Modulation of Contractile Responses in the Isolated Guinea-pig Ileum Organ Bath Preparation PART 2 - Lab Report

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1 Aim

The goal of this experiment is to analyse the effects of direct electrical stimulation along with different combinations and concentrations of drugs, including agonists and antagonists, to smooth muscle contractions in the GIT of guinea pigs, therefore providing a better understanding of the role of the ENS in the GIT as well as the effects of electrical stimulation and drugs on the ENS.

2 Methods

The method here is based on that described in the online lab manual of BPS2011.

Organ bath set up

1. The organ chamber was connected to a force transducer, which was connected to a computer and used to measure the force of the contractions.
2. The organ chamber was set by preparing and connecting a reservoir of Aerated Krebs, before it was filled and the volume was adjusted using the taps. Oxygen was supplied to the chamber from a gas tank, whose flow was also adjusted using its tap.
3. The functionality of the setup was tested by applying 1 g of tension to force transducer, which was then released. The force was measured and recorded by the computer to ensure that the setup was working properly.

Tissue set up

4. The guinea pig was stunned and exsanguinated, and we surgically removed its ileum for the experiment.
5. The ileum was cut into segments of about 1 cm in length and preserved in Aerated Krebs, which simulated the physiological environment and kept the tissue alive for the experiment.

6. A cotton loop was attached to each ends of the ileum segment, before one end was hooked into the organ chamber and the other was connected to the force transducer.
7. The tension applied to the ileum was adjusted to 1 g.
8. The tissue was washed with Aerated Krebs several times to remove any impurities.
9. The tissue was then checked for stimulation response by applying 300 μL of 10^{-6} M acetylcholine (ACh) to it, using a pipette. The response tension was measured and recorded.
10. Step 8 was repeated to remove any residual ACh, until the response tension returned to the baseline.

2.1 Experiment 1: Action of drugs on the isolated guinea pig ileum

11. Increasing concentrations of acetylcholine, starting at the lowest concentration (1x10$^-$9 M), were added to the bath, followed by the addition of histamine. This resulted in a contraction in the segments of the guinea pig ileum.
12. The maximum amplitude of the contraction was measured and recorded as relative to a response 'R' for each concentration of acetylcholine and histamine stimulation.
13. The standard dose (submaximal concentration) for acetylcholine and histamine were determined by analysis of the recordings.

2.2 Experiment 2: Dose-response curve for acetylcholine

14. A range of concentrations (including a 'no drug' blank control) of acetylcholine was added to the segments of the guinea pig ileum to determine the maximum response to acetylcholine and the lowest concentration of acetylcholine producing any contraction. The effect of each dose was tested 4 times to determine the mean response.
15. Add 4 additional tests in between and record the responses to complete the dose-response curve.
16. The ED_{50} value for acetylcholine was found according to the dose-response curve, followed by calculation of the slope for the dose-response curve and the dose for ED_{50}.

2.3 Experiment 3: Dose-response curves and the effects of antagonists

17. The effects of increasing concentrations of atropine on acetylcholine-induced contractions were tested. The tests were carried out with a standard concentration of acetylcholine (8x10^{-8} M), including a no-drug control.
18. The concentration of atropine that produced a greater than 50% reduction in response to acetylcholine was determined.
19. The concentration of atropine from the previous point was used to test the effects of a range of concentrations of acetylcholine. The curve was noted and compared to the curve produced when adding acetylcholine alone.

20. The calculation for the dissociation constant (K_B, the concentration of the drug which occupies 50% of the receptors) was noted.

21. The ED_{50} for acetylcholine in the presence of atropine was calculated (as per point 8), as well as the dose-ratio and the KB for atropine.

2.4 Experiment 4: Transmural stimulation of the guinea pig ileum

2.4.1 Part 1: Determination of the stimulus voltage for submaximal contractions

22. A stimulus electrical shock of 0.1 Hz frequency, 0.1 ms duration and 6 repeat pulses was applied to the guinea pig ileum. The voltage was from 4 V to 14 V at an interval of 2 V. The response relative to 'R' was recorded.

2.4.2 Part 2: Investigation of the effects of different drugs on the response to transmural stimulation

23. Different drugs including atropine, clonidine, morphine, naloxone and phentolamine, as well as 'no drug' control was applied to the ileum, before the application of transmural stimulation of 0.1 Hz and an optimal voltage of 10 V. The response relative to 'R' was recorded.

