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REGULATION OF RABBIT TESTICULAR CAPSULAR MOTILITY:
THE INTERACTION OF PROSTAGLANDINS, ACETYLCHOLINE
AND SYMPATHOMIMETIC AGENTS

by

James L. Hargrove

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Physiology

Approved:

Major Professor

Committee Member

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UTAH STATE UNIVERSITY
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James L. Hargrove

TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	ii
LIST OF TABLES	v
LIST OF FIGURES	vi
ABSTRACT	xii
INTRODUCTION	1
REVIEW OF LITERATURE	4
Pharmacology and physiology of the testicular capsule	4
Testicular prostaglandin production, distribution and metabolism	9
MATERIALS AND METHODS	14
Experimental animals	14
Exposure of males to females	14
Experiments <u>in vitro</u>	14
Treatments	15
Pressure recording and electrical stimulation	18
Extraction and chromatography of prostaglandin-like compounds	18
Testosterone radioimmunoassay	20
RESULTS	23
Interactions of PGE ₁ with acetylcholine, PGF _{2α} and epinephrine	23
Effects of prostaglandin inhibitors and reserpine on testicular contractility	34

TABLE OF CONTENTS (Continued)

	Page
Electrical stimulation and intratesticular pressure recordings	40
Miscellaneous pharmacological effects	46
Testosterone radioimmunoassay	54
Exposure of male rabbits to females	54
DISCUSSION	58
SUMMARY AND CONCLUSIONS	60
LITERATURE CITED	68
APPENDIX	76
VITA	94

LIST OF TABLES

Table	Page
1. Effects of treatments on the amplitude of testicular contractions <u>in vivo</u>	37
2. Bioassay data showing average prostaglandin F _{2α} equivalents recovered by extraction and chromatography procedure	45
3. Serum testosterone levels (ng/ml) obtained from male rabbits injected with indomethacin, tetraynoic acid, reserpine, prostaglandin E ₁ , or prostaglandin E ₁ plus indomethacin	56
4. Initial amplitude of contraction and prostaglandin-like material recovered from the bathing media of uteri from estradiol-treated (10 μg per day) and estradiol plus indomethacin-treated (10 mg per kg) rats	83
5. Contraction frequency (10-minute interval) of uteri from estradiol-treated (10 μg per day) rats versus those from estradiol plus indomethacin-treated (10 mg per kg) rats in response to stimulation by indomethacin, aspirin and CaCl ₂	87

LIST OF FIGURES

Figure	Page
1. Diagram showing the testicular preparation (a) <u>in vitro</u> and (b) <u>in vivo</u>	16
2. A. Response of testicular capsule <u>in vivo</u> to $1.3 \times 10^{-6}M$ acetylcholine (final concentration)	24
B. Response of the same capsule <u>in vivo</u> to $1.3 \times 10^{-6}M$ acetylcholine in the presence of $1.4 \times 10^{-8}M$ PGE_1	24
C. Absence of capsular response to $1.3 \times 10^{-6}M$ acetylcholine in the presence of $7 \times 10^{-7}M$ PGE_1	24
D. Absence of capsular response to $7 \times 10^{-7}M$ $PGF_{2\alpha}$ in the presence of $7 \times 10^{-7}M$ PGE_1	24
3. A. LDR curve for $PGF_{2\alpha}$ -induced increases in tonus of the testicular capsule <u>in vitro</u>	26
B. $PGF_{2\alpha}$ LDR curve in the presence of $7 \times 10^{-8}M$ PGE_1	26
C. $PGF_{2\alpha}$ curve in the presence of $1.1 \times 10^{-7}M$ PGE_1	26
D. Abolition of testicular response <u>in vitro</u> to $PGF_{2\alpha}$ in the presence of $1.4 \times 10^{-7}M$ PGE_1	26
4. A. LDR curve for acetylcholine stimulation of testicular tonus <u>in vivo</u>	27
B. Acetylcholine's LDR curve in the presence of $7 \times 10^{-7}M$ PGE_1 <u>in vivo</u>	27
C. Lack of stimulation by acetylcholine for the rinsed, inactive rabbit testis <u>in vitro</u>	27

LIST OF FIGURES (Continued)

Figure	Page
5. A. LDR curve for epinephrine-induced increases in tonus <u>in vitro</u> in the presence of $1.4 \times 10^{-9}M$ PGE ₁	29
B. Epinephrine LDR curve in the presence of $1.4 \times 10^{-8}M$ PGE ₁	29
C. Epinephrine LDR curve with no PGE ₁ present	29
D. Inhibitory effect of $1.4 \times 10^{-7}M$ PGE ₁ on epinephrine LDR curve	29
E. Effect of $7 \times 10^{-7}M$ PGE ₁ on epinephrine LDR curve	29
6. A. Potentiation of the <u>in vivo</u> testicular tonus increase to epinephrine by $1.4 \times 10^{-8}M$ PGE ₁	32
B. Epinephrine LDR curve <u>in vivo</u> in the absence of PGE ₁	32
C. Inhibitory effect of $1.4 \times 10^{-7}M$ PGE ₁ on the epinephrine LDR curve <u>in vivo</u>	32
D. Inhibitory effect of $7 \times 10^{-7}M$ PGE ₁ on the epinephrine LDR curve <u>in vivo</u>	32
7. A. Epinephrine LDR curve for the <u>in vitro</u> testicular capsule	33
B. PGE ₁ LDR curve <u>in vitro</u> expressed as percent of the maximum response to epinephrine	33
C. Acetylcholine LDR curve <u>in vitro</u> based on the maximum response to epinephrine	33
8. A. Partial epinephrine LDR curve in the presence of 0.1 mM theophylline and 1.2 μ M propranolol	35
B. Partial epinephrine LDR curve with no other treatment	35
C. Inhibition of epinephrine LDR curve in the presence of theophylline (0.1 mM final concentration)	35

