AN EXTENSION OF pA PRINCIPLE TO THE POTENTIATION OF DRUG EFFECTS, AND ITS APPLICATION TO THE BIOLOGICAL ASSAY OF SOME ANTICHOLINESTERASES AND CARDENOLIDES

Naohisa ISHIKAWA, Tomohiro ICHIKAWA, Hiromichi TSURU
and Tatsuro SHIGEI

Department of Pharmacology, Nagoya University School of Medicine,
Showa-ku, Nagoya 466, Japan

Accepted September 20, 1979

Abstract—A new scale for the potentiation was introduced and was termed pA1/2. The value can be calculated by using an equation: \( pA_{1/2} = pA_x + \log \left( \frac{1}{x} - 1 \right) \), which is similar to that which is used for the calculation of pA2. For the use of the equation, the parallel shift of the dose-response curve and the unchanged maximum response are prerequisites. This experiment showed the validity and the usefulness of calculating the pA1/2 values, in the biological assay. In the assay of anticholinesterases, the potentiating effects of neostigmine or physostigmine on the acetylcholine-induced contraction were examined, by using frog's rectus abdominis. The pA1/2 values obtained in the presence of two different concentrations of anticholinesterases were the same, indicating that pA1/2 is a parameter which is specific to the potentiator. The potentiating effects of some cardenolides on the K-induced contracture were examined, by using the frog's ventricular muscles. The relative potencies of four cardenolides obtained from the pA1/2 values well agreed with those reported earlier. The amount of the cardenolides required for this assay was smaller than required for other methods.

The pA2 value has been widely used as an estimate of the potency of antagonistic drugs, since Schild proposed the pA concept (1). This parameter means the affinity of the antagonist to the receptor. We have developed a new index for the potentiating agent from the pA concept, though the dose-ratios of the agonists are commonly used. The usefulness and the validity of the new index were examined in this study. A preliminary report of the results has been published (2).

Theoretical consideration: The principle for calculating a new index of the potentiating effect is as follows; it is an extension of the pA concept commonly used in a competitive antagonism of drugs. In order to obtain the pA2 value, it is required that the dose-response curve be shifted to the right in parallel without any change in the maximum response (3). When these requirements are fulfilled, the antagonism is considered to be competitive, and the pA2 value indicates the affinity of antagonistic agent to the receptor. For the calculation of the pA2 value, the following equation is used;

\[
pA_2 = pA_x + \log (x-1)
\]

where \( pA_x \) is the negative logarithm of the dose of the antagonist used and \( x \) is the ratio of the dose of agonist in the presence of the antagonist to that in its absence.
Now, assume that the dose-response curve is shifted to the left in parallel without any change in the maximum response. This potentiation is just opposite to the competitive antagonism in the direction of the shift. Then, such a relationship similar to that of the competitive antagonism should hold between the shifted dose-response curve and the control curve. The index for the potentiator may arbitrarily be termed $pA_{1/2}$ which is the negative logarithm of the dose of the potentiator that requires a halving of the dose of the agonist to produce the same response as that in the absence of potentiator. For the calculation of the value of $pA_{1/2}$, the equation 1 which gives the $pA_2$ value will have to be converted to the following equation:

$$pA_{1/2} = pA_x + \log \left( \frac{1}{x} - 1 \right)$$  \hspace{1cm} (2)

where $x$ is the ratio of the dose of agonist in the presence of potentiator to that in its absence, and is smaller than 1. Because of the opposite direction of the shift of dose-response curve, the notation $x$ in the parenthesis of equation 1 will have to be replaced with the notation $\frac{1}{x}$.

One hypothetic attempt to adapt the classic theory of drug-receptor interrelationship to the problem of potentiation will be discussed in the appendix.

**MATERIALS AND METHODS**

**Assay of the anticholinesterases:**

Frogs (*Rana nigromaculata*) were used. A rectus abdominis was suspended in an organ bath containing 20 ml of Ringer’s solution aerated with air at room temperature (22–25°C). The composition of the solution was NaCl, 111 mM; KCl, 2.7 mM; CaCl₂, 1.8 mM; NaHCO₃, 1.2 mM; glucose, 2.7 mM. The contraction was recorded on a smoked drum by an isotonic lever. The responses were induced by the injection of acetylcholine into the organ bath, every fifteen min. Each application lasted for 90 sec, and then the preparation was washed with fresh Ringer’s solution.

