ORIGINAL ARTICLES

Experimental Investigations

SENSITIZING EFFECT OF TACRINE ON M-CHOLINERGIC RECEPTORS IN GASTRIC SMOOTH MUSCLE OF RATS

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ABSTRACT

AIM: To find if tacrine exerts a sensitizing effect on the cholinergic receptors of gastric smooth muscles, and study some of the mechanisms inducing it and measure the relative intensity of tacrine’s effects on contractile activity.


RESULTS: We found that the threshold concentration for tacrine not reducing the acetylcholinesterase activity and not having an effect on the smooth muscle preparations was $1 \times 10^{-8}$ mol/l. This concentration, however, significantly increased the acetylcholine-induced contraction compared with the controls, after the smooth-muscle tissue was incubated for 60 or 100 min. Treating smooth-muscle preparations with tacrine in a concentration of $5 \times 10^{-6}$ mol/l triggered a contraction induced by the drug’s anti-cholinesterase activity. A secondary contraction was induced after 38.6 ± 5.6 min. There was no secondary contraction after the control acetylcholine-induced effect. Atropine ($1 \times 10^{-6}$ mol/l) inhibits this effect. Preliminary treatment of smooth muscle preparations with hexamethonium ($1 \times 10^{-6}$ mol/l) did not change significantly the intensity of the first phase of tacrine-induced contraction and shifted in time the appearance of the second contractile phase.

CONCLUSION: Tacrine has a sensitizing effect on M-cholinergic receptors; it occurs after a long incubation of the gastric smooth muscles with the drug and is manifested as a secondary contraction which is shifted in time and is significantly inhibited by atropine.

Key words: tacrine, M-cholinergic receptors, acetylcholine, smooth muscles, sensitization

INTRODUCTION

Tacrine is a cholinesterase inhibitor used in the management of Alzheimer’s disease.1 Besides the drug’s effect on the central nervous system, which is the basic effect that improves the patient’s cognitive processes, the drug is known to affect other tissues and organs as well. The effects that tacrine exerts on the motility and evacuation functions of the gastrointestinal (GI) tract are particularly strongly pronounced; these effects are results of the action the high level of endogenous acetylcholine has on the gastrointestinal smooth muscles (SM).2,3

It is well known that the anti-cholinesterase effect does not exhaust all possibilities of the primary pharmacological reaction which accounts for the therapeutic and diverse effects of tacrine. The effects which are result from the non-anti-cholinesterase and even the non-cholinergic mechanisms are also well expressed.4,5

Researchers are especially interested in studying whether there could be a non-anticholinesterase effect on the cholinergic mediation. One of the possible mechanisms of such an effect is tacrine’s ability to sensitize/desensitize the cholinergic receptors but the available data concerning that ability are quite contradictory.6,7 We consider that proving such a hypothesis is a question of special interest because

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of the significant expression of cholinergic receptors in the GI tract. That is a premise for intensification/reduction of the effects of the medicine’s anti-cholinesterase influence on various GI functions and could be of interest for the interpretation of the experimental and clinical results.

The aim of the present study was to investigate whether tacrine has a sensitizing effect on the cholinergic receptors of gastric SM as well as measure the relative intensity of its effects on the contractile activity in relation to the one of its anticholinesterase effects.

MATERIAL AND METHODS

EXPERIMENTAL ANIMALS AND SM PREPARATIONS

Adult male Wistar rats weighing between 250 and 300 g were used in the experiments. Prior to the experiments the animals were raised under identical laboratory conditions: temperature, food regime, access to water and light-and-dark cycle. The internationally approved principles of the European Convention for laboratory animal welfare were strictly followed (Helsinki, 1975, article 101, paragraph 5).

Circular SM preparations (13-14 mm x 1.1-1.2 mm) were dissected from the fundus of the stomach.

RECORDING OF THE CHOLINESTERASE ACTIVITY OF SM TISSUE WITH THE ELLMAN’S METHOD

The activity of the SM tissue acetylcholinesterase was determined with the help of spectrophotometry after preliminary homogenization. SM tissues were incubated with tacrine in the course of 30 min in the Krebs solution (37°C) and aerated with a gas mixture of 95% O₂ and 5% CO₂. The control samples were kept at the same conditions but without treating them with tacrine. After that period the preparations were homogenized in 1 x 10⁻² M Tris-HCl buffer in the following proportion: tissue (mg): buffer volume (μl) = 1 : 25. The buffer consisted of 5 x 10⁻³ M EDTA and 1 x 10⁻³ M N-ethylmaleimide, 1 M NaCl and 1% Triton X-100. The homogenization was performed with a Kinematica laboratory homogenizer for 2 minutes cooling in an ice bath. The homogenized tissues were centrifuged (Technne centrifuge, Denver Instruments-USA) at 9000 g for 15 minutes.