2.4.3 Part 3: Investigation of the effects of antagonists on their respective agonists in the response to transmural stimulation

24. 10^{-7} M phentolamine or naloxone, as well as 'no drug' control, was applied to the ileum, before we apply the agonists clonidine, morphine, as well as 'no drug' control, respectively. The response relative to 'R' was recorded.

2.5 Experiment 5: Effects of transmural high-frequency electrical stimulation of the ileum

2.5.1 Part1: Investigation of the effects of transmural high-frequency electrical stimulation alone on the response to acetylcholine

25. The ileum was stimulated at 0.1 Hz for 1 min to establish a baseline response. The response relative to 'R' was recorded.

26. The ileum was then stimulated at a high frequency of 10 Hz for 5 min. The response relative to 'R' was recorded.

27. The ileum was then stimulated at 0.1 Hz for 1 min to establish a post-stimulation response. The response relative to 'R' was recorded.

28. The ileum was thoroughly washed with Krebs solution to remove any residual acetylcholine.

29. The ileum was then stimulated at 0.1 Hz for 1 min for recovery. The response relative to 'R' was recorded.

2.5.2 Part2: Investigation of the effects of transmural high-frequency electrical stimulation on naloxone response

30. The ileum was stimulated at 0.1 Hz for 1 min to establish a baseline response. The response relative to 'R' was recorded.
31. The stimulation was switched off and 10^{-7} M naloxone was applied to the ileum for 5 min.
32. The ileum was stimulated at a high frequency of 10 Hz for 5 min to establish a post-stimulation response. The response relative to 'R' was recorded.
33. The ileum was then stimulated at 0.1 Hz for 1 min to establish a post-stimulation response. The response relative to 'R' was recorded.
34. The ileum was thoroughly washed with Krebs solution to remove any residual naloxone.
35. The ileum was then stimulated at 0.1 Hz for another 1 min for recovery. The response relative to 'R' was recorded.

2.5.3 Part3: Investigation of the effects of transmural high-frequency electrical stimulation on phentolamine response

36. Repeat steps 1-6 of Part 2, but with 10^{-7} M phentolamine instead of naloxone.

2.6 Cleaning up

37. The tissues were placed in the animal bags provided.
38. The organ bath was rinsed with Tyrode's silution and scrubbed with the bottle brush provided.
39. Drug dilution tubes were placed in the hard rubbish buckets provided.
40. The experimental data was analysed and the results were recorded.
41. All the rubbish on the bench was clean up.

3 Results

3.1 Tables and figures

3.1.1 Raw response data

Table 1: Action of antagonists against agonists on the ileum in experiment 1 part 2

Antagonist	Response Tension (R)											
	Acetylcholine						Histamine					
	1	2	3	4	5	6	1	2	3	4	5	6
None	43	41	45	43	41	43	47	45	45	47	47	45
Atropine	4	4	3	4	2	4	47	45	43	43	47	45
Mepyramine	41	43	45	43	43	43	6	8	4	8	6	4

Table 2: Dose-response data of the agonist acetylcholine in experiment 2 part 2

Logc	Response Tension (R)			
	1	2	3	4
-8.7	0	1	0	0
-8.1	8	10	9	8
-7.7	24	20	22	22
-7.1	43	41	45	41
-6.7	52	54	52	53
-6	59	58	60	59

Table 3: Dose-response data of the antagonist atropine on acetylcholine in experiment 3 part 1

Logc	Response Tension (R)			
	1	2	3	4
-10	44	43	43	43
-9	32	33	32	33
-8.4	17	17	15	15
-8.1	9	10	9	10
-7.7	1	2	2	1
-7.4	0	0	0	0

Table 4: Dose-response data of acetylcholine in the presence of atropine in experiment 3 part 2

Logc	Response Tension (R)			
	1	2	3	4
-8	0	0	0	0
-7.7	2	3	2	3
-7.1	16	17	15	16
-6.4	44	43	44	42
-5.7	54	53	55	55
-5.1	60	60	60	60

Table 5: The effect of electrical stimulation on the ileum in experiment 4 part 1

