SMOOTH MUSCLE CONTRACTOR ACTIVITY OF COTTON DUST AQUEOUS EXTRACT IN VITRO. I - PART OF THE COTTON-DUST-AQUEOUS-EXTRACT-INDUCED CONTRACTION OF THE ISOLATED RAT FUNDUS STRIP IS NOT DUE TO ENDOGENOUS 5-HT OR 5-HT RELEASE MECHANISMS

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Abstract

Endogenous 5-HT has been proposed as the mediator responsible for cotton dust aqueous extract (CDE)-induced contraction of the isolated rat stomach fundus. It has been previously reported that there is a residual response to CDE in the presence of concentrations of methysergide which completely block the responses to 5-HT. This study confirms the finding and shows that a combination of the cyclooxygenase inhibitor, indomethacin, and the non-selective leukotriene antagonist, FPL55712, (at concentrations which individually do not affect 5-HT or CDE-induced contractility) in combination, significantly blocked CDE-induced contractions (38%) but not those to 5-HT. Whilst the latter results cannot be readily explained, they clearly show that part of the response is not due to 5-HT or 5-HT release mechanisms.

General Introduction

Smooth Muscle Contractor Activity of Cotton Dust Aqueous Extract in vitro.

The search continues for the exact nature of the pharmacologically active agents present in cotton dust. Long-term, controlled experiments on a statistically representative sample of human subjects would be ideal. However, this is not a practical option and *In vivo* animal models are an alternative. These are often labour-intensive and time-consuming, involving a large number of potentially significant variables which are difficult to control. *In vitro* models, whilst physiologically less representative, have fewer practical problems and can provide pointers to a more directed set of *in vivo* studies. A number of reports have indicated parallels between *in vitro* findings and *in vivo* results. Some of these are outlined below.

In a comparative study, it was found that "bronchoconstriction observed in human volunteers in response to semi-purified fractions of cotton bract, corresponds exceptionally well with the *in vitro* smooth muscle [rat stomach fundus] contracting ability of these extracts but does not correlate with complement activation, endotoxin concentration, or chemotaxis." [1]

It has been reported that dust from a mill with a high prevalence of byssinosis, possessed a greater "smooth muscle contractor activity" than did dust from a mill with a low prevalence of byssinosis. [2]

Similarities have been reported between acute *in vivo* response to raw cotton and some of the cotton dust aqueous extract agents causing contraction of various smooth muscle tissues *in vitro*. For example, inhalation of cotton dust is known to be associated with an inflammatory response. The release of inflammatory mediators from a range of *in vitro* smooth muscle preparations is well documented. In an *in vivo* guinea pig model, a fraction of cotton dust aqueous extract with very low endotoxin content produced a bronchoconstrictor response significantly greater than the response to CDE one hour after exposure and was similar in magnitude to the response after two hours, even though the endotoxin concentration was 200 times lower. [3]

The acetone precipitate fraction of CDE resulted in a sustained secondary response similar to CDE in 3 out of 4 volunteers. [3] This fraction caused a bronchoconstriction in the perfused lung, whereas E. coli and E. agglomerans endotoxins led to slight bronchodialation. [5] This fraction also contained 200 times less endotoxin. The acetone-soluble fraction also led to a bronchoconstrictor response in the same 3 out of 4 subjects. The responses were smaller, but significant. Both of these results are similar to the guinea pig perfused lung studies. [5]

Studies of ether extracts of bracts in humans showed that the ether-soluble components did not have any bronchoconstrictor activity. [6] This fraction is also devoid of activity on the rat stomach fundus strip [4]. Treatment of CDE with base, but not acid, has been reported to reduce the respiratory response to cotton dust in the guinea pig. [7] Base, but not acid, treatment has also been shown to reduce cotton-dust induced cell recruitment and differentiated granulocyte count in the guinea pig. [8] We have also shown that base but not acid treatment results in a marked reduction in the contractile response to CDE in the rat fundus [4].