Before the experiment, administration of a certain amount of acetylcholine (usually 8 μg/ml) were repeated several times, until the height of the response became constant. Thereafter, one series of control responses to 1, 2, 4 and 8 μg/ml of acetylcholine was examined. The order of application was randomized. Then, an anticholinesterase was added to the organ bath, and applications of acetylcholine (2 or 4 μg/ml) were repeated. When the responses became stable, the responses to at least two concentrations of acetylcholine were examined. The dose ratio was two. We tried to match the responses with those in control, after which a higher concentration of the anticholinesterase was given, and the same procedure was repeated. At the end of the experiment, the maximum contractions were elicited by applying 10⁻³ g/ml of acetylcholine. In other preparations, the changes in maximum responses were examined in the presence and absence of the anticholinesterase.

In each experiment, the dose ratios of acetylcholine were obtained graphically in the overlapped range of responses in the three concentration-response curves, and the $pA_{1/2}$ values were calculated. The parallelism was analysed by utilizing the parallel line assay.
Assay of the cardenolides:

Preparation and experimental conditions are basically the same as those in the previous paper (6). Frogs (Rana nigromaculata) were used. A ventricular muscle strip (about 1 mm thick and 5 mm long) was suspended in an organ bath containing 10 ml of Ringer's solution aerated with 95% O₂ + 5% CO₂ at room temperature. The composition of normal Ringer's solution was: NaCl, 111 mM; KCl, 2.7 mM; CaCl₂, 1.8 mM; NaHCO₃, 15 mM; glucose, 2.7 mM. The preparation was stimulated electrically at the rate of 0.2 Hz throughout the experiment, except when K-contracture was induced and recorded. The contractile tension was recorded by means of strain-gauge transducer (Nihon Koden Kogyo, SB-1T) and carrier amplifier (Nihon Koden Kogyo, RP-5), on an ink-writing oscillograph (Nihon Koden Kogyo, RJG-3004). The resting tension of 0.3–0.5 g was applied.

K-contractures were elicited by replacing the Ringer's solution in the bath by oxygenated K-rich solution. Potassium concentrations were changed by replacing NaCl with equimolar amounts of KCl, except for the solution containing 120 mM potassium. In the latter, 111 mM NaCl was replaced by 117.3 mM KCl. K-rich solution was applied for 1–2 min, and then the preparation was washed with fresh Ringer's solution. More than 20 min elapsed before the next administration. The intervals of 20 min were long enough to obtain reproducible size of contractures.

The maximum tensions of the K-contractures were also examined, by replacing the solution which contained 5 mM CaCl₂ by that containing 5 mM CaCl₂, 110 mM KCl and 2.7 mM NaCl, in the same way as shown by Takeda et al. (6). The contractures were induced several times in the beginning of the experiment, and their tensions were compared with those observed in the presence of the test compound.

After equilibration in the maximum responses, the control responses to KCl (60, 80 and 120 mM) and control maximum response were examined. A cardenolide was then added to the organ bath. When the height of the twitch responses became stable, the responses to some concentrations of KCl, the dose ratio of which was two, and the maximum response were examined. A higher concentration of the cardenolide was then given and the same procedure was repeated.

In each experiment, the dose ratio of KCl and the pA₁/₂ value were obtained in the same way as in the assay of anticholinesterases. The relative potencies were calculated from the corresponding pA₁/₂ values, the potency of digitoxigenin being taken as 1.0. The 95% confidence limits of the relative potency were obtained on the basis of Fieller's theorem by utilizing the multiple t-distribution of Dunnett (8, 9).

Compounds used: Acetylcholine (Ovisot, Daiichi Seiyaku, ACh), two anticholinesterases and four cardenolides were used in this study. They were neostigmine bromide (Tokyo Kasei Kogyo), physostigmine sulfate (Sigma), digitoxigenin (Merck), digoxigenin*, 15β-hydroxydigitoxigenin* and a ring A-cloven cardenolide, the methyl ester of 14-hydroxy-3,5-seco-4-nor-5-oxo-14β-card-20(22)-enolid-3-oic acid* (7). Stock solutions of the cardenolides were prepared, by dissolving each compound in 70% ethanol in a concentration of
1 mg/ml. For the experiments, the stock solution was diluted with 0.6% NaCl solution. (*: kindly provided by Dr. M. Okada, Tokyo Biochemical Research Institute).

The molecular weights were 374.5 for digitoxigenin, 390.5 for digoxigenin, 391.5 for 15β-hydroxydigitoxigenin and 407.5 for the ring A-cloven cardenolide. Although molar concentrations should be used for this kind of assay, in the present paper, the concentrations of the cardenolides used were expressed as g/ml, according to the conventional way in our laboratory, since the molecular weights were quite similar.