LIST OF FIGURES (Continued)

Figure	Page
9. A. Abolition of testicular contractility <u>in vivo</u> one hour after injection with indomethacin (15 mg/kg ip)	39
B. Stimulation of testicular contractions in an indomethacin-pretreated (15 mg/kg) rabbit <u>in vivo</u> by prostaglandin $F_{2\alpha}$ ($7 \times 10^{-8}M$)	39
C. Decreased testicular contractility <u>in vivo</u> one hour after injection with 5,8,11,14-eicosatetraenoic acid (25 mg/kg ip)	39
10. A. Decreased rate of spontaneous testicular contractions <u>in vitro</u> after addition of indomethacin ($7 \times 10^{-5}M$) to the bathing medium	42
B. Inhibition of prostaglandin $F_{2\alpha}$ -induced tonus increase of rabbit testis <u>in vitro</u> by indomethacin ($7 \times 10^{-5}M$)	42
C. Lack of effect of indomethacin ($7 \times 10^{-5}M$) directly added to medium on testicular contractions <u>in vivo</u>	42
11. A. Testicular tonus increases recorded <u>in vivo</u> following two injections of epinephrine (1 mg) into the marginal ear vein	44
B. Lack of inhibitory effect of dibenamine ($9.6 \times 10^{-6}M$) <u>in vivo</u> on testicular contractions of a rabbit pretreated with reserpine (5 mg/kg)	44
C. Inhibitory effect of dibenamine ($4.8 \times 10^{-6}M$) on tonus increase and contractility induced <u>in vitro</u>	44
D. Stimulation of testicular contractility <u>in vivo</u> by acetylcholine ($1.35 \times 10^{-6}M$).	44
E. Stimulation of testicular contractility <u>in vivo</u> by atropine ($2.1 \times 10^{-4}M$)	44

LIST OF FIGURES (Continued)

Figure	Page
12. A. Pressure fluctuation recorded from rabbit testis <u>in vivo</u>	48
B. Intratesticular pressure during electrical stimulation (1.0 V, 50 Hz, 20 ms delay, 20 ms duration) of spermatic cord	48
C. Intratesticular pressure fluctuations inhibited by iv isoproterenol infusion (0.2 mg)	48
D. Intratesticular pressure increase following iv norepinephrine infusion (0.2 mg)	48
E. Intratesticular pressure following iv PGF _{2α} infusion (0.2 mg)	48
F. Intratesticular pressure following iv PGE ₁ infusion (0.2 mg)	48
13. A. Lack of a dibenamine-induced inhibition of testicular tonus increase on contractility <u>in vitro</u> caused by prostaglandin F _{2α} ($7 \times 10^{-7}M$)	50
B. Inhibitory effect of arachidonic acid ($1.65 \times 10^{-4}M$) on spontaneous testicular contractions <u>in vitro</u>	50
C. Lack of effect of aspirin ($4.2 \times 10^{-5}M$) on spontaneous contractions <u>in vitro</u>	50
14. LDR curve for prostaglandin A ₂ stimulation of testicular tonus <u>in vitro</u>	51
15. A. Stimulation of testicular tonus <u>in vitro</u> by prostaglandin E ₁ ($7.2 \times 10^{-8}M$) followed by inhibition after addition by adenosine ($10^{-4}M$)	53
B. Stimulation of tonus <u>in vitro</u> by prostaglandin E ₁ ($7.2 \times 10^{-9}M$) with subsequent inhibition by caffeine ($3.5 \times 10^{-5}M$)	53
C. Lack of effect of cyclic GMP ($10^{-4}M$) on spontaneous testicular contractions <u>in vitro</u>	53

LIST OF FIGURES (Continued)

Figure	Page
15. D. Inhibitory effect of cyclic GMP ($10^{-3}M$) on testicular contractions <u>in vivo</u>	53
16. A. LDR curve for prostaglandin $F_{2\alpha}$ -induced tonus increase <u>in vitro</u>	55
B. Inhibition of prostaglandin $F_{2\alpha}$ LDR curve <u>in vitro</u> by testosterone ($2.18 \times 10^{-5}M$)	55
C. Inhibition of prostaglandin $F_{2\alpha}$ LDR curve <u>in vitro</u> by testosterone ($4.36 \times 10^{-5}M$)	55
17. A. Reduction of rat uterine contractility <u>in vitro</u> following two changes of the bathing medium (indicated by arrows).	84
B. Restoration of contractility after initial medium from an active preparation was added back to quiescent preparation (first arrow), with reduction in activity after one rinse (second arrow)	84
C. Contractions induced by addition (at arrow) of 28 μM indomethacin, followed by tachphylaxis	84
D. Contractions in response to 0.10 mM aspirin (arrow) followed by tachphylaxis. Vertical bar is equivalent to a pen deflection caused by 1 g; preparation volume equals 20 ml	84
18. A. Stimulation of inactive rat uterus by 0.14 mM indomethacin (added at arrow)	85
B. Stimulation of inactive rat uterus by 0.28 mM aspirin (added at arrow)	85
C. Lack of inhibition of rat uterine contractility following addition (at arrow) of 0.35 mM indomethacin.	85
D. Lack of inhibition of actively contracting rat uterus after addition (at arrow) of 0.056 mM aspirin	85

