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RESEARCH ARTICLE

EFFECTS OF DRUGS AND FIELD STIMULATION ON RAT ANOCOCCYGEUS SMOOTH MUSCLE

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ABBREVIATION

Acetylcholine - ACh
Noradrenaline - NA
Guanethidine - Gua
L-arginine methyl ester - L-NAME
Tetrodotoxin - TTX
Nitric oxide - NO
Non-adrenergic non-cholinergic - NANC
Concentration-response curve - CRC

ABSTRACT

Background and Purpose: The anococcygeus muscle has a classically sympathetic noradrenaline-mediated motor innervation. Owing to its physiological importance, not much work has been done in the study of its muscular response to certain drugs and chemical mediators. The aim of the experiment was to examine the response of rat anococcygeus muscle to selected drugs and field stimulation. **Experimental Approach:** Anococcygeus smooth muscle was isolated from adult male rat. In this study, we investigated tissue response to acetylcholine (ACh), noradrenaline (NA), guanethidine (Gua), L-NAME, tetrodotoxin (TTX), and to field stimulation. The isolated muscle was suspended in an organ bath containing Krebs solution gassed with O_2/CO_2 (95%/5%) at 37°C. Responses were measured with Grass FT 03 isometric transducer, displayed on a Grass Polygraph and was statistically analysed using Microsoft® Excel 2008. **Key Results:** The muscle tissue responded to ACh and NA, with higher response mediated by NA. Nerve tissue was stimulated following field stimulation which was abolished by the presence TTX and Gua. An inhibitory innervation stimulated by nitric oxide (NO), non-adrenergic non-cholinergic (NANC) was abolished by the presence of L-NAME. **Conclusion:** α_1-adrenoceptor and muscarinic receptors are richly distributed in rat anococcygeus muscle, hence their activation by the binding of NA and ACh respectively. There appeared to be preference on NA over ACh in contracting the muscle as its effect was more profound, though its release was prevented by neuro-muscular blockers during the field evoked response. There was complete inhibition of the NO pathway due to the presence of L-NAME.

INTRODUCTION

Smooth muscles widely differ in their physiological characteristics. Though they principally contain fibres of actin and myosin necessary for functioning of cells, smooth muscles are supported by a framework of different proteins (Lodish *et al*., 2008). The anococcygeus muscle tissue is located in the urogenital tract, with a classically sympathetic noradrenaline-mediated motor innervation, alongside transmitter NO which has just been recently identified to mediate relaxation of the muscle (Gibson and McFadza, 2001). The anococcygeus muscle originates from the upper coccygeal vertebrae closely aligned one to another, with the muscles passing caudally and to the colon, joining together to form a bar, millimetres away from the anus (Gillespie, 1972). The geometry in which the cells of the anococcygeus muscle are arranged (parallel bundles) is to allow for proper diffusion of drugs following administration. It has recently been reported that anococcygeus muscle have a rich supply of adrenergic innervations with no cholinergic innervations (Rang *et al*., 2010). This can be demonstrated in the laboratory using adrenergic (α_1-adrenoceptor) agonist, NA, to mediate contractile response on the tissue.

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Drugs such as exogenous ACh may be used to experiment for its contractile ability on the rat anococcygeus muscle as the muscle does not possess cholinergic nerve. Gillepsie (1972) has reported that anococcygeus muscles undergo inhibition innervation that is thought to be NANC. Thus, the muscle-relaxing property of NO characterised as being NANC inhibitory transmitter has been emphasized (Liu *et al*., 1991).

Aim:the aim of the experiment was to examine the response of rat anococcygeus muscle to drugs (noradrenaline, acetylcholine, guanethidine, tetrodotoxin and L-NAME) and to field stimulation.

MATERIALS AND METHODS

The drugs used for the investigation were stock solutions of acetylcholine (ACh) ($1x10^{-2}$M), noradrenaline (NA) ($1x10^{-2}$M), guanethidine (Gua) ($1x10^{-2}$M), L-NAME ($1x10^{-2}$M) and tetrodotoxin (TTX) ($1x10^{-2}$M).

Animal model and muscle preparation: The investigation conforms to the United Kingdom Animal procedures act 1986, and with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication, 8th Edition, 2011). Ethical approval was granted by the University Ethics Committee.