RESULTS

Assay of the anticholinesterases: The control responses to ACh were approx. 15–75%, when the maximum response was taken as 100%. After addition of the lower concentration (10⁻⁷ M) of neostigmine, the ACh-induced responses were gradually potentiated, and became stable in approx. 2.5 hr. Then, the concentration-response curves in the presence of neostigmine were obtained. A typical result is shown in Fig. 1 (left). The higher concentration of neostigmine (3×10⁻⁷ M) was then applied. The concentration-response curves were shifted to the left and in parallel. As for physostigmine, the same responses were also shown in Fig. 1 (right). Four experiments were performed with neostigmine and four with physostigmine.

The maximum responses were not affected by the treatment with neostigmine or physostigmine.

Fig. 1. Potentiation of ACh-induced contracture of the frog rectus abdominis. A typical experiment. The concentrations of the anticholinesterases are indicated below the concentration-response curves.

<table>
<thead>
<tr>
<th>Neostigmine</th>
<th>Physostigmine</th>
</tr>
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<tbody>
<tr>
<td>6.96±0.07 (10⁻⁷ M)</td>
<td>5.85±0.15 (3×10⁻⁷ M)</td>
</tr>
<tr>
<td>6.95±0.02 (3×10⁻⁷ M)</td>
<td>5.87±0.03 (10⁻⁸ M)</td>
</tr>
</tbody>
</table>

Table 1. The pA₁/₂ values for neostigmine and physostigmine. Potentiation of ACh-induced contracture of the frog rectus abdominis. Data are expressed as mean±S.E. (n=4). Each value was obtained in the presence of the potentiator, the concentration of which is indicated in the parenthesis.
physostigmine.

In each experiment, two pA₅₀ values were calculated separately on the basis of the two concentration-response curves obtained. The results are summarized in Table 1. It was noted that, for both neostigmine and physostigmine, the values obtained in different degrees of potentiation were exactly the same.

**Assay of the cardenolides:** The typical experiment is shown in Fig. 2. The maximum responses were first observed, and after equilibration, K-contractures as in control were induced by three concentrations of KCl, i.e., 60, 80 and 120 mM. The tension development reached a plateau in 1–2 min. The maximum response was again examined. Thereafter, K-contractures in the presence of digoxigenin were studied. When $3 \times 10^{-8}$ g/ml of the compound was applied, the twitch tension was increased slightly, and reached a steady level in about 90 min. The K-rich solutions were then applied, and we tried to match the responses with those in the control. The required concentrations of KCl were predicted by preliminary experiments. When a higher concentration ($10^{-7}$ g/ml) of digoxigenin was applied, the required concentrations of KCl were much lower. The experimental procedures for the other cardenolides were the same as in Fig. 2.

The maximum responses obtained in the presence of cardiotonic steroids were approx. 112%, when those in control were taken as 100%. Such slight increases were also observed in the absence of the cardenolides, confirming the observation of Takeda et al. (6). There were no significant differences of the increases in maximum responses between them, when analysed by the analysis of covariance (10).

Figure 3 shows the typical concentration-response curves of K-induced contracture. Administration of digitoxigenin or 15β-hydroxydigitoxigenin caused the dose-dependent shifts of concentration-response curves to the left. The same phenomena were also observed in the other compounds tested. The shifted concentration-response curves were parallel
to the control curves. For each compound, four experiments were performed.

In each experiment, two pA$_{1/2}$ values were calculated, one from the dose ratio of potassium in the presence of the lower concentration of the cardenolide, and the other from

![Graph](image)

**FIG. 3.** Potassium-induced contracture of the frog ventricular muscle. A typical experiment. Shift of the concentration-response curves caused by digitoxigenin and 15β-hydroxydigitoxigenin. The concentrations (g/ml) used are indicated below the curves.

**TABLE 2.** Comparison of the results obtained by the three different methods.

<table>
<thead>
<tr>
<th></th>
<th>Potentiation of K-contracture</th>
<th>Straub’s method$^{(1)}$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>the present study</td>
<td>Takeda et al$^{(1)}$</td>
</tr>
<tr>
<td></td>
<td>pA$_{1/2}$</td>
<td>relative potency</td>
</tr>
<tr>
<td>I</td>
<td>7.12±0.03</td>
<td>1.0</td>
</tr>
<tr>
<td>II</td>
<td>7.05±0.01</td>
<td>(0.72~1.0)</td>
</tr>
<tr>
<td>III</td>
<td>5.80±0.05</td>
<td>(0.035~0.065)</td>
</tr>
<tr>
<td>IV</td>
<td>5.92±0.05</td>
<td>(0.046~0.086)</td>
</tr>
</tbody>
</table>

|                  |                  |                           |
| B                 |                  |                           |
| I                 | $3 \times 10^{-8}$ | $3.6 \times 10^{-7}$   | $3 \times 10^{-7}$ |
| II                | $3 \times 10^{-8}$ | $9.0 \times 10^{-7}$   | $3 \times 10^{-7}$ to $10^{-6}$ |
| III               | $3 \times 10^{-7}$ | $5.4 \times 10^{-6}$   | $3 \times 10^{-6}$ to $10^{-5}$ |
| IV                | $3 \times 10^{-7}$ | *                      | $3 \times 10^{-6}$ to $10^{-5}$ |