LIST OF FIGURES (Continued)

Figure	Page
19. A. Stimulation of rat uterine contractility by 7 mM CaCl ₂ (added at arrow).	88
B. Lack of effect of 7 mM CaCl ₂ (arrow) on quiescent uterus from indomethacin-pretreated rat.	88
C. Lack of effect of 7 mM CaCl ₂ (first arrow) followed by contractions induced by addition of 71 nM prostaglandin F _{2α} (second arrow).	88
D. Slight inhibition of actively-contracting rat uterus caused by addition of 50 λ of ethanol (42 mM final concentration) at arrow.	88
E. Lack of stimulation of quiescent rat uterus by 50 λ of ethanol (added at arrow).	88
20. A. Log dose-response (LDR) curve for the acetylcholine-caused contraction rate in uteri from estradiol-pretreated rats	89
B. LDR curve for acetylcholine stimulation of uteri from estradiol- and indomethacin-pretreated rats, in the presence of 7 nM prostaglandin F _{2α}	89
C. LDR curve depicting the response of uteri from estradiol- and indomethacin-pretreated rats to acetylcholine. Vertical bars equal ± the standard error of the mean	89

ABSTRACT

Regulation of Rabbit Testicular Capsular Contractility:
The Interaction of Prostaglandins, Acetylcholine
and Sympathomimetic Agents

by

James L. Hargrove, Doctor of Philosophy

Utah State University, 1975

Major Professor: Dr. LeGrande C. Ellis
Department: Biology

The regulation of the contractility and intratesticular pressure of rabbit testes was studied. The effects of, and interactions among, prostaglandins (PGs) E_1 and $F_{2\alpha}$, testosterone, acetylcholine, epinephrine and norepinephrine were ascertained both in vitro and in vivo. All of these compounds, except for high concentrations of PGE_1 and testosterone, stimulated the testicular capsule to contract. PGE_1 at low concentrations potentiated the contractions produced by epinephrine and acetylcholine. Above $10^{-7}M$, PGE_1 inhibited contractions caused by $PGF_{2\alpha}$, epinephrine or acetylcholine. Testosterone inhibited contractions caused by $PGF_{2\alpha}$ in vitro.

Preinjections of rabbits with the PG synthetase inhibitor, indomethacin, significantly reduced testicular contractility in vivo. A second inhibitor of PG synthetase, 5,8,11,14-eicosatetraenoic acid (TYA), had no such effect. Reserpine failed to

alter testicular contractions in vivo. Attempts to stimulate the spermatic nerve electrically using Ag-AgCl electrodes did not alter intratesticular pressure.

Exposing male rabbits to females had no significant effect on testicular contractions in vivo or in vitro. Serum testosterone measurements were made after injections of indomethacin, TYA, reserpine and combinations of indomethacin and PGE₁. No significant changes were noted among groups due to large variability.

Intratesticular pressure showed spontaneous, rhythmical fluctuations in some preparations. Intravenous injections of norepinephrine increased overall pressure, whereas isoproterenol decreased it. The inhibition of rhythmical pressure changes after iv infusion of isoproterenol persisted at least half an hour. During this time norepinephrine failed to increase intratesticular pressure.

(107 pages)

INTRODUCTION

Prostaglandins (PGs) constitute a family of hormones derived from linoleic and arachidonic acid. They are unsaturated, acidic lipids, 20 carbons in length, that possess a cyclopentano ring with keto or hydroxyl substituents. Kurzrock and Lieb (1930) and Goldblatt (1933) noted both stimulatory and inhibitory influences of human seminal fluid on uterine strips. Von Euler (1936) found that extracts of semen and prostate glands of some species also affected blood pressure; he termed the responsible factor "prostaglandin." The isolation, crystallization and ultimate identification of the PGs (Nugteren et al., 1966) is discussed comprehensively by Bergström (1967).

One of the original suggestions for a physiological function of PGs was that they might activate smooth muscle in ducts and facilitate emptying of accessory genital organs (Von Euler, 1936; Eliasson, 1959). Other roles might include maintenance of spermatozoal activity (Hawkins and Labrum, 1956), regulation of autonomic neuro-transmission (Hedqvist, 1973), and effects on the output of hypothalamic gonadotropins or gonadal steroids (Flack, 1973).

A role for PGs in the testis was first investigated by Ellis and Baptista (1969). They found that PGA_1 and PGE_1 significantly reduced testosterone production in minced rat testes, and that

PGA₁ decreased total androgen production. In addition, lipid peroxidation (which includes PG production) was subject to hormonal control, since hypophysectomy decreased output of peroxides. Antioxidants such as D- α -tocopherol had similar effects (Ellis and Baptista, 1969). Aging also increased lipid peroxidation in their study. Subsequent work showed that rat testes produce PGs from labelled precursors (Carpenter and Wiseman, 1970; Carpenter, Manning and Wiseman, 1971) as well as from unlabelled substrate (Ellis, Johnson and Hargrove, 1972; Ellis, 1972). Rabbit testes also contain PGs (Hargrove et al., 1973; Seeley, 1973), as do swine testes (Michael, 1973).