A male rat was maintained in a cage, on saw dust bedding, and subjected to a 12 h–12 h light/–dark cycle with food and water provided *ad libitum*. In this study, anococcygeus muscle was used. The rat was killed by stunning and exsanguinations and the abdomen was cut open. Care was taken to ensure that the muscle tissue was not damaged. The colon was located and cut through the top of the pelvic. Connective tissues were removed and the anococcygeus muscle was isolated and set up for simulation in a 20 ml organ bath containing warm (Krebs) physiological salt solution gassed with O_2/CO_2 (95%/5%) at 37^oC.

Experimental protocol: Before the start of the experiment, tension was measured using Grass FT 03 isometric transducer and displayed on a Grass Polygraph (Grass Instruments Co., Quincy, Massachusetts, USA), producing the initial tension of 0.5 g. The tissue was allowed to achieve equilibration for 15 minutes before administration of the drug was commenced, starting with least concentrated drug. At equilibrium, field stimulation on the nerve fibre was applied as muscle was suspended through a pair of electrodes, as has been described by Burn and Rand (1960). A 40Hz frequency at 0.5msec pulses was initially used for the nerve stimulation. Subsequent variation in the frequency was recorded. The required doses of ACh, NA, Gua, L-NAME, and TTX were administered on the rat tissue in the organ bath with increasing concentrations of one log unit (table 1).Starting with ACh, 20 µl of $1x10^{-5}$ M was added to the organ bath to yield a bath concentration of $1x10^{-8}$M. After 3 minutes when a plateau must have been achieved, the drug was washed off several times using distilled water. Furthermore, 20 µl of $1x10^{-4}$ M was added to the organ bath to yield a bath concentration of $1x10^{-7}$M and was allowed to plateau before washing. The process was repeated to yield bath concentrations of 10^{-6}M, $1x10^{-5}$M and 10^{-4}M using drug concentrations of $1x10^{-2}$M, $1x10^{-3}$M and $1x10^{-4}$M with the required volumes.

The response trace and concentration-response curve (CRC) to ACh was constructed (figures 1 and 2). After obtaining the responses for ACh, the preparation was washed 6 times with warm PSS until the tone returned to the baseline. Muscle contraction was investigated by administering NA, starting with the least concentrated drug. The cycle was followed as was the case of ACh, but this time to a bath concentration of 10^{-5}M. A response trace and CRC to NA was constructed (figures 3 and 4). The effect of field stimulation was determined at different frequencies of 1,2,4,10,20 and 40 Hertz supramaximal voltage with 0.5msec pulse width. The effect of constant duration of stimulation (2 sec at different frequencies) was also compared with fixed number of pulses (80 pulses at different frequencies). The trace and responses were constructed (figures 3 and 4).The anococcygeus muscle was then stimulated with 2 sec trains of pulses at 10 Hz and then 60 µl of adrenergic neurone blocker, Gua ($1x10^{-2}$M), and was added to the organ bath (to a concentration of $3x10^{-5}$M) to abolish the nerve-evoked contractions. The muscle was stimulated for 2 second trains of pulses at 10 Hz until relaxation was stable. The traces and responses were constructed (figures 3 and 4). The effect of TTX ($1x10^{-6}$M) on the inhibitory responses was also measured, trace and responses were constructed and (3 and 4).

RESULTS

Serial dilution of the drugs:In determining the concentrations of the drug to be administered to produce the required responses on their respective receptors, serial dilutions were carried out as follows:

To make $1x10^{-3}$M ACh from $1x10^{-2}$M stock,

$V_1C_1=V_2C_2$ was used.
V_1= volume of stock Ach

Table 1. Drug (ACh and NA) concentrations added to organ bath to yield the required bath concentration

Drug concentrations (M)	Organ bath concentrations (M)				
	$1x10^{-8}$	$1x10^{-7}$	$1x10^{-6}$	$1x10^{-5}$	$1x10^{-4}$
$1x10^{-5}$	20 µl				
$1x10^{-4}$		20 µl			
$1x10^{-3}$			20 µl		
$1x10^{-2}$				20 µl	200 µl