I = digitoxigenin, II = digoxigenin, III = 15β-hydroxydigitoxigenin, IV = methyl ester of 14-hydroxy-3,5-seco-4-nor-5-oxo-14β-card-20 (22)-enolid-3-oic acid (1) referred from the report by Takeda et al. (6). *Amount was too little to be examined.

A: The indices (pA$_{1/2}$, pD$_2$ = the negative logarithm of ED$_{50}$, pD$_c$ = the negative logarithm of the concentration in which systolic contracture was induced) and the relative potencies in each assay method. Figures in parentheses are the 95% confidence limits.

B: The minimum concentrations (g/ml) of the compounds required for each assay method.
that in the higher concentration of the cardenolide. The former value for each compound and the potency relative to digitoxigenin are summarized in Table 2. As can be seen, the 95% confidence limits or the range of relative potencies obtained by the three different assay methods overlapped. In the lower parts (B) of the Table, the minimum concentrations of drugs required for each assay method are shown. The concentrations for the present method were the smallest, compared with the other two methods. Another set of \( pA_{1/2} \) values which were obtained in experiment with higher concentrations of cardenolides were as follows: Digitoxigenin = 7.05 ± 0.05; digoxigenin = 6.95 ± 0.04; 15β-hydroxydigitoxigenin = 5.87 ± 0.07; the compound IV = 5.95 ± 0.09.

**DISCUSSION**

The \( pA \) concept was devised by Schild (1). The value of \( pA_2 \) is a negative logarithm of the molar concentration of competitive antagonist, which requires a doubling of the concentration of agonist to compensate for the action of the antagonist. The meaning of \( pA_2 \) is the affinity of the antagonist to the receptor (4). In the same way, the value of \( pA_{1/2} \) is a negative logarithm of the concentration of a potentiator, which requires a halving of the concentration of agonist so as to induce a given response. As shown in the appendix, the meaning of \( pA_{1/2} \) seems to be the affinity of the potentiator to a substance which weakens the effect of agonist by inactivation or uptake of the agonist, etc.

In the experiments of the reversible anticholinesterases, the concentration-response curves were shifted to the left without any change in maximum responses, and in parallel. These phenomena fulfilled the two requisites required for the type of potentiation, where the \( pA_{1/2} \) values can be calculated, by utilizing the equation 2. The \( pA_{1/2} \) values obtained in the presence of two different concentrations of neostigmine, or physostigmine, were close to each other, indicating that the \( pA_{1/2} \) value was independent on the concentration in the scope of the experiment. Therefore, it may be concluded that the index, \( pA_{1/2} \), expresses some specific property of the potentiator. The meaning of \( pA_{1/2} \) may be the affinity of the anticholinesterases to the acetylcholinesterase, in the same way as the \( pA_2 \), since the fact that acetylcholinesterase is inhibited competitively by reversible anticholinesterases is well known (5, 11). Furthermore, it must be noted that the \( pA_{1/2} \) value of neostigmine (6.95), obtained in this experiment, is very close to the negative logarithm of the \( K_m \) value (6.80) reported for the binding of cholinesterase and neostigmine (12–14).

The same type of potentiation as described above was also observed in the assay of the cardenolides. The concentration-response curves were shifted to the left in parallel. The maximum responses increased slightly, perhaps time-dependently, but no significant change was caused by the application of the cardenolides. The \( pA_{1/2} \) values obtained in the presence of the two different concentrations of a cardenolide were also similar.

Previously, Takeda et al. (6) reported a new method for the biological assay of cardiotonic steroids, in which the potentiating effects of some cardenolides on the K-induced contracture of frog ventricular muscle were compared, utilizing the parallel line assay technique. In the present study, the method was improved introducing a new scale for the
measurement of drug potentiation. For the comparison among the present method and
the other two methods reported previously, the relative potencies and 95% confidence
limits are shown in Table 2, as well as the smallest concentrations required for each method.
As can be seen, the 95% confidence limits of the relative potencies were close to each other,
indicating that the \( pA_{1/2} \) value is a useful indicator of the potentiating effect. Furthermore,
the present method requires much lower concentrations of the compounds than the other
two methods, indicating that it can be applied to the assay of compounds having lower
potency or lower solubility.