Testicular smooth muscle may be a target for PG activation. The capsule of rat and rabbit testes contracts in response to exogenously-administered autonomic agents (Davis, Langford and Kirby, 1970). The rabbit testis contracts spontaneously in vitro and in vivo (Davis and Langford, 1970). These spontaneous contractions appear to be mediated by prostaglandins in vitro (Hargrove et al., 1973). The factors that control in vivo capsular activity probably include nerves in the rat (Bell and McLean, 1973) and the dog (Rikimaru et al., 1972) but not the human (Shirai, Suzuki and Rikimaru, 1973). Catecholamines of adrenal origin may also be important in regulating activity of the testicular capsule (Cross, 1958). Epinephrine, norepinephrine, acetylcholine and PGF_{2 α} all contract the testicular capsule (Davis and Langford, 1969a and b; Johnson, Hargrove and Ellis, 1971), while PGE₁ (Hargrove, Johnson and Ellis, 1971) and

testosterone (Seeley et al., 1972) inhibit it. The interactions of these agents may be important in the determination of sperm output, testicular blood flow, and return of lymphatic fluid to the body (Hargrove, Seeley and Ellis, 1973).

REVIEW OF LITERATURE

Pharmacology and physiology of the
testicular capsule

Physiological investigations concerning the smooth muscle of rabbit testicular capsules were begun by B. A. Cross (1959). The capsules contracted rhythmically when the testis was left in the scrotal sac in vivo and contracted tonically after an iv infusion of norepinephrine. In addition, electrical stimulation of the sympathetic centers in the hypothalamus caused capsular contraction (Cross, 1958). With light microscopy smooth muscle cells were later observed in the tunica albuginea of testes from men and rabbits and found to have uneven distribution in the human capsule (Holstein, 1967). In addition, pressure fluctuations equal to 0.25 cm of water occurred, with a frequency of 0.7 per minute (Holstein and Weiss, 1967). These observations were corroborated for the rabbit and extended to the laboratory rat by Davis, Langford and Kirby, 1970; and Davis and Langford, 1969a and b.

The first pharmacological study of capsular smooth muscle indicated that acetylcholine and norepinephrine produced contractures of long duration (20 minutes) in isolated rat testicular capsules at concentrations of 10^{-8} M or less (Davis and Langford, 1969a). These workers postulated, as did Holstein (1967)

and Holstein and Weiss (1967), that such contractions might affect sperm transport. In more extensive studies, Davis and Langford (1970, 1971) noted that parasympathomimetic agents (carbachol and pilocarpine) and sympathomimetic agents (epinephrine and norepinephrine), as well as ganglionic stimulating agents (tetramethyl ammonium), histamine, and $BaCl_2$ all produced contractile responses. Isoproterenol relaxed the capsule (Davis and Langford, 1971). Two layers of muscle were found by microscopic examination, an outer longitudinal layer parallel to the longitudinal axis of the testes with a deeper circular layer (Davis and Langford, 1970). In reviews of their work, Davis, Langford and Kirby (1971) pointed out that radioactive lysine diffused from the outer to the inner compartment of isolated rat testes within 3-4 hours. They suggested that tissue fluid or testicular metabolites may move across the capsule similarly to lysine. Rabbit testes, in contrast to rat testes, evolved rhythmical contractions both in vitro and in vivo, but both showed similar responses to various pharmacological agents. Strips of human capsules from biopsy specimens also contracted autorhythmically in vitro (Davis and Langford, 1970). Examination of human capsules showed that the outer layer (tunica vaginalis visceral) was a serous membrane. The middle layer of the tunica albuginea consisted predominantly of smooth muscle, while the inner tunica vaginalis vasculosa was densely supplied with blood vessels (Langford and Heller, 1973). In the same study,

neighboring muscle cells were shown to approach within 50 Å of each other by way of cytoplasmic extensions. Such close connections may provide the anatomical basis for the concerted, autorhythmic contractions noted in both rabbit and human preparations.

Other pharmacological agonists also affected rabbit testicular contractions. Dimethyl-4-phenylpiperazinium (DMPP), a postjunctional stimulant of autonomic ganglia, induced a contraction, but this could be abolished by bretylium (an agent that prevents adrenergic transmitter release). On the other hand, the preparation was not affected by atropine or hexamethonium (Rikimaru and Suzuki, 1972). These data suggest that there is an adrenergic innervation of the rabbit tunica albuginea.

Tetrodotoxin did not affect autorhythmic contractions but reduced the contractile response to electrical field stimulation. This response was abolished by 1 mM Mn^{++} in the bathing medium (Rikimaru and Suzuki, 1972). One interpretation of these data would be that sodium is less important than calcium ion in producing these contractions, since tetrodotoxin blocks permeability of cells to sodium, and Mn^{++} can antagonize the effects of calcium ion. The lack of effect of bretylium, atropine or tetrodotoxin on spontaneous contractions also implicated substances other than catecholamines or acetylcholine in engendering autorhythmicity. More recent observations on the human capsule failed to detect either catecholamine-containing nerves or

acetylcholinesterase activity (Shirai et al., 1973), although rat capsules appeared to have both types of nerves (Bell and McLean, 1973). In addition, electrical field stimulation of the perivascular nerves of dogs produced a pressure rise equivalent to 50 mm H₂O within the testis (Rikimaru et al., 1972).

Nonnervous factors have recently been implicated in the control of spontaneous rhythmicity of rabbit testicular capsules. Hargrove, Johnson and Ellis, 1971, and Johnson, Hargrove and Ellis, 1971, found that PGE₁ and PGF_{1α}, respectively, inhibited or stimulated rabbit testicular contraction. Later work showed that PGE₁ and E₂ stimulated inactive testes at low doses but inhibited them at high doses, while PGF_{1α} and PGF_{2α} stimulated the preparation at all doses (Hargrove et al., 1973; Seeley et al., 1974). Compounds that had PG-like properties (Hargrove et al., 1973), that co-chromatographed with authentic PGs, and possessed smooth muscle-stimulatory properties (Seeley, 1973), were released into the bathing medium in vitro and in vivo (Seeley et al., 1974). Changing the original bathing medium abolished spontaneous rhythmicity in vitro, while bathing the inactive preparation in the original media restored activity (Hargrove et al., 1973). These effects, however, could not be demonstrated in vivo (Seeley et al., 1974). In addition, both rat and rabbit testes yielded extractable, PG-like material when whole testes were homogenized (Seeley, 1973). PGs added to the bathing medium of rabbit testicular capsules not only induced contractions in most

quiescent preparations, but also potentiated the responses of the testes to exogenous epinephrine and histamine (Hargrove, Seeley and Ellis, 1973).

Investigation into the mechanism by which PGs stimulate rabbit testicular capsules showed that calcium ion (Ca^{++}) stimulated active, but not inactive, preparations (Hargrove, Seeley and Ellis, 1973). PGs could restore this Ca^{++} stimulation in a dose-dependent manner and were themselves dependent on Ca^{++} for their action. Thus, a reciprocal dependency was observed between PGs and Ca^{++} . Seeley et al., 1974, investigated the inhibitory action of PGEs and suggested that inhibition was due to the accumulation of cyclic adenosyl 3',5' monophosphate (cyclic AMP). A tonus decrease or complete relaxation of the capsule could be produced by either dibutyryl cyclic AMP or theophylline, the latter being a compound that causes cyclic AMP to accumulate intracellularly by blocking cyclic nucleotide phosphodiesterase. In summary, low concentrations of PGEs apparently stimulate capsular contractility by allowing increased Ca^{++} into the cell, and higher doses inhibit testicular motility by causing cyclic AMP to build up. PGFs, however, have no apparent effect on cyclic AMP and do not inhibit the testis.

Other studies on the testicular capsule showed that testosterone and other steroids inhibited contractility in vitro (Seeley et al., 1972). However, these steroids had no effect in vivo (Seeley et al., 1973). Some mechanism apparently prevents steroid

inhibition of the capsule's activity in the live rabbit, a result of interest, since the testis is the site of testosterone production.

Some results from other laboratories may suggest roles for testicular capsular contractions. Free and Jaffe, 1972, noted rhythmical pulsations of blood pressure in rat testes, possibly implicating the capsule as a regulator of hemodynamics. Concerning the tunica albuginea's speculative role in sperm transport, it has been shown in rabbits that injections of $\text{PGF}_{2\alpha}$ and PGE_2 can affect sperm numbers in the deferent duct (Hafs, Louis and Stellflug, 1974) and reduce sperm transport time by two days (Hunt and Nicholson, 1972).

Testicular prostaglandin production, distribution and metabolism

Early attempts failed to detect PGs in testes (Von Euler and Hammarstrom, 1937; Horton and Thompson, 1964) or to show entry of tritiated PGE_1 into rat or dog testes (Nakano, 1970). However, PGs E_1 , E_2 , and $\text{F}_{2\alpha}$ were reported to be present endogenously and to be synthesized by rat testes injected with ^{14}C -linoleic acid (Carpenter and Wiseman, 1970; Carpenter et al. 1971). Acidic lipids that co-chromatographed with authentic PGs E_1 , E_2 , $\text{F}_{1\alpha}$ and $\text{F}_{2\alpha}$ and possessed smooth muscle-stimulatory properties were found to be released by rabbit testes into saline solution during pharmacological studies (Hargrove

et al., 1973; Seeley, 1973). Larger quantities of such PG-like material were extracted from rabbit and rat testes (Seeley, 1973) and from swine testes (Michael, 1973). Studies employing bioassay (Seeley, 1974), gas chromatography, or radioimmunoassay (Carpenter, 1974) to detect PGs all suggest that the major PG in rat testis is $F_{2\alpha}$. Carpenter (1974) pointed out that the principal PGs produced by a tissue often correspond to the amount of precursor polyenoic fatty acid present and that this generalization holds for the testis, since arachidonic acid predominates there. Thus the quantities of $PGF_{2\alpha}$ and E_2 exceed those of $F_{1\alpha}$ and E_1 (Seeley, 1973; Carpenter, 1974). In addition, Carpenter extracted about five times as much $PGF_{2\alpha}$ from immature, as compared to mature, organs. Rabbit testicular homogenates convert approximately one percent of substrate into PGE_1 (Christ and Van Dorp, 1972); comparable studies have not been carried out for conversion of arachidonate into $PGF_{2\alpha}$ or E_2 .

PGB and material that may represent other PG metabolites also appear to be present in rat testes (Carpenter, 1974). PGs are rapidly metabolized by testicular homogenates of either rat or swine testes. Both 15-hydroxy-PG dehydrogenase and prostaglandin- δ^{13} -reductase are present in the supernatant, particle-free fractions (Anggard, Larsson and Samuelsson, 1971; Nakano and Prancan, 1971; Nakano, Montague and Darrow, 1971). The dehydrogenase enzyme was more active in the testicular preparation than in the ovary, uterus or placenta, and the reductase enzyme

was comparable in activity to homogenates from such sources (Nakano, Montague and Darrow, 1971). In view of the suggested importance of PGs for ovarian and uterine function (Labhsetwar, 1974), the greater metabolic capacity of the testis for these compounds could indicate equivalent functional importance in the male gonad. There is increasing evidence that points to a dependence of testicular and seminal vesicular PG production on androgens and pituitary gonadotropins. Ellis and Baptista (1969) observed that testicular production of malonaldehyde (a by-product of PG synthesis and lipid peroxidation) decreased following hypophysectomy in rats. In addition, PG synthetase activity decreased significantly five weeks after hypophysectomy, and possibly after adrenalectomy as well (Ellis, Johnson and Hargrove, 1972). In corroboration of pituitary control of PG synthesis, Sturde (1971a) found that a synthetic androgen, methyl androstano-
lone, increased seminal PG concentration from 0.9 to 6.7 mg/ml in human males attending a fertility clinic. He also noted a doubling of sperm concentration from 8.2 to 16.4 million/ml. Later studies showed that treatment with human chorionic gonadotrophin (HCG) increased seminal prostaglandins without altering sperm numbers in 24 subjects (Sturde and Bohm, 1971). Paradoxically, administering an antiandrogen (Cyproterone; 200 to 400 mg) for 4-16 weeks also increased seminal PGs, possibly due to increased gonadotrophin output following abolition of negative feedback by androgens (Sturde, 1971b). Homogenates of

seminal vesicles collected from rats one day after hypophysectomy have only 30 percent of the capacity for PG biosynthesis shown by controls. Hypophysectomy also inhibited the conversion of arachidonic acid to PGE_2 in vitro and depleted rat seminal vesicles of their stores of arachidonate (Tan and Privett, 1973). These investigators noted that hypophysectomy, like essential fatty acid deficiency, can produce dermal scaling despite dietary supplements of these fatty acids; moreover, essential fatty acid deficiency mimicks hypophysectomy in that it leads to testicular atrophy. In addition, mating increased the PG content of vasa deferentia from mice compared to tissue from isolated, nonexposed animals (Marley and Smith, 1974). Barcikowski, Saksena and Bartke (1973) administered testosterone propionate to castrated rats and found an increase of plasma PGF levels from 0.9 to 4.1 ng/ml. A low dose of cyproterone acetate decreased plasma PGF, while a high dose increased it (in agreement with Sturde, 1971b).

PGs also decrease testosterone output, a result similar to the luteolytic capacity of PGs in the female. Such an effect has been observed following injections of $\text{PGF}_{2\alpha}$, E_2 , A_1 or A_2 (Bartke et al., 1973; Saksena, El Safoury and Bartke, 1974). The site of action probably is testicular, since prostaglandins induce the release of pituitary gonadotropins (Harms, Ojeda and McCann, 1974). The site of action for reduction of testosterone output may be cholesterol esterase, an enzyme that cleaves the testosterone precursor, cholesterol, from fatty acids that are

bound to it. Bartke et al. (1973) noted that 200 μg of $\text{PGF}_{2\alpha}$ injected sc into mice lowered testosterone and increased the concentration of esterified cholesterol in the testis. Free and Tillson (1973) suggested that the depressed testosterone secretion rate they measured following iv PGE_2 infusion in rats may have been caused by the depressed testicular blood flow that they also observed. However, other work suggests a stimulatory effect of PGs on steroid synthesis. For instance, Eik-Nes (1971) noticed an increased testosterone outflow following high PGE_2 infusion rate (1 $\mu\text{g}/\text{min}$). One rationalization for such an effect may be that PGEs can increase 3',5'-cyclic adenosine monophosphate (cyclic AMP) in minced rat testes (1 mM caffeine was also present), according to Butcher and Baird (1968). No effect of PGE_1 or $\text{F}_{1\alpha}$ was observed for cyclic AMP accumulation in seminiferous tubules (Kuehl et al., 1970a, so the Leydig cells would be the presumed site of action. Kuehl and coworkers (1970b) noted that a proposed PG antagonist, 7-oxa-13-prostynoic acid, blocked the rise of cyclic AMP induced by follicle-stimulating hormone (FSH) in rat testes. This important observation may indicate a natural role for PGs in mediating or contributing to testosterone production that follows gonadotropin stimulation.

MATERIALS AND METHODS

Experimental animals

Mature male rabbits of mixed breeding (Dutch Belted, New Zealand, or crosses of these strains) were either obtained from local sources or raised in a small animal laboratory. The rabbits were isolated from females, under natural photoperiod, and at a temperature range of 70 to 80 degrees Fahrenheit (approximately 21 to 27 degrees C) in a small animal laboratory. Rabbits were provided with water and feed (Purina rabbit chow) ad libitum. Data recorded from 63 rabbits are presented in this investigation.

Exposure of male rabbits to females

Eight male rabbits were transferred to large dog cages in which rabbit cages containing females had been placed. These buck rabbits were maintained with the females for two weeks prior to sacrifice. Conditions of temperature, photoperiod and feed were similar to those given in the preceding paragraph.

Experiments in vitro

Testes used for studies in vitro were obtained by anesthetizing the animals with sodium pentobarbital (20 to 60 mg per kg ip or iv; Abbott Laboratories), supplemented with ether when necessary. The experimental rabbits were restrained with their ventral sides

up on a small animal operating table. An incision was made through the scrotum and tunica vaginalis parietal, the blood supply was excluded with a ligature, and the testis was excised. Cotton ligatures were attached to the testicular capsule at both the superior and inferior poles. This preparation was then suspended in a water-jacketed muscle warmer that contained oxygenated Tyrode's solution at a temperature of 35 degrees C. The lower thread was secured to a rubber stopper, while the upper one passed to a sensitive myograph transducer (Statham Co.) attached to a recording Gilson minipolygraph (Figure 1A).

Experiments in vivo

Once the testis used for in vitro studies had been removed from the rabbit, the incision in the scrotal sac was closed with wound clamps, and the opposite scrotum was similarly opened. Cotton threads were attached to the two poles of the testis and used to draw it through a celluloid dam into a second water-jacketed muscle warmer that was not provided with oxygenation. A circular wire tie was passed around the base of the muscle warmer, thereby securing the scrotal sac, celluloid dam and inferior cotton thread to the base. This arrangement sealed the peritoneal cavity against leakage of Tyrode's solution from the muscle warmer. The rabbit's anterior end was then elevated, the preparation was checked for signs of ischemia, and the superior thread was finally attached to a myograph transducer as described above (Figure 1B).

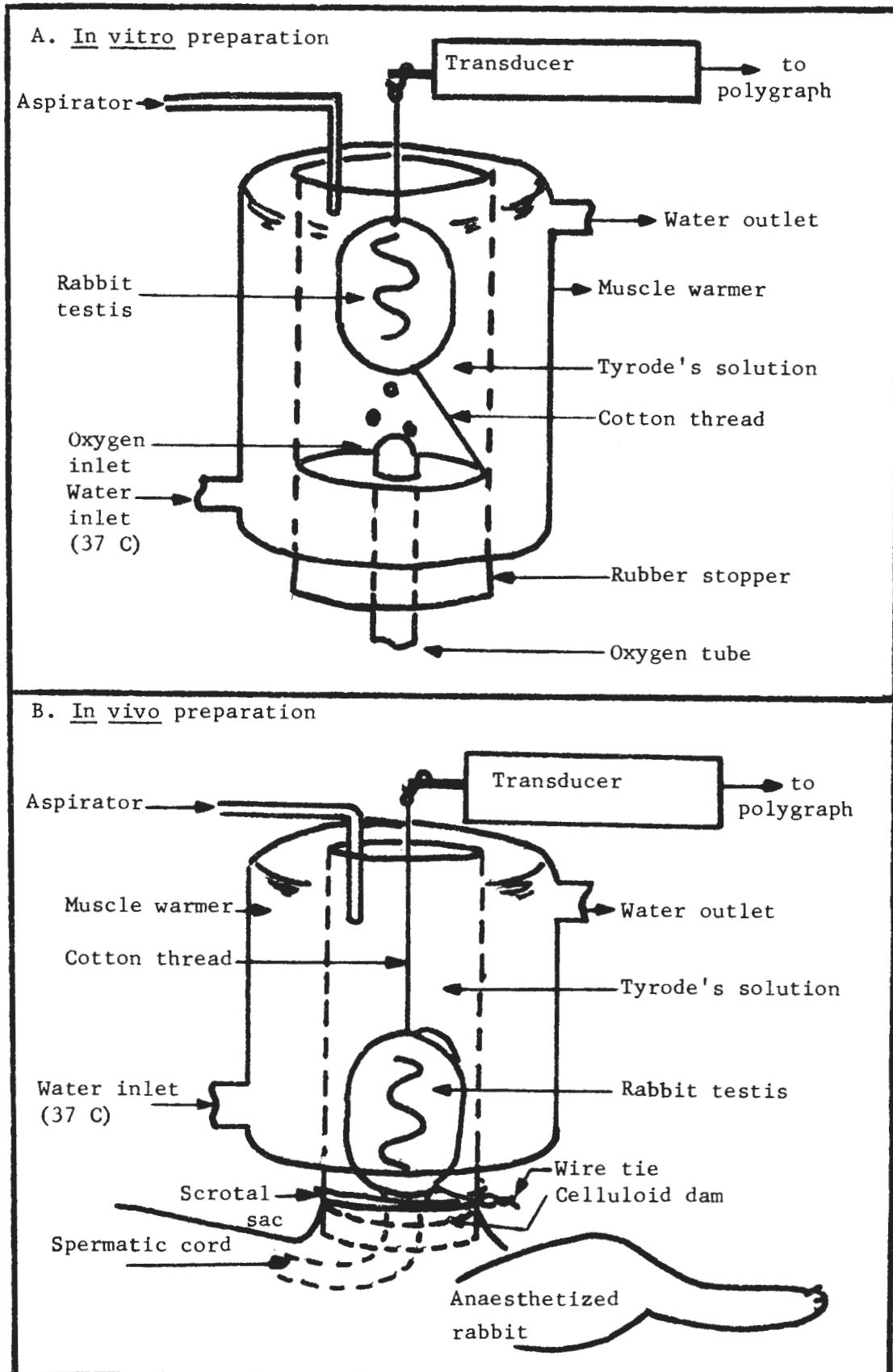


Figure 1. Diagram showing the testicular preparation (a) in vitro and (b) in vivo.

Treatments

Prostaglandins E_1 and $F_{2\alpha}$ (Upjohn Co.), testosterone propionate (Sigma) and indomethacin (Merck, Sharpe and Dohme, Inc.) were dissolved in 95 percent ethanol and added directly to the bathing medium. Mixing the drug into the medium was accomplished by the oxygen bubbles for in vitro studies, and manually for in vivo investigations. L-epinephrine, norepinephrine and acetylcholine (Sigma), L-propranolol (Ayerst Laboratories), and theophylline (K & K Laboratories, Inc.) were dissolved in distilled water and added to the bathing medium. The epinephrine stock solution was acidified with HCl in order to solubilize the epinephrine.

Groups of four animals each were injected ip with the following agents: 2 ml corn oil (this served as the vehicle for all other agents), or 15 mg/kg indomethacin, 25 mg/kg of 5,8,11,14-eicosatetraenoic acid (Hoffmann-La Roche, Inc.), 5 mg/kg reserpine (Sigma), or 15 mg/kg indomethacin plus 0.5 mg/kg PGE_1 . Each agent was injected ip in 2 ml of corn oil. Blood samples were taken prior to injection and at 1, 4 and 12 hours thereafter by cardiac puncture. The samples were centrifuged for 5 minutes in a clinical centrifuge, and the serum was pipetted for storage at -20 degrees C. These samples were analyzed for testosterone by radioimmunoassay (see below). The following day the rabbits were anesthetized and testicular contractions were measured in vivo. The contralateral gonad was frozen in a small volume of Tyrode's solution at -20 degrees C and later assayed for endogenous PGs.

Pressure recording and electrical stimulation

Intratesticular pressure changes were recorded from eight rabbits. A 21-gauge needle was attached to a Statham pressure transducer (range: 0-15 mm Hg) that contained heparinized Tyrode's solution in the recording chamber. The needle was inserted into the testis directly through the scrotal sac. After half an hour of recording basal contractility, norepinephrine or isoproterenol (0.2 mg) was infused into the marginal ear vein. In six rabbits electrical stimulation of the perivascular nerve in the spermatic cord was also attempted. An incision 1 cm in length was made through the scrotum at the juncture of the vas deferens and epididymal fat pad. A pair of silver-silver chloride electrodes was touched to the vas and electrical current (0.5 to 4.0 volts, 50 Hz, 20 ms duration and 20 ms delay) was passed through the tissue. Pressure changes were simultaneously recorded. A Grass stimulator served as a source for electrical current.

Extraction and chromatography of prostaglandin-like compounds

Argentaffin thin-layer chromatography was carried out, using plates prepared as described in Green and Samuelsson (1964) with a coating of silica gel (Merck type H, without gypsum binder). A slurry was prepared with the following proportions: 30 g of silica gel, 60 ml of water and 6 g silver nitrate. This slurry was

spread onto glass plates that had been washed in acidic methanol and rinsed with deionized distilled water. The gel formed a layer approximately 0.25 mm thick. These plates were activated by heating for 30 minutes at 110-115 degrees C, and were thereafter stored in a dessicator in a darkened cabinet. Residues from acetone-extracted testicular homogenates were applied to these plates in 50 μ l of ethanol. The plates were developed in the A-II solvent system of Green and Samuelsson (1964), comprised of ethyl acetate, acetic acid, methanol, 2,2,4-trimethylpentane and water in the ratio of 110:30:35:10:100 v/v. The resulting mixture was equilibrated for two hours and the less-polar phase was used to develop the plates.

Rabbit testes were extracted as follows: Testes were decapsulated by opening the tunic with a razor blade, holding the capsule tightly with forceps, and scraping the contents out with the razor blade. The tubules were weighed and homogenized in a heavy glass tube, using a drill with a Teflon pestle attachment, and 20-ml aliquots of acetone. The homogenate was centrifuged at 10,000 rpm for five minutes and the supernatant was transferred to an evaporation flask. After two further washes and centrifugations of the residue, the acetone extracts were evaporated under negative pressure in a rotary evaporator at a temperature of 40 degrees C. The resulting residue was taken up in 20 ml of acidic phosphate buffer (pH, 3.0) and subsequently partitioned three times against equal volumes of ethyl acetate. After the

organic solvent was evaporated, the residue was chromatographed as described above.

Once the chromatograms were developed, zones that migrated with authentic PGE₂, E₂, F_{1α} or F_{2α} were scraped from the plates and eluted three times with 5-ml volumes of methanol. The resulting extract was dried under nitrogen gas at 40 degrees C, taken up in 5 ml of phosphate buffer (pH, 3.0), and partitioned three times against ethyl acetate. The ethyl acetate was evaporated under nitrogen gas to yield a residue carrying prostaglandin-like compound. This residue was dissolved in 20 ml of prewarmed Tyrode's solution and bioassayed on the rabbit testicular capsule.

Testosterone radioimmunoassay

Sequential blood samples were taken by cardiac puncture just prior to and at 1-, 4- and 12-hour intervals after injection with indomethacin (15 mg/kg), PGE₁ (0.5 mg/kg), indomethacin plus PGE₁ (same doses), 5,8,11,14-eicosatetraenoic acid (25 mg/kg) or reserpine (5 mg/kg). The unanaesthetized rabbits were restrained by an assistant while about 1-2 ml of blood was drawn from the heart into a 5-ml syringe equipped with a 21-gauge disposable needle. The blood was centrifuged for 10 minutes before the serum was pipetted off and transferred to vials for storage at -20 degrees C.

Radioimmunoassay for plasma testosterone levels was carried out according to the procedure of Mongkonpunya and Niswender, 1973.

Duplicate aliquots of serum or plasma (0.1 ml) were placed in disposable culture tubes (15 x 80 mm). To account for procedural losses, 2000 dpm of ^3H -1,2-testosterone (New England Nuclear; 45 c/mM, repurified by column chromatography) was added to a third aliquot from a representative number (10 to 20 within each assay) of unknowns. Samples of serum with ^3H -1,2-testosterone were vortexed for 10 sec and endogenous and labelled hormones were allowed to equilibrate for 30 min. Serum was extracted by vortexing with 2.0 ml of nanograde diethyl ether:hexane (1:2) for 30 sec. Tubes were then stored at -20 degrees C for at least 1 hr to freeze the aqueous phase. With precautions taken to avoid thawing the aqueous phase, extracts destined for radioimmunoassay were decanted into disposable culture tubes (12 x 75 mm) and those for procedural losses were decanted into scintillation vials. