

Muscarinic
acetylcholine
receptors in the pig
urinary bladder
biology essay



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Background and purpose. This investigation sought to identify the principal muscarinic receptor subtype associated with contraction of the pig bladder. Additionally, comparisons of muscarinic receptor expression in the pig bladder and caudate nucleus were conducted.

Experimental approach. Contractility of isolated strips of pig bladder was assessed using isotonic tension recordings in an organ bath. Radioligand binding to particulate preparations from pig caudate nucleus and bladder detrusor was assessed using [3H]-quinuclidinylbenzilate.

Key results.

The results obtained from the contractile response experiment showed that the best antagonists for pig bladder contractions were methoctramine, pirenzepine and oxybutynin, this was according to the pEC₅₀ data. These results led to the identification of the presence of M1 and M3 receptor subtypes in the bladder. Whereas M1 and M2 receptor subtypes were found to occur in the brain. Pirenzepine exhibited the smallest R_{max} value, and was therefore the most successful antagonist. Whereas 4-DAMP had the largest R_{max} value, identifying this as the worst antagonist.

Conclusions and Implications.

It is clear that M3 muscarinic receptors are found in the bladder, but are absent in the brain. This is made more certain as 4-DAMP showed fairly low affinity for muscarinic receptors in the bladder, but its affinity was higher than that recorded in the brain which is known to contain a low amount of M3 receptors. 4-DAMP also has the highest affinity recorded in the brain, due to binding at the M1 muscarinic receptor.

Introduction

There are five distinct types of muscarinic receptors (M1, M2, M3, M4, M5), all of which belong to the same family of G-protein-coupled receptors. M1 receptors are found mainly in the cortex and hippocampus of the brain, but also on the CNS and peripheral neurons. These receptors are excitatory, this excitation is produced by a decrease in potassium ions which causes membrane depolarization.

M2 receptors exert inhibitory effects; they are present in the heart and so are of little importance in this experiment.

M3 receptors reside in smooth muscle. These receptors are excitatory and produce vasodilatation and bronchodilatation, via an increase in intracellular calcium levels.

As this experiment includes analysis on the effects of muscarinic antagonists on muscarinic receptors in the brain and bladder, it is mainly the M1 and M3 receptors which are being concentrated on.

The aim of the experiment was to add a range of antagonists pig bladder tissue, and then recording the effect each had on the tissue's contraction. The five antagonists used in the experiment were atropine, pirenzepine, methoctramine, 4-DAMP and oxybutynin. Each of the antagonists used are able to bind to muscarinic receptors, but they bind to different subtypes as they have differing affinities.

Atropine is a non-selective antagonist, which acts via competitive inhibition of muscarinic acetylcholine receptors. It is a naturally occurring alkaloid

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which can be found in solanaceous plants, for example the deadly nightshade (*Atropa belladonna*). Atropine causes anti-cholinergic effects such as mydriasis, salivary inhibition, tachycardia, inhibition of GI motility and smooth muscle relaxation. It can be used clinically to treat anticholinesterase poisoning, bradycardia and GI hypermotility.

Pirenzepine is a muscarinic receptor antagonist, and is slightly selective for the M1 sub-type. It is used clinically for the treatment of peptic ulcers, whereby it inhibits gastric acid secretion.

Methoctramine is a slightly M2 selective antagonist. It has also been found to show selectivity for cardiac M2 muscarinic receptors, but to have low affinity for both vascular M2 and ganglionic M1 receptors.

4-DAMP is a slightly M3 selective antagonist, although it has only low affinity. There is no clinical use for 4-DAMP, it is mainly used in experiments for the analysis of muscarinic receptors.

Oxybutynin is a non-selective muscarinic antagonist. It is used clinically to decrease muscle spasms of the bladder in patients suffering from frequent urination or urge incontinence.

During the contractile experiment these antagonists will be used to determine which of the muscarinic receptors are present in the bladder, and which occur in the greatest amount. The same antagonists will then be used in the radioligand experiment, to compare each of their effects on brain tissue. Once both experiments are performed, the results can be used to compare the presence of muscarinic effects in the bladder and brain tissue.