In this experiment, \( pA_{1/2} \) was revealed to be a useful parameter, which implies some
specific property of the potentiator. The \( pA_{1/2} \) value was obtained by the equation 2.
Whether or not this equation can generally be used for other potentiators remains undeter-
mined until \([B]_T\) and \(K_B\) introduced in the appendix are estimated or some other theoretical
formulation can be achieved.

**APPENDIX**

**Process I:** It is assumed that responses are induced by the binding of agonist A and
receptor R, the total amount of which is \([R]_T\), in the biophase. The concentration \([A]\) of
agonist A is not changed by the binding \((AR)\). Then, the following equation can be
obtained,

\[
\frac{[AR]}{[R]_T} = \frac{[A]}{[A]+K_A}
\]

(1),

where \(K_A\) is the dissociation constant of the binding of A and R.

**Process II:** Before the connection with receptor R, agonist A is inactivated quickly
by binding \((AB)\) with substance B. Therefore, the concentration of A in the neighborhood
of the receptor R is not \([A]_T\), which is that in the absence of substance B, but \(([A]_T-[AB])\).
The inactivation of agonist A depends on the amount of substance B, the concentration of
which \([B]\) is not affected by binding with A. Then, the amount of the complex \((AB)\) can
be obtained from the equation,

\[
\frac{[AB]}{[A]_T} = \frac{[B]}{[B]+K_B}
\]

(2),

where \(K_B\) is the dissociation constant of the binding of A and B. Since \([A]=[A]_T-[AB],\)

\[
\frac{[A]}{[A]_T} = \frac{K_B}{[B]+K_B}
\]

(2).

**Process III:** When agent C is given into the biophase, agent C reacts to substance B
with low dissociation constant or irreversibly. The amount of substance B decreases to
\([B]\), while in the absence of agent C, it is \([B]_T\). The concentration \([C]\) of agent C is not
changed by binding with substance B. Since the dissociation constant \(K_C\) is much lower
than \(K_B\), it may be assumed that formation of the complex \((AB)\) is much less than that of
the complex \((BC)\), and is negligible. This corresponds to the fact that the \(K_m\) value in the
binding of cholinesterase and neostigmine ($2 \times 10^{-7}$) (12-14) was much smaller than that of ACh and cholinesterase ($2-4 \times 10^{-4}$) (12). Then, the following equation can be given,

$$\frac{[BC]}{[B]_T} = \frac{[C]}{[C]+K_C},$$

therefore,

$$\frac{[B]}{[B]_T} = \frac{K_C}{[C]+K_C} \text{ .........................................................(3).}$$

When all these processes are present, the following equation can be obtained,

$$\frac{[AR]}{[R]_T} = \frac{1}{1 + \frac{K_A}{[A]_T} \left(1 + \frac{[B]_T}{K_B} \times \frac{K_C}{[C]+K_C}\right)} \text{ .............................................(4).}$$

When processes I and II are present and process III is absent, i.e., agent C is absent,

$$\frac{[AR]}{[R]_T} = \frac{1}{1 + \frac{K_A}{[A]_T} \left(1 + \frac{[B]_T}{K_B}\right)} \text{ .........................................................(5).}$$

To obtain the same responses in the presence and absence of agent C, the concentration of agonist A in the presence of agent C is required to be x times of that in the absence. Then, the following equation can be obtained,

$$\frac{[C]}{K_C} = \frac{[B]_T}{K_B} \left(1-x\right) + \left(1-x\right) \text{ .........................................................(6).}$$

If $[B]_T$ is much larger than $K_B$,

$$\frac{[C]}{K_C} = \frac{1}{x} - 1, \quad (0<x<1) \text{ .........................................................(7).}$$

If $[B]_T$ is just the same as $K_B$,

$$\frac{[C]}{K_C} = \frac{1}{2x-1} - 1, \quad (0.5<x<1) \text{ .........................................................(8).}$$

If $[B]_T$ is much smaller than $K_B$, the equation (4) does not differ from the equation (5). In this experiment, the equation (7) may be more acceptable, since x can be smaller than 0.5. Consequently,

$$\log([C]) - \log K_C = \log \left(\frac{1}{x} - 1\right),$$

$$pA_{1/2} = pA_x + \log \left(\frac{1}{x} - 1\right).$$
REFERENCES


8) DUNNETT, C.W.: New tables for multiple comparisons with a control. Biometrics 20, 482 (1964)