In this series of studies (parts I-V, these proceedings), we report on a number of experiments investigating the physicochemical nature, and the pharmacological mechanism of action, of the agents in aqueous extracts of raw cotton mill dust possessing spasmogenic activity on the isolated guinea pig trachea and the isolated rat stomach fundus.

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Introduction to Part I

Part of the Cotton-Dust-Aqueous-Extract-Induced Contraction of the Isolated Rat Fundus Strip is not Due to Endogenous 5-HT or 5-HT Release Mechanisms.

Endogenous 5-HT has been proposed as the agent responsible for cotton dust aqueous extract (CDE)-induced contraction of the isolated rat stomach fundus.

The presence of 5-HT in CDE has been reported: "Post column derivatisation [with o-phthalaldehyde (OPT)] produces a highly selective and sensitive detection technique for serotonin." [9] Using this method, respirable cotton dust (<20µm) was found to contain 10.1µg/g and total cotton dust 17.2µg/g of 5-HT [9]. Whilst providing strong evidence for the existence of endogenous 5-HT, this technique may not be totally conclusive. The method may be suitable for the detection of serotonin in animal tissues. However, a much wider range of indole derivatives is found in plants. In the original paper describing OPT derivatisation for fluorimetric determination of indole derivatives, it is stated that "the combination of -OH or $-OCH_3$ substituents in the 5-position with a variety of 2- or 3-carbon side chains in the 3-position yields good fluorescence." [10]

Pharmacological evidence is based on findings that methysergide(100ng/ml), which blocks 100% of the action of 5-HT on the rat fundus, blocked contractor activity of cotton bract extract by 83%. [11] In the present series of studies it was found that in an isolated guinea pig tracheal preparation, insensitive to 5-HT, methysergide blocked 90% of the CDE-induced contractions. In an earlier study [12], methysergide was shown to partially block $PGF_{2\alpha}$ and arachidonic acid induced contractions in the rat fundus.

These findings prompted further investigation of the possible involvement of eicosanoids in CDE-induced contractions in this tissue.

Materials and Methods

Table 1 gives summary information of the chemical tools used in this series of five reports. "Fw" is the formula weight of the *derivative used.*

Cotton Dust Aqueous Extract: - In the literature, the method of preparation of CDE varies amongst different investigators; ranging from a single extraction to multiple extractions, separation after leaving the mixture at 15°C overnight and immediate separation, centrifugation at 1000 xg and at 3000 xg, freeze drying followed by reconstitution and direct application.

Solvent extraction and TLC studies indicated that the rat fundus active constituents had a high affinity for water, suggesting that leaving the mixture to stand overnight is unnecessary. By virtue of physicochemical considerations, in any extraction procedure, per unit volume of solvent, multiple extractions with small aliquots are more effective than a single extraction. Higher speed centrifugation reduces the possibility of contamination with residual particulate matter, yielding a clear supernatant. This allows for a more ready determination of complete dissolution of active matter when reconstituted from a freeze-dried form (one cannot be sure whether all soluble matter is dissolved if a cloudy solution is to be expected). In exploratory studies, using carbachol as a reference spasmogen, it was found that freeze-dried extracts of cotton dust could be kept at 4°C for many weeks without reduction in unit activity. Sets of interconnected experiments were carried out using the same batch of extract. In order to standardise experiments in different laboratories, a "standard" dust, collected in 1984 was distributed by Cotton Inc. of USA. Extracts of this dust sample have been used throughout this investigation.

CDE Extraction Procedure: - The quantities given here are for the preparation of approximately 2g of CDE. Cotton dust (20g) was mixed with distilled water (100ml) in a mortar (15-20 mins). The mixture was transferred to a large Buchner funnel mounted on a vacuum flask. Aided by squeezing the mixture, the solution was vacuum extracted. With the vacuum switched off, a further aliquot of water (50 ml) was uniformly poured over the surface of the cotton. After allowing to stand for a few minutes, the solution was again sucked out. This procedure was repeated with two further aliquots (50ml) of water. The solution was then centrifuged (3000 g,20 mins). The supernatant was freeze-dried overnight. Yield(±SEM) = 95.2(±5.1)mg/g (n=6 batch preparations).

The Rat Fundus Preparation: -The left ventral quartile of the rat stomach fundus was prepared as described by Riazi-Farzad(1995) [12]. Responses were measured isotonically, under a tension of 1g.

Carbachol as an Internal Standard - The carbachol concentration-response relationship was determined (fig. 1). A concentration of 5ng/ml was chosen as an internal standard to normalise data amongst the different tissues. There is an inherent assumption here that any inter-preparation variation, due to slight differences in resting length, recording equipment and more significantly, biological sensitivity, would affect responses to carbachol and other agonists to a similar degree. Also, it has been assumed that "selective" antagonists for other receptor sites would not interfere with the carbachol response. Carbachol was also used to confirm tissue viability at various stages of the experiments. The response to the first dose of carbachol was often significantly less than that to subsequent doses. Doses were, therefore, repeated until consistent responses were obtained (3 doses were usually sufficient). Responses to single doses of other agonists were expressed as a percentage of the respective carbachol responses, before and after antagonist treatment. At the end of each

experiment, a further dose of carbachol was given to ensure tissue viability. If, during this procedure, the response to the standard carbachol dose diminished, all tests after the previous carbachol dose would be repeated with a fresh preparation.

Statistical Analysis - Inter-tissue variations and experimental errors were assumed to be normally distributed. Standard errors were calculated accordingly. Where an inhbitory effect was expected, confidence limits were established by means of a one-tailed t-test by comparing the response as a percentage of the carbachol (5ng/ml)-induced contraction before and after antagonist treatment.

5-HT - A concentration-response curve was constructed for 5-HT and the ED_{50} was determined by correlation analysis. Often, 5-HT was used as a reference with which the mechanism of action of CDE was compared. Carbachol was used as a standard. Hence, in such cases, responses to both 5-HT and CDE were normalized by expressing them as a percentage of the carbachol response and then the (remaining) differences were compared.

Arachidonic Acid - Since arachidonic acid metabolites have also been implicated in byssinosis [13,14,15,16], as well as part of the smooth muscle contractile response [11], the mode of action of this agent was also investigated. A concentration-response relationship was obtained to identify suitable dose levels for study. Also, its action in the presence of inhibitors of its metabolic pathways and antagonists of its metabolites were compared.

$PGF_{2\alpha}$ - Although arachidonic acid is a useful tool, its actions are not specific. $PGF_{2\alpha}$, a metabolite of arachidonic acid causing contraction in the rat fundus, was used for investigation of the specificity of compounds modifying arachidonic acid action. A concentration-response relationship was determined and the possible effects of some antagonists on $PGF_{2\alpha}$ were studied.

CDE - A concentration-response relationship was obtained so that suitable sub-maximal doses could be chosen for single dose studies.

Methysergide - In pilot studies, spiperone and ketanserin ($5\text{-}HT_2$ antagonists) did not modify the activity of CDE. Methysergide, on the other hand, had profound effects on both 5-HT and CDE responses. Its effect on $PGF_{2\alpha}$ and arachidonic acid was also tested.

Indomethacin - The possible effects of indomethacin on 5-HT and CDE were investigated. Although known as an **inhibitor** of the cyclo-oxygenase pathway, in one report [11], indomethacin was used as a direct **antagonist** of $PGF_{2\alpha}$ at the receptor level without explanation. This possibility was also investigated.

FPL55712 - In attempting to discover the possible involvement of metabolites of the lipoxygenase pathway in CDE response, the effect of the non-selective leukotriene antagonist, FPL55712 [19,3], on 5-HT, CDE, $PGF_{2\alpha}$ and arachidonic acid were investigated.

Results

Agonists' Concentration-Response Curves In the left-ventral section of the rat fundus strip, the following ED_{50}(±SEM) values were obtained: Carbachol, 8.5(±2)ng/ml (fig. 1), CDE, 10(±2.5) μg/ml (fig. 2), 5-HT, 3.5(±1)ng/ml (fig. 3), arachidonic acid, 1.8(±0.4)µg/ml and $PGF_{2\alpha}$, 0.7(±0.4)ng/ml.

