schild's plot for blocking effects of p-fluoro- hexahydro-sila-difenidol (pFHHSiD) on tonic contractions of feline stomach longitudinal muscle produced by acetylcholine (see Tables 1 and 2). pA2 value (intercept on x-axis)=7.93±1.09 (SD), and slope=1.01±0.17 (SD). EC50 ? agonist concentration producing half the maximal response of intact tissue; EC50* ? agonist concentration producing half the maximal response of intact tissue incubated with certain antagonist concentration. [view this figure]

However, although this regular procedure should be completed whenever possible, it cannot always be performed on human isolated organs. Isolated organs are often under significant ischemic stress during surgical procedures, which results in their low viability and responsiveness. Usually experimentators are not able to test the effects of more than one antagonist concentration on the responses produced by an agonist. They have to make an estimate of these values from effect of only one antagonist concentration, using Schild's equation:

$$\frac{EC_{50a}}{EC_{50c}} - 1 = \frac{[B]}{K_B}$$

Where EC50a=concentration of the agonist producing half the maximal response in the presence of the antagonist; EC50c=concentration of the agonist producing half the maximal response in the absence of the antagonist; [B]=concentration of the antagonist used; and KB=dissociation constant of the antagonist. PA2 values could then be calculated as -log KB (4).
However, this estimate may differ significantly from actual values that could be obtained by the complete Schild's plot procedure. The aim of our study was to find out which antagonist concentration is best for such estimation: those producing minimal, medium or maximal blocking?

Material and Methods

Experimental Procedures

We used our experiments on isolated gastric smooth muscles for comparison and calculation. The data comprised the effects of 9 muscarinic receptors blockers (atropine, pirenzepine, telenzepine, hexacyclium, trihexyphenydil, p-fluoro-hexahydro-sila-difenidol (pFHHSiD), pancuronium, scopolamin-butilbromide, and methoctramine) on concentration-dependent tonic contractions of isolated preparations caused by muscarinic agonists acetylcholine, bethanechol or carbachol (Table 1). There where 5 types of isolated preparations: (a) isolated strips of the longitudinal muscle of the human gastric corpus; (b) isolated strips of the circular muscle of the human gastric corpus; (c) isolated strips of the longitudinal muscle of the feline gastric corpus; (d) isolated strips of the circular muscle of the feline gastric corpus, and (e) isolated strips of the rat gastric fundus. All blockers used in the study (except when tried on the rat gastric fundus) behaved as competitive antagonists of acetylcholine, bethanechol or carbachol: the slopes of the Schild's regression line were always between 0.8 and 1.2.

Table 1: EC50 values and antagonist concentrations (mol/L) from experiments on isolated gastric smooth muscles. [view this table]
Table 2: pA2 values calculated by Schild's plot (first column) or Schild's equation (second to fourth columns). [view this table]
Table 3: Dissociation constants of antagonists (mol/L) calculated by Schild's plot (first column) or Schild's equation (second to fourth column). [view this table]

By means of classic Schild's procedure (using three or four concentrations of an antagonist) we calculated pA2 values and dissociation constants for antagonists (the first columns in Tables 2 and 3). By means of the Schild's equation we then calculated pA2 values and dissociation constants for each concentration of an antagonist (columns II to IV in Tables 2 and 3).

Statistics

Each column in Table 2 is a group of calculated pA2 values. We had four groups: the first (column 1) with values calculated in a classical way; the second (column 2) with values calculated by the Schild's equation using the lowest concentration of an antagonist; the third (column 3) with values calculated by the Schild equation using medium concentration of an antagonist; and the fourth (column 4) with values calculated by the same equation using the highest concentration of an antagonist. By means of the Friedman's test (5) we tested if all groups were taken from the same population of pA2 values or not. The Friedman's test was used because it makes no assumptions about the distribution of the data (e.g., normality). Then, the significance of difference between each group of pA2 values calculated by Schild's equation and the first group (calculated in a classical way) was tested by the t-test for two small dependent samples (6,7).

Results

Four groups of pA2 values differed significantly from each other. The value of Friedman's chi-square was 8.27 ($p<0.05$).
The mean difference between pA2 values in the first and second columns of Table 2 was 0.08±0.28 (SD). This difference was not significant ($t=1.36$; $p>0.05$).
The mean difference between pA2 values in the first and third columns of Table 2 was 0.16±0.34. It was significant ($t=2.22$; $p<0.05$).
The mean difference between pA2 values in the first and fourth columns of Table 2 was 0.24±0.37. It was also significant ($t=3.08$; $p<0.01$).

Discussion

The estimates of the pA2 value and dissociation constant from the effect of on only one antagonist concentration are not always appropriate. Friedman's test showed that estimates in our studies based on different concentrations and actual values calculated in a classical way were not from the same population. However, the comparison between pA2 and dissociation constants calculated in a classical way and the values estimated on individual antagonist concentrations showed that only pA2 values estimated on the basis of the lowest antagonist concentration were not significantly different. It means that the most accurate estimate of the pA2 value could be done using the antagonist concentration that produces a minimal significant shift of the agonist dose-response curve to the right. In practice, when an experimentator checks the effect of an antagonist for the first time, he starts with very low concentrations (usually in a nanomolar range) and gradually increases them until the first inhibition of agonist effects is observed. According to our study, the first concentration of an antagonist producing significant inhibition of agonist's responses gives the best estimate of the pA2 value for the antagonist. However, the minimum shift of an agonist concentration-response curve in the presence of the antagonist should be at least 3-fold (EC50a/EC50c>3), since a very small shift may have disproportionately large influence on the pA2 value calculated by the Schild's equation. For example, in the human stomach longitudinal muscle, where acetylcholine was the agonist and hexocyclium the antagonist, the lowest concentration of the antagonist produced very small shift to the right (1.4-fold, Table 1) and the pA2 values calculated by the Schild's equation and Schild's plot differred by as much as 0.92 units.

Schild's calculus of the pA2 value is based on the assumption that an antagonist behaves competitively: concentration-response curves for the agonist in the presence of increasing concentrations of the antagonist have to be parallel and the slope of the regression line for the antagonist has to be approximately 1. However, this is not what happens in reality (8). Even the most competitive antagonist never behaves ideally, and its higher concentrations always tend to lessen the slopes of concentration-response curves for the agonist (9,10). Although there are mathematical manipulations to adjust this and make concentration-response curves for the agonists parallel, this is considered a sort of violence over the true results [11]. In the lower concentration range, antagonists behave more competitively than in the higher concentration range, so that a Schild's equation for the calculation of pA2 and KB based on low antagonist concentration may give an estimate of pA2 and KB not much worse from the one calculated with the Schild's plot procedure. However, Schild's plot procedure gives a lot of additional information which could not be obtained by the Schild's equation (slope of the plot, significance of linear regression for the plot, etc.), remaining the "golden standard" in the analysis of drug effects on isolated organs and tissues.

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