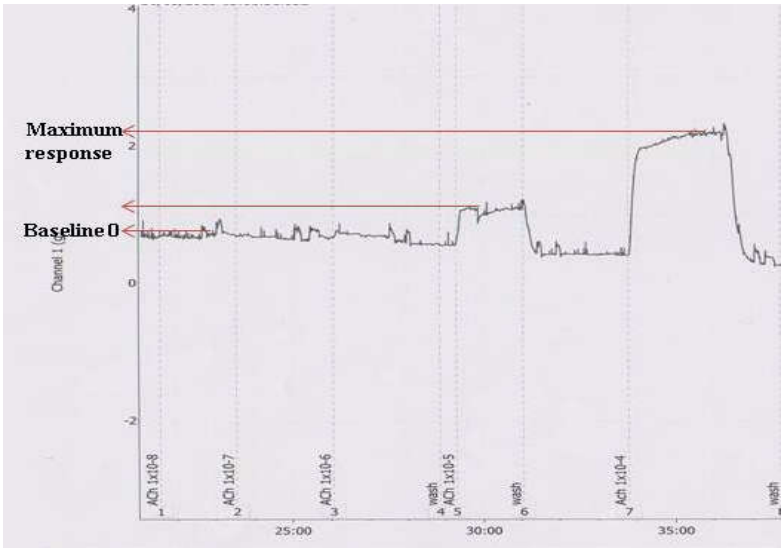

Figure 1. The response of the rat anococcygeus muscle to ACh. The record shows the increasing contractions in response to increasing molar concentrations of ACh. Increasing [ACh] induces maximum response. Time 05sec

Table 2. [ACh] % response curve

Concentration (M)	Log concentration	Tension (mm)	% response
$1x10^{-8}$	-8	0	0
$1x10^{-7}$	-7	0	0
$1x10^{-6}$	-6	0	0
$1x10^{-5}$	-5	13	30
$1x10^{-4}$	-4	43.5	100

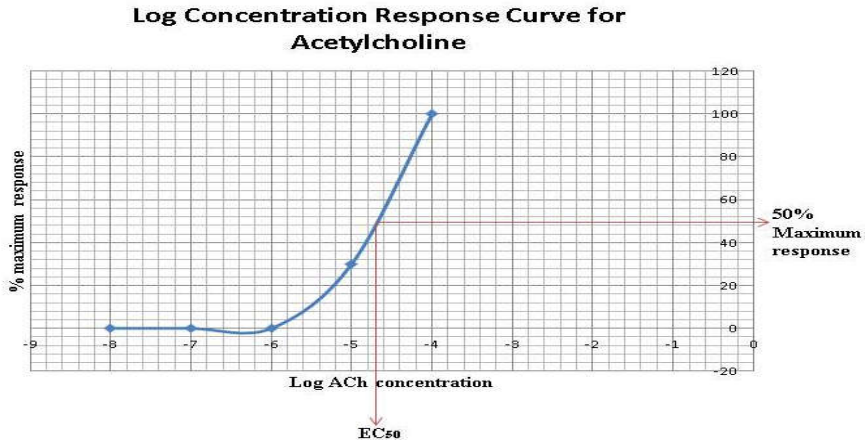

Figure 2. Graph of concentration-response curve showing a general increase in % maximum response of anococcygeus muscle contraction as the concentration of log [ACh] agonist increases from -8 to -5. The EC_{50} = -4.7

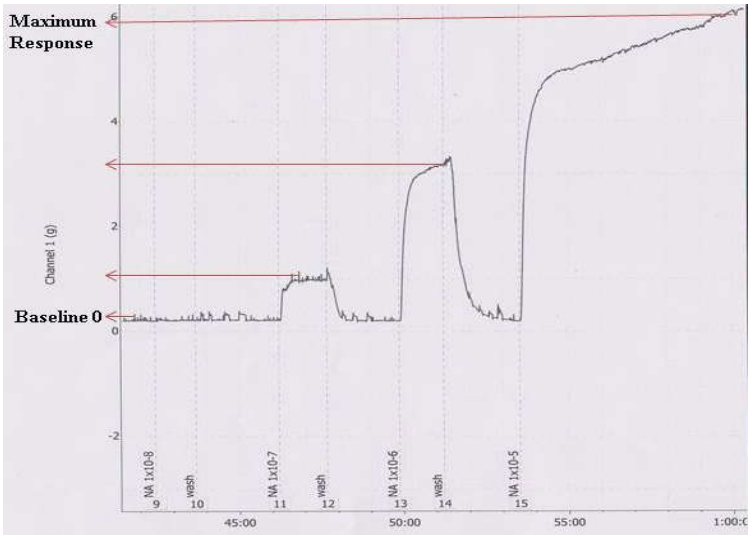

Figure 3. The response of the rat anococcygeus muscle to NA. The record shows the increasing contractions in response to increasing molar concentrations of NA. Increasing [NA] induces maximum response. Time 05sec