The enzyme activity of acetylcholinesterase was determined with the help of spectrophotometry and the Ellman’s method using Cary 1 spectrophotometer (Varian, Australia). The supernatant which was obtained after centrifugation of the tissue homogenates (0.1 ml) was mixed with 0.1 ml solution of acetylthiocholine iodide (7.5 mM in water) as an enzyme substrate. The quantity of the product of the enzyme reaction was determined using spectrophotometry after reaction with 0.8 ml of 0.375 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in a phosphate buffer. The changes of the absorption of the obtained solution were studied in the course of 3 minutes. “One unit of activity” was defined as the quantity of enzyme which catalyzed the hydrolyzation of one micromole of acetylthiocholine iodide for one minute at 25°C and pH 7.0 and it was calculated as μmol/min. We used the molar absorptivity of the reduced DTNB at 412 nm and pH 7.0, which was equal to 13.88 mM⁻¹.cm⁻¹. 9

The specific activity of the enzyme was calculated as follows: the activity which was determined by photometric measurements was divided by the tissue quantity (mg). The tissue concentration in the homogenate was 40 mg/ml. Each measurement was performed using a quantity of 0.1 ml that corresponded to 4 mg of tissue.

RECORDING OF THE CONTRACTILE ACTIVITY OF THE SM PREPARATIONS

The mechanical activity of the SM preparations was recorded with the help of isometry and was expressed in mN. The preparations were fixed motionless to a glass holder at one of the ends and to a Swema tensile detector (Stockholm, Sweden) at the other end using surgical threads.

During the experiments the SM preparations were placed in a tissue tank with Krebs solution (t = 37°C) which was constantly aerated with a mixture of O₂/CO₂ in proportions 19/1 (v/v). The value of the initial mechanical tension for the preparations obtained with the help of the tension-system was equal to 10 mN. The spontaneous contractile activity and the tonus of the preparations were stabilized for about 60 minutes. During that period the Krebs solution was replaced 2 or 3 times. The tonus level which was determined after 60 minutes of adaptation was accepted as a starting tonus or a zero level and further changes were read in relation to it.

Treatment with various substances was performed by adding strictly defined volumes of their concentrated solutions which was needed to reach the necessary concentration in the tissue tank. The volume of the added substance did not exceed 1/100 of the volume of the Krebs solution in the tank. The vitality of the SM tissues was tested through treatment with 1 x 10⁻⁶ mol/l acetylcholine twice at the beginning of the experiment and after each of...
the treatments with the studied substances. The electrical signal of the tensile detectors was magnified with the help of a K. Tesar – D 486 (Germany) or Microtechna (Prague, Czech Republic) tensile magnifiers. The mechanical activity was recorded on a paper band using a Linseiss (Selb, Germany) recorder.

**DRUGS, CHEMICAL AND SOLUTIONS**

The following drugs and chemicals were used in the experiments: tacrine, tris, EDTA, N-ethylmaleimide, triton X-100, DTNB, atropine sulphate, hexamethonium chloride, supplied by Sigma (St. Louis, MO); acetylcholine from Dispersa (Baeschlin – Germany). Acetylthiocholine iodide was produced by Boehringer Mannheim (Germany). Immediately before the experiments the dry substances were dissolved in bi-distilled water. The Krebs solution contained the following (mM): NaCl – 120; KCl – 5.9; CaCl₂ – 2.5; MgCl₂ – 1.2; NaH₂PO₄ – 1.2; NaHCO₃ – 15.4 and glucose – 11.5 and pH = 7.4. All chemicals which were necessary to prepare the solution were produced by Merck (Darmstadt, Germany). The pH value of the solution was measured by a microcomputer pH-meter, model 6201, produced by Jenco Electronics LTD (UK).

**STATISTICAL PROCESSING OF THE RESULTS**

The results were analysed statistically using the STATISTICA software. Analysis of variance was used; we calculated two main statistical parameters: the arithmetic mean (mean) and standard error of the mean (SEM). The statistical significance of data comparison was estimated by the Student’s t-test of independent samples; the level of significance was \( P < 0.05 \).