Methysergide - [For a results summary see table 1] In preliminary studies using methysergide (0.3ng/ml), the mean reduction in responses to 5-HT and CDE were comparable. Using a higher concentration of methysergide (1.0ng/ml), the mean reduction in response to 5-HT appeared to be much greater compared with CDE. However, it was found that at these very low concentrations, the inter-tissue variations were very high and the differences between methysergide's effect on these two agonists were not significant (fig. 4). Consequently, higher concentrations were employed.

The effects of methysergide (5ng/ml) on responses to single doses of 5-HT (1.5ng/ml), CDE (10µg/ml), $PGF_{2\alpha}$ (1.5ng/ml) and arachidonic acid (1.5µg/ml) were investigated. This revealed a comparable reduction in responses to 5-HT and CDE and it was interesting to note that the responses to $PGF_{2\alpha}$ and arachidonic acid were also reduced significantly (fig. 5). The response to the dose of carbachol employed as reference did not appear to be affected by methysergide (5ng/ml). At a much higher concentration (100ng/ml), methysergide almost completely blocked 5-HT and CDE as expected. However, this did not lead to any significant enhancement of the degree by which $PGF_{2\alpha}$ and arachidonic acid were blocked. (fig. 6)

Methysergide did not alter the baseline tissue tone when it was administered prior to the agonists.

Indomethacin - [For a results summary see table 2] In a similar experiment using indomethacin (2.5µg/ml), 5-HT and CDE were not affected whilst $PGF_{2\alpha}$ was significantly blocked. In an exploratory study using a higher concentration of indomethacin (10µg/ml) 5-HT and CDE remained unaffected whilst $PGF_{2\alpha}$ and arachidonic acid were further reduced and this pattern of activity was retained even when the indomethacin concentration was increased to 25μg/ml.

FPL55712 (1µg/ml) did not significantly modify 5-HT, CDE, or $PGF_{2\alpha}$ responses (92(±11)% (n=8,p>0.5), 97(±11)% (n=8,p>0.9), and 89(±12)% (n=8,p>0.6) of control respectively). The response of arachidonic acid, however, was reduced significantly (46(±13)% (n=8,p<0.004) of control) (fig. 8).

FPL55712 Plus Indomethacin - A combination of FPL55712 (1µg/ml) and indomethacin (2.5µg/ml) almost completely abolished the arachidonic acid (1.5µg/ml) response (to 0.5(±0.4)% (n=7,$p \approx 0$) and significantly blocked (to 40(±15)% (n=7,$p<0.005$)) the $PGF_{2\alpha}$ (3ng/ml) response. In contrast, the response to 5-HT (1.5ng/ml) was not blocked (106(±10)% (n=7, $p>0.5$) of control) whilst the response to CDE (10µg/ml) was blocked significantly (63(±9)% of control (n=6,$p<0.01$) (fig. 9).

Discussion

Methysergide - The effects of very low concentrations of methysergide (0.3 & 1.0ng/ml) were variable but at 5ng/ml, methysergide antagonised both 5-HT and CDE, suggesting that their mechanism of action may be similar. However, a considerably higher methysergide concentration (100ng/ml) almost totally abolished the residual effect of 5-HT but not that of CDE. Thus, methysergide may have a large, but incomplete antagonistic effect on the CDE-induced contractile mechanism. On the other hand, there may be more than one contractor component in CDE, the major one of which, although being methysergide-sensitive, produces its effects via a different mechanism to 5-HT. It is, however, more likely that CDE contains a major contractile component operating via the same mechanism as 5-HT in addition to a minor, methysergide-resistant component. A dose of methysergide (5ng/ml) that did not completely abolish the response to a sub-maximal dose of 5-HT, partially but significantly, antagonised both $PGF_{2\alpha}$ and arachidonic acid. However, this effect, although remaining significant, was not increased by higher concentrations of methysergide (100ng/ml).