Voltage	Response Tension (R)					
	1	2	3	4	5	6
4	8	8	8	8	8	8
6	11	15	15	15	15	15
8	30	30	30	30	30	30
10	56	56	56	56	56	56
12	60	60	60	60	60	60
14	60	60	60	60	60	60

Table 6: The effect of drugs on the ileum in experiment 4 part 2

Drug	Response Tension (R)					
	1	2	3	4	5	6
None	56	56	56	56	56	56
Atropine	1	0	1	0	0	0
Clonidine	20	19	15	12	12	10
Morphine	25	23	15	13	11	8
Naloxone	56	56	56	56	56	56
Phentolamine	56	56	56	56	56	56

Table 7: The effect of drug combinations on the ileum in experiment 4 part 3

Drug Combination	Response Tension (R)					
	1	2	3	4	5	6
None	56	56	56	56	56	56
Clonidine alone	20	19	15	12	12	10
Clonidine + Naloxone	20	19	15	12	12	10
Clonidine + Phentolamine	56	56	56	56	56	56
Morphine alone	25	23	15	13	11	8
Morphine + Naloxone	56	56	56	56	56	56
Morphine + Phentolamine	25	23	15	13	11	8

3.1.2 Response curves

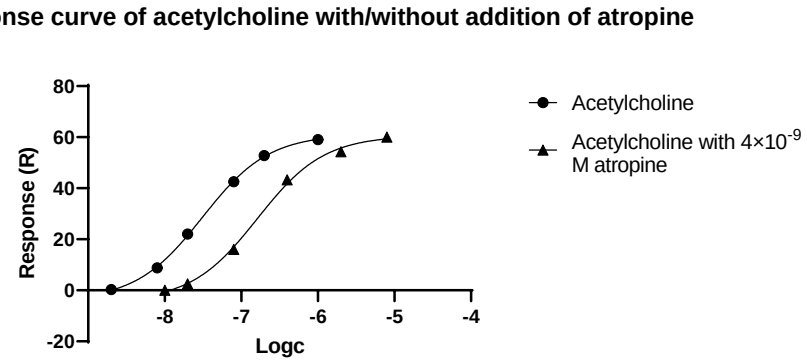

Figure 1: Dose-response curve of acetylcholine with/without addition of atropine The concentration of the antagonist atropine used in this experiment is the optimal 4×10^{-9} M. For each of the curves, the concentration of acetylcholine was increased gradually so that the response tension rises from minimum visible to submaximal. The concentration of the agonist is represented in logarithm and the curve was fitted with a non-linear regression model to determine the ED_{50} and the corresponding dose of acetylcholine.

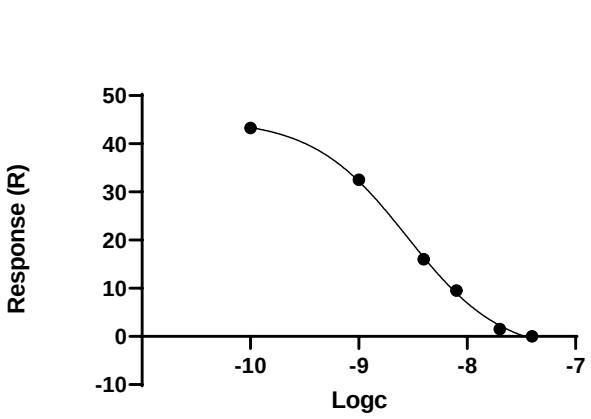

Figure 2: Dose-response curve of antagonist atropine on 8×10^{-8} M acetylcholine The concentration of the agonist acetylcholine used in this experiment is the optimal 8×10^{-8} M. Atropine was increased gradually so that the response tension drops from maximal to fully inhibited. The concentration of the antagonist is represented in logarithm and the curve was fitted with a non-linear regression model to determine the dose-ratio and the K_B of acetylcholine.

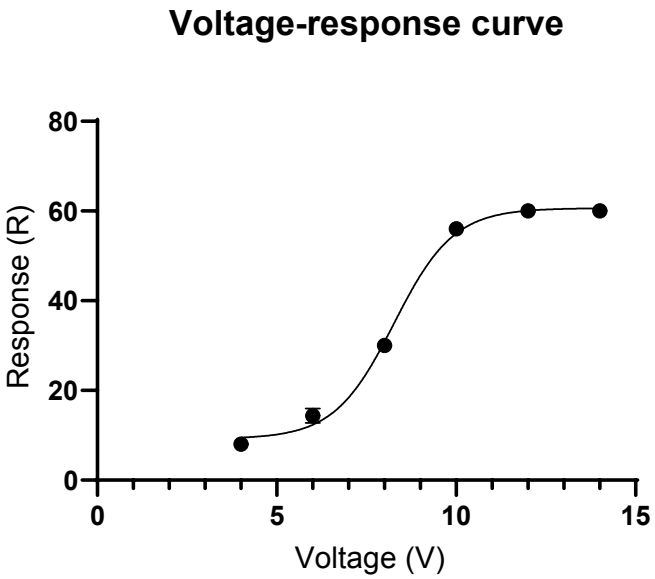

Figure 3: Voltage response curve of Guinea-pig ileum to electrical stimulation with drugs The voltage was increased from 4 to 14 V in steps of 2 V. The response tension was measured and recorded for each voltage, in relative units. The curve was fitted with a non-linear regression model to determine the maximum response tension, half-maximal voltage, etc.

3.2 Analysis

Two-way ANOVA analysis of responses to acetylcholine or histamine, in the presence of no-drug and the antagonists, was performed. The results are shown in Table 12 and Table **??**.

Table 8: Two-way ANOVA analysis of responses to acetylcholine, in the presence of no-drug and the antagonists

Source of Variation	% of total variation	P value	P summary	Significant?
Interaction	73.64	<0.0001	****	Yes
Row Factor	25.38	<0.0001	****	Yes
Column Factor	0.4887	<0.0001	****	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	9246	2	4623	F (2, 30) = 2280	P<0.0001
Row Factor	3187	2	1594	F (2, 30) = 785.8	P<0.0001
Column Factor	61.36	1	61.36	F (1, 30) = 30.26	P<0.0001
Residual	60.83	30	2.028	-	-

Mean of Acetylcholine	Mean of Histamine	Difference between means	SE of difference	95% CI of difference
29.72	32.33	-2.611	0.4747	-3.581 to -1.642

From the ANOVA analysis, we found that most of the variations is explained by the interaction, rather than the row factor or the column factor. Therefore, we suggested that there is a corresponding relationship between the agonist and the antagonist. Therefore, a Dunnett's multiple comparison analysis was conducted:

Table 9: Dunnett's multiple comparison analysis of responses to acetylcholine, in the presence of no-drug and the antagonists

Group		Mean Diff.	95% CI of diff.	Rejected	Summary	Adjusted P
Acetylcholine	None vs. Atropine	39.17	37.26 to 41.07	Yes	****	<0.0001
	None vs. Mepyramine	-0.3333	-2.241 to 1.575	No	ns	0.8880
Histamine	None vs. Atropine	1.000	-0.9081 to 2.908	No	ns	0.3810
	None vs. Mepyramine	40.00	38.09 to 41.91	Yes	****	<0.0001

The null hypothesis, which states that there is no difference between the responses to acetylcholine in the presence of no-drug and the antagonists, was rejected for the responses to acetylcholine in the presence of atropine and mepyramine. The null hypothesis was not rejected for the responses to histamine in the presence of no-drug and the antagonists. Therefore, we suggested that atropine is an antagonist for acetylcholine, while mepyramine is an antagonist for histamine.

Non-linear fit of the responses to acetylcholine with/without atropine was performed to calculate the ED_{50} values. The results are shown in Table 12.

Table 10: Non-linear fit of the responses to acetylcholine with/without atropine

Group	Best-fit values					Goodness of Fit			
	Bottom	Top	$LogEC_{50}$	EC_{50}	Span	df	R^2	SS.	Sy.x
ACh	-3.926	61.12	-7.508	3.103×10^{-8}	65.05	21	0.9974	30.91	1.213
Ach + Atropine	-4.363	60.63	-6.789	1.624×10^{-7}	64.99	21	0.9976	33.12	1.256

Group	95 % CI (profile likelihood)				Number of points	
	Bottom	Top	$LogEC_{50}$	EC_{50}	X	Y analysed
ACh	[-5.393, -2.500]	[59.94, 62.33]	[-7.555, -7.462]	$[2.788\times10^{-8}, 3.454\times10^{-8}]$	24	24
ACh + Atropine	[-5.668, -3.092]	[59.55, 61.73]	[-6.836, -6.743]	$[1.459\times10^{-7}, 1.808\times10^{-7}]$	40	24

Non-linear fit of responses to acetylcholine stimulation with/without atropine, with increasing dose of acetylcholine, showing the difference in dose-response parameters. The bottom and top values are the minimum and maximum response, respectively. The EC_{50} value is the concentration of acetylcholine that produces a response halfway between the bottom and top values. The span is the difference between the top and bottom values. The 95% confidence intervals (CI) are shown for each parameter. The goodness of fit is shown as the R^2 value, the sum of squares (SS) and the standard error of the estimate (Sy.x). The number of points is the number of X values and the number of Y values analysed.

From fitted function, we can find the ED_{50} values for acetylcholine with and without atropine, which are 3.103×10^{-8} and 1.624×10^{-7}, respectively. The ED_{50} value of acetylcholine with atropine is higher than that of acetylcholine without atropine, which means that atropine increases the ED_{50} of acetylcholine, i.e. seemingly exerts an inhibitory effect of the stimulating ability of acetylcholine. To further confirm that inhibitory effect, we have to perform a two-way ANOVA. Since GraphPad Prism 9 does not offer an option to perform ANOVA analysis within each column (which stands for a group with/without the antagonist) if there's experiment data for only one of the two groups at any certain dose, we had to make sure that the test for each selected dose was carried out, or the result is deducible as it went before the minimum response or after the submaximal.

Here is the result of the two-way ANOVA analysis on the completed data set:

Table 11: Two-way ANOVA analysis of responses to acetylcholine stimulation with/without atropine, with increasing dose of acetylcholine. ($\alpha = 0.05$)

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	4.101	<0.0001	****	Yes
Row Factor	90.46	<0.0001	****	Yes
Column Factor	5.351	<0.0001	****	Yes

Source of Variation	SS	DF	MS	F (DFn, DFd)	P value
Interaction	3079	15	205.2	F (15, 96) = 316.5	P<0.0001
Row Factor	67904	15	4527	F (15, 96) = 6981	P<0.0001
Column Factor	4016	1	4016	F (1, 96) = 6194	P<0.0001
Residual	62.25	96	0.6484	-	-

Mean of Acetylcholine	35.95
Mean of Acetylcholine+Atropine	24.75
Difference between means	11.20
SE of difference	0.1424
95% CI of difference	[10.92, 11.49]

Two-way ANOVA analysis of responses to acetylcholine stimulation with/without atropine, with increasing dose of acetylcholine. The row factor is the existence of the antagonist atropine, while the column factor is the dose of acetylcholine. Both factors are significant explanatory variables for the response, which means that there is a positive correlation between acetylcholine dose with/without atropine (from columns), while the inhibitory effect of atropine is also significant (from rows).

Non-linear fit of the inhibitory effect of antagonist atropine on 8×10^{-8} M acetylcholine was performed to determine the K_B for atropine. The results are shown in Table **??**.

Table 12: Inhibitory effect of antagonist atropine on 8×10^{-8} M acetylcholine

Best-fit values					Goodness of Fit			
Bottom	Top	LogIC_{50}	IC_{50}	Span	df	R^2	SS.	Sy.x
-3.705	45.06	-8.554	2.794×10^{-9}	48.76	21	0.9978	13.37	0.7979

95 % CI (profile likelihood)				Number of points	
Bottom	Top	LogEC_{50}	EC_{50}	X	Y analysed
[-4.575, -2.858]	[44.18, 45.95]	[-8.593, -8.514]	$[2.552\times10^{-9}, 3.060\times10^{-9}]$	24	24

Non-linear fit of the inhibitory effect of antagonist atropine on 8×10^{-8} M acetylcholine. The bottom and top values are the lower and upper asymptotes of the curve, while the LogIC_{50} and IC_{50} are the logarithm and the value of the concentration of atropine that produces half-maximal inhibition of the response, respectively. The span is the difference between the top and bottom values. The 95% CI (profile likelihood) are the 95% confidence intervals of the parameters. The number of points are the number of points used for the fit.

One-way ANOVA of the responses to electrical stimulation for each part of the experiment was performed. The results are shown in Table 13.

Table 13: One-way ANOVA of the responses to electrical stimulation

	Statistics				
	F	P value	P summary	S.D. among means (P < 0.05)?	R squared
Part1	5292	<0.0001	****	Yes	0.9991
Part2	377.2	<0.0001	****	Yes	0.9843
Part3 Morphine	211.5	<0.0001	****	Yes	0.9658
Part3 Clonidine	615.0	<0.0001	****	Yes	0.9880

Part1: One-way ANOVA analysis of responses to electrical stimulation under control conditions, with increasing voltage. Part2: One-way ANOVA analysis of your responses to electrical stimulation for the control group and drug-treated groups, including atropine, clonidine, morphine, naloxone and phentolamine. Part3: One-way ANOVA analysis of your responses to 1. control vs morphine alone vs morphine + naloxone, 2. control vs clonidine alone vs clonidine + phentolamine.

For each ordinary one-way ANOVA analysis conducted, we found that the F-value is rather large, and the P-value is <0.0001, indicating the significance of the results. The P-value summary is ****, indicating that the results are highly significant. The R-squared value is close to 1, indicating that the model fits the data well. Therefore, we can conclude that the results are reliable.

4 Discussion

4.1 Interpretation

As for the

From the voltage-response curve, we can see that the response tension increases with the voltage, with a maximum response at 12 V. This is consistent with the fact that the ileum is sensitive to electrical stimulation to produce a contraction.

By comparing the response to electrical stimulation under the control groups and drug-treated groups, we can see that atropine treatment reduced the response to around zero, which means a total block on the contraction of the ileum; clonidine and morphine reduce the response significantly, but not to zero, which means partial block on the contraction of the ileum; naloxone and phentolamine have no direct effect as the response is the same as the control group.

However, by comparing among the control group, morphine alone, morphine + naloxone, as well as the control group, clonidine alone and clonidine + phentolamine, we can see that the effects of morphine and clonidine are antagonistic. If we add naloxone to morphine-treated group, the response is the same as the control group, which means that naloxone can reverse the effect of morphine as an antagonist; the same is true for adding phentolamine to clonidine-treated group. This is consistent with the fact that naloxone and phentolamine are antagonists of morphine and clonidine, respectively.

The statistical tests tell us that the results are reliable, as the F-value is rather large, the P-value is <0.0001, the P-value summary is ****, and the R-squared value is close to 1. These all indicate that the results are highly significant, and the model fits the data well.

4.2 Challenges and limitations

One of the challenge of this experiment is that the materials, i.e. the ileum, are very fragile, which could be not showing consistent results as we perform multiple times of electrical stimulation. To overcome this challenge, we have to be very careful when handling the ileum, and make sure that we provide ideal conditions as well as gentle stimulation to the ileum. Even though we have done our best to overcome this challenge, we still cannot guarantee a consistent result.

The limitations of this methodology could be that it cannot show the mechanism of drug interaction or drug-drug interaction. Also, there could be a difference in the response between tissues in our organ bath that *in vivo*.

4.3 General discussion

The role of ACh in the GIT is both on smooth muscle cells, where it binds to muscarinic receptors to cause contraction, and on nerve endings, where it binds to nicotinic receptors to cause stimulation. Therefore, it is a vital neurotransmitter in the non-adrenergic control of gut contraction.

Other neuron transmitters that might also cause contraction in the gut includes Substance P and serotonin, and we can also perform the same experiment to investigate the effect of these neurotransmitters on the ileum, where we observe the response to electrical stimulation after the application of these neurotransmitters, compared to the control group.

Based on our experiment and knowledge of these drugs, morphine and histamine might be acting on receptors on the smooth muscle cells and/or ENS neurons. (in fact, morphine is an opioid analgesic that act on μ-opioid receptors on the end of ENS neurons, while histamine acts on H1 receptors on smooth muscle cells as well as on the ends of ENS neurons.)

5 Conclusion

During the experiment, I have successfully investigated the effect of voltage and some certain drugs on the ileum. The results show that the ileum is sensitive to electrical stimulation, and that the response tension increases with the voltage, with a maximum response at 12 V; atropine exerts a total block on the contraction of the ileum, clonidine and morphine partially block the contraction, and naloxone and phentolamine have no direct effect on it; however, naloxone and phentolamine can reverse the effect of clonidine and morphine as antagonists, respectively. These clearly relate back to the aim of the experiment.