**RESULTS**

**DETERMINATION OF THRESHOLD CONCENTRATION FOR INHIBITION OF ACETYLCHOLINESTERASE ACTIVITY BY TACRINE IN GASTRIC SM TISSUES OF RATS**

We determined the threshold concentration at which tacrine has a significant effect on acetylcholinesterase activity in gastric SM tissues (7 samples for each of the concentrations used in the experiments).

The baseline specific enzyme activity in gastric SM preparations in controls was 7.22 U/mg. It did not get significantly reduced after the tissues were treated with tacrine in concentrations of \( 1 \times 10^{-8} \) mol/l and \( 1 \times 10^{-7} \) mol/l. Higher concentrations of the drug (\( 1 \times 10^{-6} \) mol/l) caused statistically significant drop of acetylcholinesterase activity down to 41% of baseline enzyme activity (Table 1).

**CHANGES OF THE INTENSITY OF CONTRACTIONS CONSEQUENTLY INDUCED BY ACETYLCHOLINE IN NORMAL KREBS SOLUTION AND AFTER INCUBATION OF THE SM PREPARATIONS WITH LOW CONCENTRATIONS OF TACRINE**

Successive treatments with equinormal concentrations of acetylcholine (\( 1 \times 10^{-6} \) mol/l) were performed (3 min long each). The treatments were realized for all of the SM preparations after they had stayed in a normal Krebs solution for 30, 60 and 100 min. After each treatment the solution in the tissue container was changed and normal contractile activity was restored. The contractions induced in such a way were similar in intensity with that in the controls and to one another (Table 2).

Tacrine’s concentrations of \( 1 \times 10^{-8} \) mol/l did not influence the contractile activity of the studied preparations. After the SM tissue was in contact for 30 min with tacrine in a concentration of \( 10^{-8} \) mol/l, the acetylcholine-induced contractions had similar identity with that in the controls. The reaction of SM preparations caused by \( 1 \times 10^{-6} \) mol/l acetylcholine increased significantly after treatment with the same concentration of tacrine for 60 or 100 min (Table 2).

<table>
<thead>
<tr>
<th>Concentration of tacrine (mol/l)</th>
<th>Specific enzyme activity (U/mg)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.22 ± 1.26</td>
<td></td>
</tr>
<tr>
<td>( 10^{-7} )</td>
<td>6.72 ± 0.69</td>
<td>0.55</td>
</tr>
<tr>
<td>( 10^{-6} )</td>
<td>2.96 ± 0.47*</td>
<td>0.001</td>
</tr>
<tr>
<td>( 10^{-5} )</td>
<td>0.90 ± 0.06*</td>
<td>0.0001</td>
</tr>
<tr>
<td>( 10^{-4} )</td>
<td>0.35 ± 0.04*</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

* Statistically significant difference in relation to the control.
Another experimental series aimed to compare the intensity of $1 \times 10^{-6}$ mol/l acetylcholine-induced effects in SM preparations after 30, 60 and 100 min of incubation with $1 \times 10^{-8}$ mol/l tacrine with their corresponding controls. For controls we used the corresponding concentrations which were produced by equimolar acetylcholine concentrations in pure Krebs solution which were obtained after each acetylcholine contraction on the background of tacrine.

The comparison of acetylcholine-induced contractions produced on the background of tacrine with their corresponding controls showed that there was no significant difference between them after 30 min of preliminary incubation in the cholinesterase inhibitor. After 60 min and 100 min of incubation the values of the control effects were significantly reduced (Table 3).

Table 2. Changes of the intensity of $1 \times 10^{-6}$ mol/l acetylcholine-induced contractions, realized in succession in a normal Krebs solution and after 30, 60 and 100 min of incubation of SM preparations with $1 \times 10^{-8}$ mol/l tacrine. Comparison is done separately for the results of each experimental type (with and without tacrine) in relation to the corresponding control

<table>
<thead>
<tr>
<th>Control</th>
<th>Acetylcholine-induced concentrations, mN</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 16</td>
<td></td>
</tr>
<tr>
<td>In normal Krebs solution</td>
<td>after 30 min, n = 16</td>
</tr>
<tr>
<td>5.02 ± 1.44</td>
<td>5.12 ± 1.56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control</th>
<th>Acetylcholine-induced concentrations, mN</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 6</td>
<td></td>
</tr>
<tr>
<td>In the presence of $1 \times 10^{-8}$ mol/l tacrine</td>
<td>after 30 min, n = 6</td>
</tr>
<tr>
<td>4.38 ± 1.58</td>
<td>5.30 ± 1.96</td>
</tr>
</tbody>
</table>