Table 3 [NA] % response curve

Concentration (M)	Log concentration	Tension (mm)	% response
$1x10^{-8}$	-8	0	0
$1x10^{-7}$	-7	18	17
$1x10^{-6}$	-6	59	55
$1x10^{-5}$	-5	107	100

Table 4. Frequency response curve obtained from average of the three spikes at each frequency in mm

Frequency (Hz) at 0.5msec pulse	Tension (mm)				% maximum response
	1	2	3	Average	
1Hz	2	2	3.5	2.5	3
2Hz	7	7	6	6.6	7
4Hz	23	23	21	22.3	24
10Hz	59	57	54	56.6	60
20Hz	84	84	84	84	89
40Hz	95	93	95	94.3	100

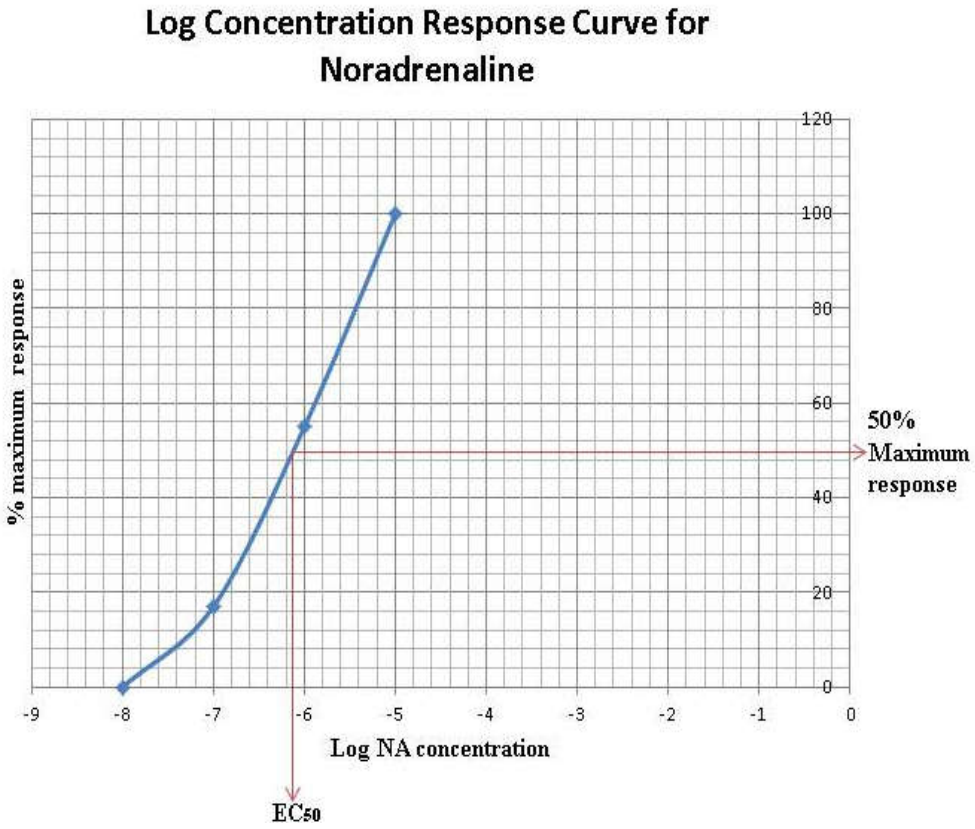

Figure 4. Graph of concentration-response curve showing a general increase in % maximum response of anococcygeus muscle contraction to NA as the concentration of log [NA] agonist increases from -8 to -5. The EC_{50} = -6.15.

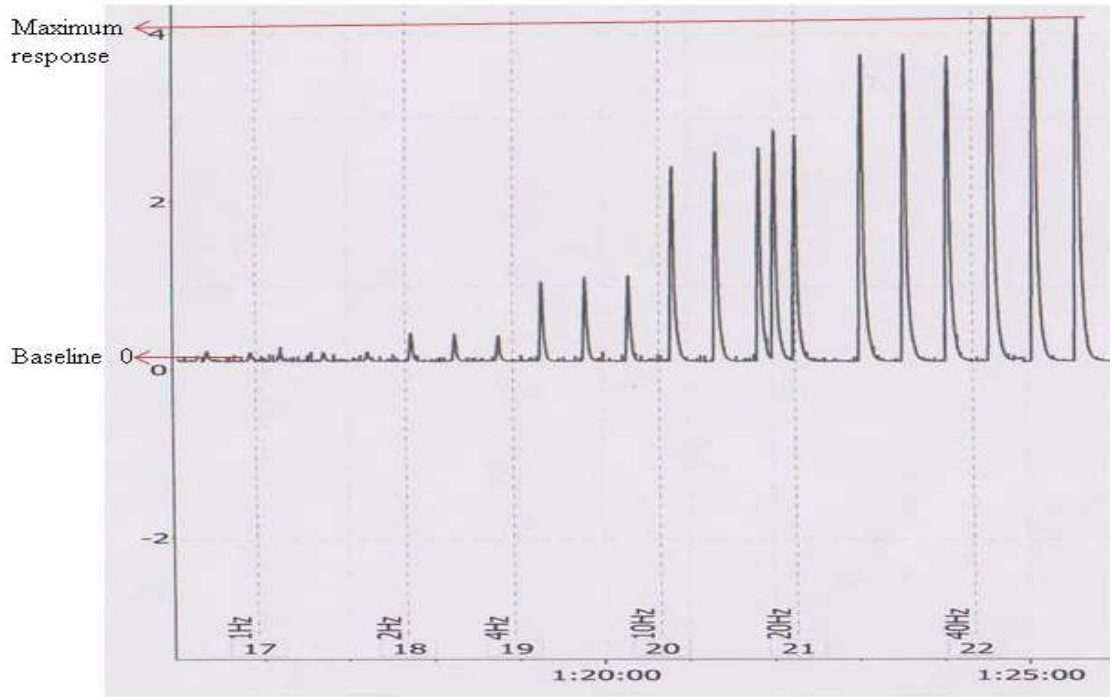

Figure 5. The response of the rat anococcygeus muscle to field stimulation. The record shows the effect of variations in frequencies (Hz) to muscle response.

C_1= concentration of stock ACh= $1x10^{-2}$M
V_2= V_1 + cold PSS = 1000µl
C_2= $1x10^{-3}$M.

$V_1 = \frac{V2C2}{C1}$

$V_1 = \frac{1000x(1x10^{-3})}{1x10^{-2}}$

$V_1 = \frac{1}{1x10^{-2}}$

V_1= 100ml
V_1 + PSS = 1000µl
PSS = 1000µl-100µl = 900µl.

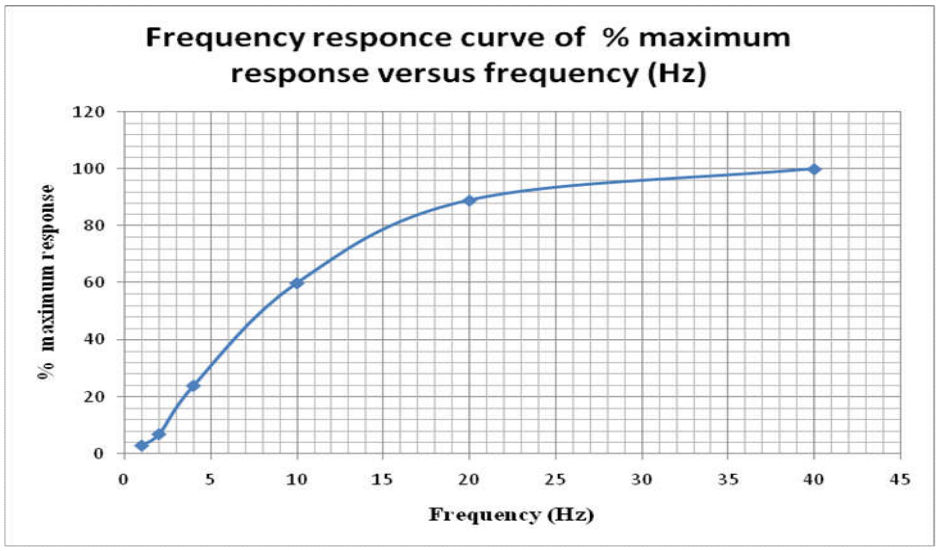

Figure 6. Graph of maximum response versus frequency showing a general increase in % maximum response of anococcygeus muscle contraction as the frequency of field stimulation increases from 1Hz to 40Hz.

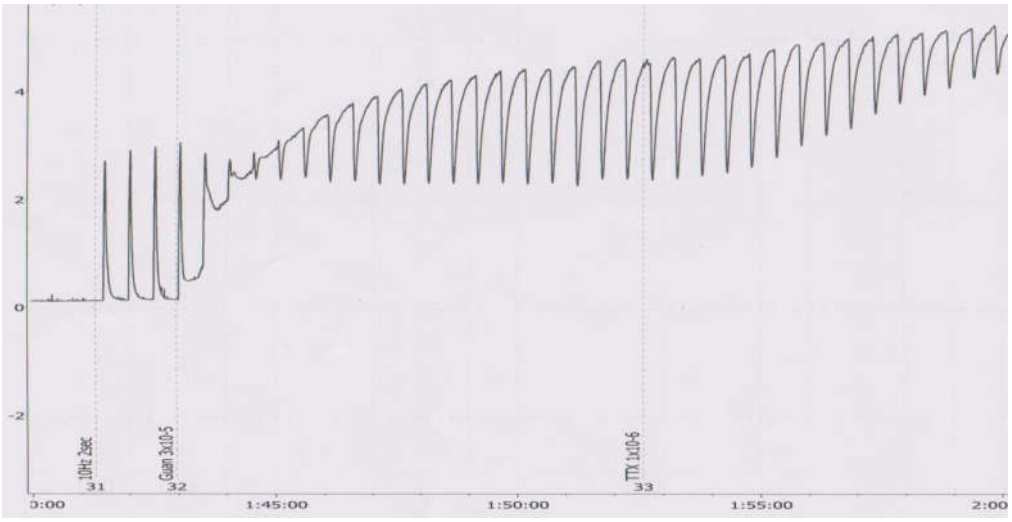

Figure 7. Response of the rat anococcygeus muscle to fixed frequency and pulse of field stimulation in presence of adrenergic neurone blocking drugs, Gua and TTX

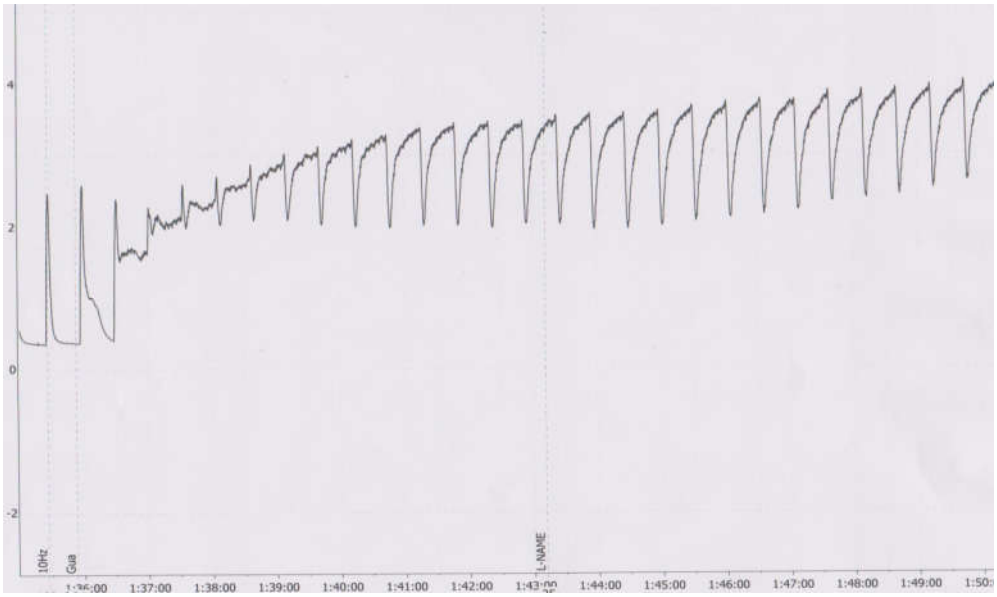

Figure 8. Response of the rat anococcygeus muscle to fixed frequency and pulse of field stimulation in the presence of adrenergic neurone blocking drug Gua and NO synthase inhibitor, L-NAME

Hence, 900µl of cold PSS was added to 100µl of stock ACh to yield $1x10^{-3}$M of diluted ACh. Same method was used to obtain the diluted concentrations of $1x10^{-4}$M and $1x10^{-5}$M. Therefore, the volume of ACh to add to the organ bath to yield bath concentration of $1x10^{-8}$M was made as follows using the relation $V_1C_1=V_2C_2$.

V_1= volume of stock ACh to be added
C_1= concentration of ACh = $1x10^{-5}$M
V_2= volume of organ bath = 20000µl
C_2= organ bath concentration $1x10^{-8}$M.
$V_1=\frac{V2C2}{C1}$
$V_1=\frac{20000\,x(1x10^{-3})}{1x10^{-5}}$ = V_1= 20µl

Therefore, the volume of ACh added to produce an organ bath concentration of $1x10^{-8}$M was 20 µl. The same method was used to obtain the required bath concentrations (table 1).

Effect of ACh on anococcygeus muscle: From figure 1, the maximum response was 43.5mm = 100% (at bath concentration of $1x10^{-4}$M). At bath concentration of $1x10^{-5}$M, the response was at 13mm, therefore,

% response = $\frac{13}{43.5}$x 100 = 30%.

This method was similarly used in calculating for the percentage response of other concentrations (table 2).

Effect of NA on anococcygeus muscle: Effect of anococcygeus muscle excitatory responses to field stimulation: Effect of anococcygeus muscle excitatory responses to a neurone blocker

Data and Statistical Analysis: The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al*., 2015). Statistical analysis was done using Microsoft® Excel 2008 to generate graphical presentation of the generated responses of the muscle tissue.

DISCUSSION AND CONCLUSION

The present study demonstrates the effects of different drugs on rat anococcygeus muscle, a smooth muscle located in the urogenital tract.
The *in vitro* muscle has its advantages which have already been stated in the introductory part of this paper. The observed contractile responses of ACh and NA (figures 1 and 3 respectively) on the rat anococcygeus muscle were very prominent. When the log CRC (figures 2 and 4 respectively) for both drugs were compared, the EC_{50} for ACh (-4.7) was lower than that of NA (-6.15). This suggests that lower amount of NA is needed to bind to the α-adrenoceptorsto produce 50% maximum contractile response in the anococcygeus muscle compared to ACh on muscarinic receptors. The muscle shows higher preference to NA stimulation than ACh at lower molar concentration. The response to the aforementioned drugs and chemical mediators exhibited by the muscle tissue points to the fact that smooth muscles are richly supplied with receptors for ACh (muscarinic receptors) and NA (α-adrenoceptors). We cannot rule out the possibility of the presence of ß-adrenoceptorsat this stage as the investigation of drugs which selectively binds to ß-adrenoceptors was beyond the scope of this study.

The extrinsic nerves of the muscle were stimulated by field stimulation, and triggers contraction of the muscle (figure 5 and 6) with a general increase in % maximum response as the frequency of field stimulation increases from 1Hz to 40Hz.A very critical deduction from the study was the inhibition of the smooth muscle contraction following administration of Gua (figure 7) in the presence of field stimulation, suggesting that the contraction of the muscle was as a result of nerve stimulation. The raised tone (contraction) response of anococcygeus muscle to fixed frequency and pulse of field stimulation (10Hz at 2 seconds) (figure 7) was achieved and the effects of the inhibitors was observed. Gua and TTX, adrenergic neurone blockers were used to block the adrenergic terminal which prevented the release of NA in response to arrival of an action potential (figure 7). The leakage of NA from the adrenergic nerve was mainly due to the effect of electrical stimulation, in line with the findings of Gillpsie (1971). The study went further to characterise NANC innervation as an inhibitory innervation. The conversion of L-Arginine to NO by neuronal nitric oxide synthase was competitively inhibited by the presence of L-NAME (figure 8). This inhibition is due to the prevention of NO synthesis which brings about muscle relaxation, hence, allowing for the sustained contraction of the smooth muscle.

Finally, the rat anococcygeus smooth muscle responded to the effect of ACh and NA by contracting via the α-adrenoceptors and muscarinic receptor respectively. The α-adrenoceptors appears to have higher affinity for NA as it produced higher contractions with similar doses to ACh, and the field-evoked response was summarily abolished by Gua, TTX while L-NAME prevented NO-mediated muscle relaxation.

Recommendation

Selective drugs to ß-adrenoceptor should be tested to ascertain the true distribution of the receptor in the rat anococcygeus muscle. Further studies on the antagonising effect of TTX and L-NAME should be carried out on the muscle whilst under the effect of ACh and NA. This would enable us to observe critically their antagonistic strength on the muscle tissue.

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