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Materials and Methods

Contractile response studies

Strips of urinary detrusor were stored overnight at 4 °C in Kreb's Ringer solution [composition (mM): NaCl (118); KCl (4.9), MgCl₂ (1.2); KH₂PO₄ (1.2); D-glucose (12); NaHCO₃ (25); CaCl₂ (1.3); gassed with O₂: CO₂ (95: 5)], as previously described (Lot and Wilson, 1994). On the morning of the experiment, tissue was removed from the refrigerator and allowed to equilibrate to room temperature. Thereafter, strips were mounted in an organ bath at 37 °C in Kreb's Ringer solution. Contractility was monitored using isotonic transducers connected to CED 1502 amplifiers and recorded on a personal computer running Spike 2 software (CED, Cambs, UK).

A steady baseline was then achieved for 20 minutes before 1ml 3M KCl was added to 50ml of Kreb's solution (in water bath), to achieve a final concentration of 60mM. The tissue was then washed out three times once a maximal response was recorded, and this was then left for a further 20 minutes until a steady baseline was achieved once again.

10 dilutions containing varying concentrations of carbachol were then prepared in LP4 tubes, using 3-fold dilutions. A cumulative-concentration curve was created by adding the preparation containing the lowest concentration of carbachol to the organ bath first. After waiting for 7 minutes, the next preparation with a higher concentration was added. This process was repeated without washing out the tissue, until there was no further increase in tissue tone recorded.

Once this part of the experiment was completed, the tissue was washed out 3 times with warm Krebs's solution. This was followed by the addition of the putative antagonist and the tissue was left for 60 minutes. After achieving a steady baseline, a cumulative-concentration curve was created by carrying out the same process with increasing concentrations of carbachol as before; but this time in the presence of the putative antagonist. The results were printed off once the process was completed.

Radioligand binding studies

Radioligand binding to total particulate preparations from the pig was conducted essentially as previously described (Alexander et al., 1994). Briefly, tissue was homogenised in 10-30 volumes of 0.1 M phosphate buffer (pH 7.5) using an Ultra-Turrax homogeniser. After centrifugation at 30 000 g for 15 minutes at 4°C, the supernatant layer was discarded. This homogenisation/centrifugation cycle was repeated twice more and the resulting pellet was re-suspended in 10 volumes. After storage at -20 °C, thawed tissue was incubated in a total volume of 500 µL 0.1 M phosphate buffer (pH 7.5), containing 0.25 nM [³H]-QNB for 30 minutes at 37 °C. Rapid filtration with repeated washing allowed isolation of bound radioligand, which was then estimated using liquid scintillation counting.

A dilution curve was prepared using 10-fold dilutions once the drug was provided, this contained the highest concentration. 32 LP4 tubes were labelled 1-32 and these were placed in test tube racks. 50 µl aliquots of different concentrations of drug were then added to tubes 5-28, with each being made up to 500 µl by adding 450 µl of buffer.

50 µl of buffer was added to tubes 1-4, these acted as a control whilst atropine was added to the remained tubes 29-32.

400 µl of the radioligand was added to each of the tubes, with 50 µl of particulate preparation being added to the tubes in groups of 10 at 12 minute intervals. Once this process was completed, the tubes were incubated in a water bath at 37°C for 30 minutes.

The Whatman GF/B filters were placed in the 10-place manifold and were wet using the buffer solution. Ten of the tubes were then removed from the water bath, and 3ml of ice-cold buffer was added to each of them. They were filtered immediately after this process. Once two additions of 3ml of ice-cold buffer had taken place on each filter paper, they were washed. This was repeated for each of the LP4 tubes, once incubation in the water bath was halted on their removal.

The filter papers were then placed into labelled scintillation vials, they were then analysed.

Data analysis and statistical procedures

There are equations which can be used to analyse the data collected in the experiment, once the information has been put into a concentration curve graph. The first equation used is the Gaddum equation:

$$pK_i = \log (CR - 1) - \log [Ant]$$

In order to make use of this equation it is necessary to work out the EC₅₀, which is the effective concentration of drug required to give 50% of the

maximum response. This EC50 value can be obtained from the concentration curve, as it is at 50% of the maximum response on the graph. The results collected from the graphs were Molar, these can be converted to μM by multiplying the values by 106. These values can now be used to produce a concentration ratio, indicating the EC50 response with and without the presence of the antagonist.

Graph 1 shows bladder tissue contractile responses to histamine in the presence of different concentrations of promethazine. The EC50 values recorded for histamine and promethazine were -6.5 and -5 respectively. By converting these Molar values to μM by using the technique stated above, histamine 0.316 μM and promethazine 3.16 μM can be inputted into the Gaddum equation. The pKi value which is calculated in this equation is an indication of potency of the antagonist used, and a high pKi indicates a high affinity for the receptor. The pKi in this case was -8.5.

As the radioligand was being carried out, calculations were made in order to work out the Kd and Bmax. The equation used in the radioligand binding experiment was the Cheng-Prusoff equation:

$$\text{IC}_{50}/\text{K}_i = 1 + [\text{A}]/\text{K}_d$$

To work out the pKi, the same process as the contractile response experiment is used. Then the pIC50, the concentration of the antagonist which displaces 50% of the ligand, can be calculated. It is then necessary to work out the IC50 value to be used in the Cheng-Prusoff equation, this is achieved by -log of the pIC50 value. Finally, -log of the Ki obtained from the equation gives a pKi value of 8.2.

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Drugs, chemicals, reagents and other materials

Porcine material (from pigs of the modern Hybrid white strain, either sex, approximately 50-70 kg) was obtained from an abattoir and transported rapidly to the laboratory on ice.

[3H]-QNB (specific activity 1591 GBq mmole⁻¹) was obtained from Amersham Pharmacia Biotech (Herts, UK), while muscarinic receptor ligands were all obtained from Sigma (Dorset, UK). All drug and molecular target nomenclature conforms to the British Journal of Pharmacology's ' Guide to Receptors and Channels' (Alexander et al., 2008).

Results

Table 1. Contractile response results:

Drug

pEC50

Rmax

Concentration Ratio

pKi

Atropine

4. 5

131

20. 9

9. 1

Pirenzepine

4. 3

109

42. 6

7. 4

Methoctramine

4. 3

50. 2

7. 1

4-DAMP

4. 6

139

37. 9

9. 2

Oxybutynin

4. 3

210.5

8.1

Water

4.7

116

5.0

Table 1 shows the results obtained from the contractile response experiment, whereby the pig bladder tissue was exposed to five antagonists and the contractility of the tissue was measured.

Table 2. Radioligand binding results:

Brain

Bladder

Drug

pKi

SEM

pKi

SEM

Atropine

9.8

0.1

9.8

0.1

Pirenzepine

7.7

0.2

8.0

0.6

Methoctramine

8.0

0.0

7.7

0.1

4-DAMP

9.2

0.0

8.4

0.1

Oxybutynin

7.4

0.0

7.8

0.0

Carbachol

4.8

0.1

4.3

0.0

Table 2 shows the results obtained from the radioligand experiment, which identified the different muscarinic receptors found in the brain and bladder tissue.

Discussion and conclusions

The conclusions that can be made from the results are that the main types of muscarinic receptor involved in the contraction of the bladder are the M1 and M3 receptor. Whereas, M1 and M2 muscarinic receptor subtypes occur in the brain. Therefore, an ideal drug for therapeutic treatment of urge

incontinence and bladder dysfunction would be M3 selective. This would not have any adverse effects in the brain, as M3 receptors are not present in this part of the body.

Bladder contractions occur due to activation of muscarinic receptors leading to an increase in intracellular calcium, which causes contraction of the smooth muscle. The results collected in the contractile response experiment were due to antagonism of M1 and M3 receptor subtypes. 4-DAMP recorded a pKi value of 9.2, a value which corresponded with the M3 subtype and was the highest of all the antagonists. This antagonist is M3 selective but also has affinity for the M1 muscarinic receptor; this may have caused its high pKi value to be due to binding at this muscarinic receptor subtype.

Atropine recorded the second highest pKi, 9.1. This was to be expected as it is a non-selective antagonist, with high affinity for each of the muscarinic receptor subtypes able to cause contraction of the bladder tissue.

Oxybutynin recorded the third highest pKi value, 8.1. Oxybutynin has a slightly higher affinity for the M3 muscarinic receptor, therefore the pKi value is due to binding at this receptor subtype.

Pirenzepine, being an M1 selective antagonist, would be expected to have a similarly high pKi to 4-DAMP. This was not the case as pirenzepine only recorded a pKi of 7.4, a value expected to be obtained from M3 selective antagonists.

Methoctramine recorded a pKi value of 7.1, the lowest of all the antagonists.

Although it is an M2 selective antagonist, the pKi value leads to the

conclusion that contractile response is due to the presence of M1 or M3 receptors.

The results obtained in the radioligand experiment revealed that mostly M1 and M2 muscarinic receptors occur in the brain. Methoctramine has low affinity at the M1 receptor, even so, the results recorded in the experiment showed the antagonist to have high affinity. The pKi range of the M2 receptor subtype for methoctramine is 7.8-8.3. Therefore, the recorded pKi value of 8.0 suggests the presence of M2 receptor subtypes in the brain. The pKi of 8.0 is quite far from the pKi range of methoctramine for the M3 receptor subtype, leading to the conclusion that there are a small number of M3 receptors in the brain.

Atropine, pirenzepine, 4-DAMP and oxybutynin antagonists are able to act at the M1 muscarinic receptor and each of these antagonists possess similar affinities for the receptor. Atropine (pKi 9.8) and oxybutynin (pKi 7.4) are both non-selective antagonists, so as in the contractile response will have fairly high affinities for any of the muscarinic receptor subtypes present in the brain. Pirenzepine recorded a pKi value of 7.7; this failed to fall into any of the pKi ranges expected for the muscarinic receptors. The value was closest to the M1 subtype range (7.8-8.5). The SEM recorded was the highest of all the antagonists (0.2) concluding that some of the results may have been anomalous, with most of the pKi values falling within the M1 range. The pKi range of pirenzepine for the M3 receptor subtype is 6.7-7.1. The pKi recorded, much like that of methoctramine, was quite far from the M3 range.

4-DAMP recorded a high pKi of 9.2. This pKi value fell into the ranges for both the M1 and M3 muscarinic receptors, showing high affinity of the antagonist for both subtypes. In this experiment the pKi value recorded was with respect to the M1 receptor, not the M3 subtype.

The non-selective muscarinic antagonist oxybutynin is the principle drug used to treat urge incontinence. This antagonistic drug possesses anticholinergic and antispasmodic properties, which together act on the bladder to inhibit micturition. However, there are significant adverse effects associated with this choice of therapeutic treatment; such as dry mouth, constipation and blurred vision. These side effects highlight the non-selectivity of oxybutynin as each occurs due to antagonism at the M1 receptor. The radioligand binding experimental results show that oxybutynin has a pKi of 7.8 in the bladder, which is only slightly higher than the pKi of 7.4 recorded in the brain. These pKi values indicate that oxybutynin is slightly more selective towards the M3 receptor subtype present in the bladder. However, it is evident that the antagonist also has significant affinity towards the M1 and M2 receptor subtypes which are present in the brain.

There were a few limitations encountered in both parts of the experiment. The contractile response experiment was carried out for a fairly short amount of time, more reliable results could be obtained by increasing the amount of time that the experiment is undertaken. As not all of the tissues used in the experiment were of the same source or size, the results obtained were inconsistent the responses recorded were of varying degrees. By ensuring every piece of tissue is the same size, more accurate and reliable could be obtained. Human error when collecting and interpreting the data in <https://assignbuster.com/muscarinic-acetylcholine-receptors-in-the-pig-urinary-bladder-biology-essay/>

the experiment could have caused considerable variations in the results recorded.

During the radioligand binding experiment complications arose due to contamination, with various external factors such as temperature and buffer strength affecting the results.