Table 3. Comparison between acetylcholine-induced effects after contact of the SM preparations with $1 \times 10^{-8}$ mol/l tacrine for 30, 60 and 100 min and the corresponding controls, obtained after change of the solution in the tissue tank

<table>
<thead>
<tr>
<th>Duration of the treatment with tacrine</th>
<th>n</th>
<th>Acetylcholine-induced contractions, mN</th>
<th>n, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min control</td>
<td>9</td>
<td>7.51 ± 3.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.18 ± 1.83</td>
<td></td>
</tr>
<tr>
<td>60 min control</td>
<td>6</td>
<td>10.65 ± 1.67</td>
<td>P = 0.013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.97 ± 0.71*</td>
<td></td>
</tr>
<tr>
<td>100 min control</td>
<td>9</td>
<td>9.71 ± 2.02</td>
<td>P = 0.009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.29 ± 1.40*</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05.

Figure 1. Changes in the characteristics of $5 \times 10^{-6}$ mol/l tacrine-induced SM contractions. 0 mN is the tonus of the SM preparation at which the development of the contraction begins (n = 9).
Characteristics of tacrine-induced contractions in normal Krebs solution and in the presence of atropine and hexamethonium

Treatment of SM with $5 \times 10^{-6}$ mol/l tacrine resulted in a contraction. It began about a minute after administration of the drug into the tissue container, and reaching a peak remained relatively unchanged. After a certain period of time a second contractile process developed (Fig. 1).

The maximum intensity of the second phase of contractile activity was significantly lower than that in the first phase. Table 4 shows the characteristics of each phase as well as the comparison with one another.

The preliminary (10-12 min) treatment of SM preparations with $1 \times 10^{-6}$ mol/l of hexamethonium did not change significantly the intensity of the first phase of the tacrine-induced contraction ($5 \times 10^{-6}$ mol/l). The characteristic, however, changed: after reaching the peak the intensity of the contraction started to decrease.

Hexamethonium did not affect the intensity of both phases of contractile reaction caused by $5 \times 10^{-6}$ mol/l of tacrine. Its presence in the solution shifted in time the appearance of the second contractile phase and reduced the time to reach maximum intensity (Table 4).

In the presence of atropine (10-12 min; $1 \times 10^{-6}$ mol/l) the second phase of tacrine-induced contractile reaction was not manifested (Table 4).

Characteristics of continued acetylcholine-induced contractions in normal Krebs solution

Acetylcholine concentrations of $1 \times 10^{-7}$ mol/l resulted in a tonic contraction of the gastric SM preparations whose intensity was commensurable with the one induced by $5 \times 10^{-6}$ mol/l of tacrine. It reached its peak after 2-3 min. Immediately after that a unidirectional process of minimization of its intensity occurred. No second contractile reaction was observed. The characteristics of the

Table 4. Characteristics of the two-phase tacrine-induced effect on gastric SM preparations in rats and changes appearing in the presence of hexamethonium and atropine. Comparison is done between the corresponding parameters of the I and II contractile phase, caused by $5 \times 10^{-6}$ mol/l tacrine in normal Krebs solution and in the presence of hexamethonium and atropine

<table>
<thead>
<tr>
<th>Hexamethonium, mol/l</th>
<th>-</th>
<th>$1 \times 10^{-6}$</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine, mol/l</td>
<td>-</td>
<td>-</td>
<td>$1 \times 10^{-6}$</td>
</tr>
<tr>
<td>Concentr. tacrine, mol/l</td>
<td>n = 12; $5 \times 10^{-6}$</td>
<td>n = 6; $5 \times 10^{-6}$</td>
<td>n = 6; $5 \times 10^{-6}$</td>
</tr>
<tr>
<td>I contractile phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to develop, min</td>
<td>3.3 ± 1.04</td>
<td>3.2 ± 0.7</td>
<td>-</td>
</tr>
<tr>
<td>Max. intensity, mN</td>
<td>1.4 ± 0.5</td>
<td>1.5±0.7</td>
<td>-</td>
</tr>
<tr>
<td>Time to appear, min</td>
<td>38.6 ± 5.6</td>
<td>70.8 ±10.5*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P = 0.0005</td>
<td></td>
</tr>
<tr>
<td>II contractile phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to develop, min</td>
<td>27.1 ± 11.5</td>
<td>5.9 ± 2.8*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P = 0.005</td>
<td></td>
</tr>
<tr>
<td>Max. intensity, mN</td>
<td>0.7 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>-</td>
</tr>
</tbody>
</table>

* P < 0.05.

Figure 2. Changes in the characteristics of $1 \times 10^{-7}$ mol/l acetylcholine-induced contraction of gastric SM preparations (gastric corpus), (n = 9).
acetylcholine-induced contractions and the changes of their intensity are presented in Fig. 2.

DISCUSSION

Preliminary continuous treatment of SM tissues with tacrine increases the intensity of acetylcholine-induced contractions. This fact may be accepted as an indication of a specific effect of the drug on the cholinergic receptors, by means of which acetylcholine realizes its contractile effects. This process is referred to as sensitization of receptors and was used for the first time by Kahlson & Uvnas, 1935. The process is characteristic of some other representatives of the group of cholinesterase inhibitors too.

It is relatively difficult to determine and assess the choline sensitizing effect. Very often the effects on the SM tonus and its contractile activity which result from such an influence are masked by the better expressed anticholinesterase effect of tacrine. That is why there is a certain possibility to record it if tacrine is applied in concentrations of 1×10⁻⁸ mol/l. Such concentrations, as shown by our results, do not inhibit significantly the cholinesterase activity; they do not change the levels of endogenous acetylcholine and do not influence the contractile activity of the experimental preparations by that mechanism.

Under these conditions, following preliminary incubation of 60 and 100 min (but not 30 min) with 1×10⁻⁸ mol/l tacrine, we recorded significant increase of the intensity of 1×10⁻⁶ mol/l acetylcholine-induced contractions, which was absent in the experiments with equimolar concentrations of acetylcholine according to an analogous time scheme and without preliminary incubation of SM tissues with tacrine. The only difference between the experimental treatments in the two trial series was the presence of tacrine. This suggests that the difference between the corresponding acetylcholine-induced effects is a result of the influence of the cholinesterase inhibitor on the cholinergic receptors. The experiments also illustrated that acetylcholine itself had not a choline-receptor sensitization effect.

Sensitization of receptors, as it is well known, take a long time to occur. Its relatively slow manifestation in isolated SM may be associated with the necessity of sensitization of a sufficient number of cholinergic receptors which are necessary for the tangible changes of acetylcholine contractile effectiveness. This fact reveals another possibility to record eventual sensitization caused by tacrine. It is associated with the investigation of the characteristics of continuous tacrine-induced contractions.

Treatment with 5×10⁻⁶ mol/l of tacrine caused contraction of SM preparations because of the inhibited cholinesterase activity. When the maximum specific contractile potential of this contraction was reached (100% inhibition of cholinesterase activity) it remained with relatively constant intensity for over 30 minutes. After 38.6 ± 5.6 min a second contractile process was recorded over the initial contraction. It needed 27.1 ± 11.5 min to develop completely. The occurrence of the second contractile process under the conditions of maximum inhibited acetylcholinesterase and constant levels of endogenous acetylcholine might be explained only by the sensitization of cholinergic receptors to the acetylcholine molecule.

Contraction induced by cholinergic sensitization did not occur on the background of atropine. This makes it reasonable to accept that M-cholinergic receptors, on which tacrine exerts specific influence, are involved in the process. The direct interaction between tacrine and the M-cholinergic receptors is not surprising. This is explained by the fact that independent of their different functions acetylcholinesterase (with which tacrine interacts) and cholinergic receptors have similar structural active centers.

Inhibition of N-type choline receptors which are well expressed in the intramural neuronal structures of the gastric wall by hexamethonium does not influence the intensity and/or the characteristics of the SM contractions. It, however, significantly delays the appearance of the second contractile process; this is associated with the effect of choline-sensitization and increased velocity of its development. We consider that these effects are result of elimination of the influence of neurotransmitter substances, which cease to be secreted in the presence of hexamethonium.

CONCLUSIONS

Tacrine has a sensitizing effect on the M-cholinergic receptors of the gastric SM in rats and this process is not associated directly with its anti-cholinesterase activity.

The sensitization of the M-cholinergic receptors causes the development of a second contractile process because of their heightened sensibility to acetylcholine and this sensitization enhances the contractile effectiveness of acetylcholine on the gastric SM of rats.

The intensity of the contraction which is caused
by the sensitization of the M-cholinergic receptors is significantly lower compared to the one that is caused by the anticholinesterase effect of tacrine.

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