Considering the present findings, that methysergide antagonises the action of arachidonic acid metabolites directly, some of the previous reports which assume that methysergide is 5-HT selective need be re-examined. For example, inhibition of PGE_1 derived mast cell degranulation [18], and indomethacin sensitive uterine contractions [17], which were all attenuated by methysergide might be more plausibly explained by direct receptor antagonism by arachidonic acid metabolites and not the concluded 5-HT related mechanism.

Indomethacin had a significant effect on arachidonic acid responses, probably via inhibition of cyclooxygenase metabolite production. However, the results also show that indomethacin antagonises $PGF_{2\alpha}$ at the receptor level at concentrations below 3µg/ml. Since, in this study, indomethacin inhibits production of $PGF_{2\alpha}$, its antagonism of $PGF_{2\alpha}$ receptors is unimportant. However, it is conceivable, that other agonists, such as lipoxygenase metabolites, may also be antagonised by indomethacin at the receptor level.

Indomethacin did not affect CDE responses at concentrations as high as 25µg/ml, indicating that CDE's effects are unlikely to be due to mechanisms involving the cyclooxygenase pathway.

FPL55712, a leukotriene antagonist, partially blocked arachidonic acid whilst 5-HT, CDE, and $PGF_{2\alpha}$ were unaffected. This suggests that leukotrienes are not involved in the rat fundus contractor effects of CDE.

FPL55712 Plus Indomethacin - A single-dose arachidonic acid response was blocked (75%) by indomethacin (2.5µg/ml). In combination with FPL55712 (1µg/ml) this response was completely abolished. This suggests that the residual 25% response in the presence of indomethacin alone is due to leukotrienes. Since higher concentrations of indomethacin also reduce this residual response (not reported here), it might be concluded that indomethacin and FPL55712 have some common inhibition sites. Although the indomethacin/FPL55712 combination did not affect the 5-HT-induced contractions, the effect of CDE was blocked by 37% ($p=0.012$). The reason for the difference between the effect of the combination of these two antagonists and the sum of their individual effects is uncertain. However, this does provide further evidence that at least part of the CDE effect is probably not mediated via 5-HT receptors.

The possible involvement of released inflammatory agents, as well as direct action at the receptors via 5-HT related mechanisms, as part of the CDE-induced contraction of the rat fundus suggests that this preparation may be an informative initial model for the investigation of both the chronic and acute phases of byssinosis. However, there seem to be other "smooth muscle contractor" agents which are not detected by this preparation (see part III of this series of reports, also presented at this conference). Consequently, a variety of *in vitro* models need be considered for the elucidation of pointers to appropriate approaches to the study of the complex *in vivo* pharmacology of cotton dust.

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Table 1. Data on Compounds Used in This Report (I) Plus Parts II-V, Also Presented at This Conference

COMPOUND (deriv. used)	Fw (Deriv)	STRUCTURAL NAME	ACTION	SOURCE
Arachidonic acid	**304.5**	*5,8,11,14-eicosatetraenoic acid*	Source of inflammatory agents	Sigma
Bradykinin **(acetate salt)**	**1118.2**	*H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH (all L-isomers)*	Pharmacologically active peptide	Sigma
Carbachol	**182.6**	*2-[(aminocarbonyl)oxy]-N,N,N-trimethyl-ethanaminium chloride*	Cholinergic agonist	Sigma
Chlorpheniramine **(maleate salt) [Piriton]**	**390.9**	*γ-(4-chlorophenyl)-N,N-dimethyl-2-pyridinepropanamine*	Histamine antagonist	Sigma
Dioxan		*1,4-dioxane*	Solvent	BDH
E. Coli **endotoxin**		*heat-stable lipopolysaccharide protein complex of cell wall of gram-negative bacteria*	Pyrogenic, increase capillary permeability & other	Sigma
FPL55712	**559.7**	*7-3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylate*	Lipoxygenase inhibitor	Fisons
GR38032F (Ondansetron)	**293.4**	*1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl)methyl]-4H-carbazol-4-one*	5-HT3 Antagonist	Glaxo
Histamine **(diphosphate salt)**	**307.1**	*1H-imidazole-4-ethanamine*	H-Receptor agonist	Sigma
5-Hydroxytryptamine **(creatinine sulphate complex)**	**387.4**	*3-(2-aminoethyl)-1H-indole-5-ol*	5-HT Receptor agonist	Sigma
Indomethacin	**357.8**	*1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid*	Cyclooxygenase inhibitor	Sigma
Ketanserin	**395.4**	*3-[2-[4-(fluorobenzoyl-1-piperidinyl]ethyl]-2,4-(1H,3H)-quinazolinedione*	5-HT2 Receptor antagonist	Janssen
Methysergide	**353.5**	*[8β(S)]-9-10-didehydro-N-[1-(hydroxymethyl)propyl]-1,6-dimethyl-ergoline-8-carboxamide*	5-HT2 Receptor antagonist	Sandoz
PGF2α **(tris salt)**	**475.6**	*(5Z,9α,11α,13E,15S)-9,11,15-trihydroxy-prosta-5,13-dien-1-oic acid*	Inflammatory cyclooxygenase product	Sigma
Piriprost	**423.55**	*[4R-4α(1E,1S*),5β]-1,4,5,6-tetrahydro-5-hydroxy-4-(3-hydroxy-1-octenyl)-1-phenyl-cyclopenta[b]pyrrole-2-pentanoic acid.*	Lipoxygenase inhibitor	Upjohn
Pyridine			Solvent	BDH
Spiperone	**395.5**	*8-[4-(4-fluorophenyl)-4-oxobutyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one*	5-HT2 Receptor antagonist	Janssen

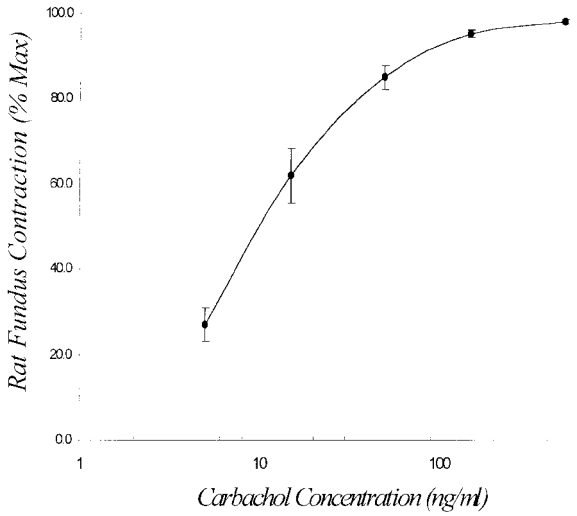

Figure 1. Carbachol Concentration-Response Relationship in the Modified Rat Fundus, Expressed as a Percentage of Its Maximum Response.

In the left-ventral section of the rat fundus strip, Carbachol had an ED_{50} of 8.5(±2)ng/ml.

A carbachol concentration of 5ng/ml was used as a standard internal reference.

By calculating responses to other agonists as a percentage of the carbachol response, the responses were normalized amongst the different tissues in the hope of reducing the possibility of real changes being masked by inter-tissue variation.

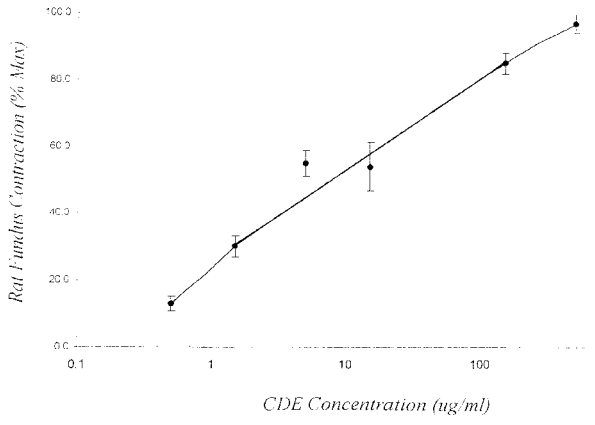

Figure 2. CDE Concentration-Response Relationship in the Modified Rat Fundus, Expressed as a Percentage of Its Maximum Response.

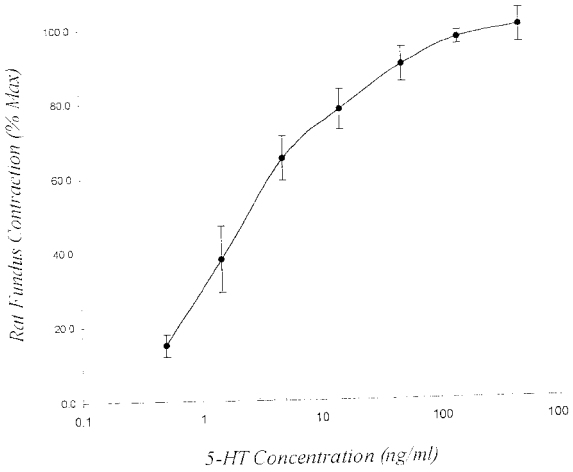

Figure 3. 5-HT Concentration-Response Relationship in the Modified Rat Fundus, Expressed as a Percentage of Its Maximum Response.

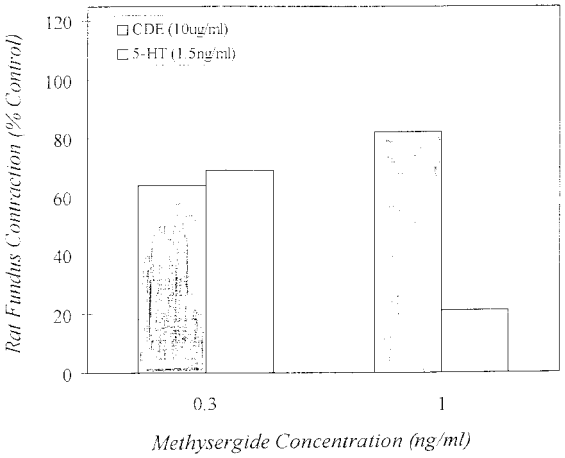

Figure 4. Pilot Study of the Effects of Methysergide on Contractions Induced by 5-HT and CDE in the Modified Rat Fundus Preparation.

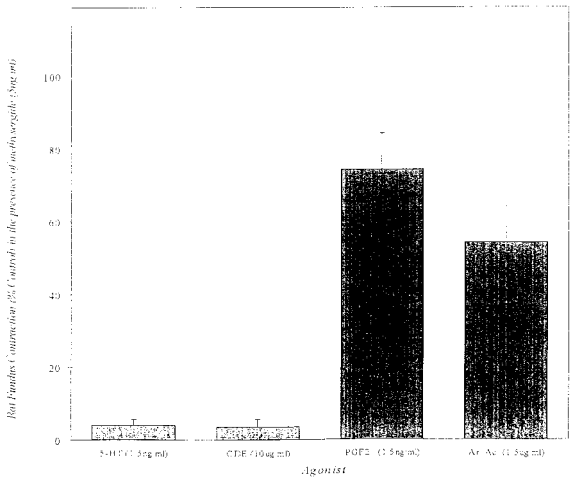

Figure 5. The Effects of Methysergide (5ng/ml) on Contractions Induced by Single Doses of 5-HT, CDE, $PGF_{2\alpha}$ and Arachidonic acid in the Modified Rat Fundus Preparation.

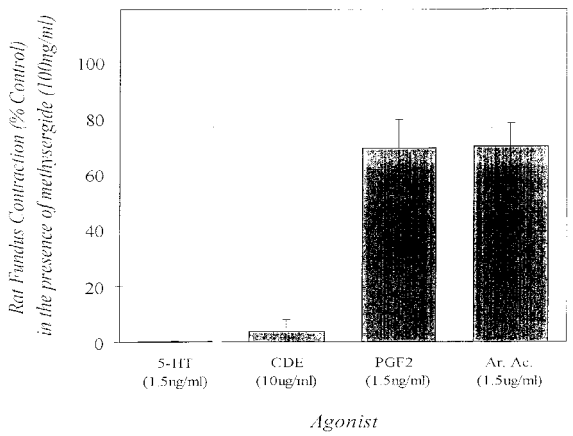

Figure 6. The Effects of Methysergide (100ng/ml) on Contractions Induced by Single Doses of 5-HT, CDE, $PGF_{2\alpha}$ and Arachidonic Acid.

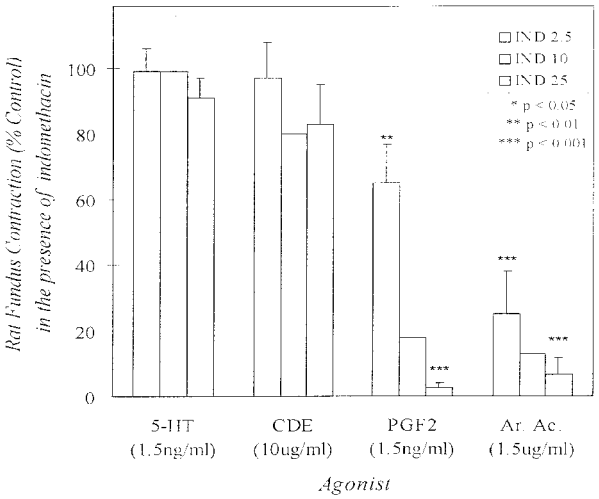

Figure 7. The Effects of Indomethacin (2.5, 10, 25 μg/ml) on Contractions Induced by Single Doses of 5-HT, CDE, $PGF_{2\alpha}$ and Arachidonic Acid in the Modified Rat Fundus Preparation.

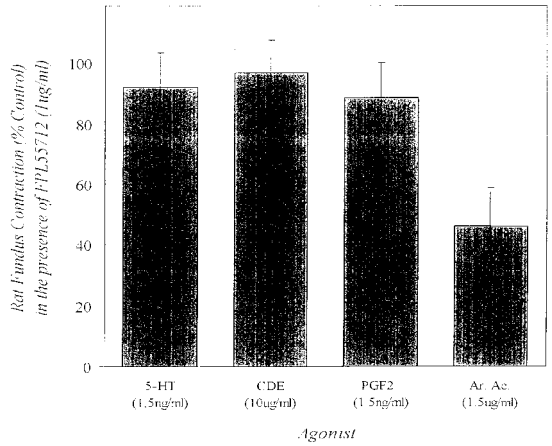

Figure 8. The Effects of FPL55712 (1 μg/ml) on Contractions Induced by Single Doses of 5-HT, CDE, $PGF_{2\alpha}$ and Arachidonic Acid in the Modified Rat Fundus Preparation.

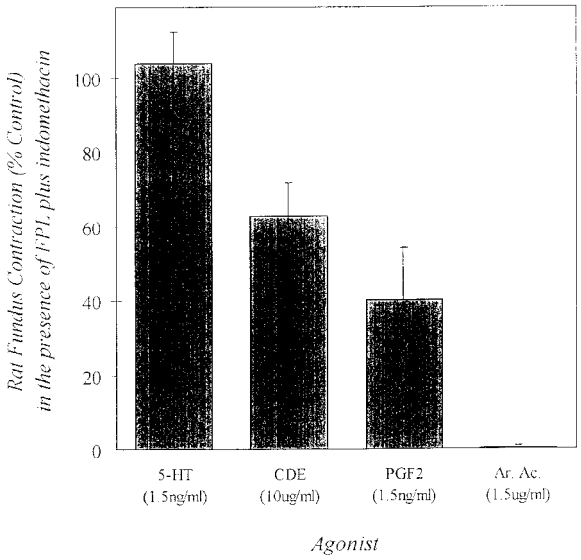

Figure 9. The Effects of FPL55712 (1 μg/ml) and Indomethacin (25μg/ml) on Contractions Induced by Single Doses of 5-HT, CDE, $PGF_{2\alpha}$ and Arachidonic Acid in the Modified Rat Fundus Preparation.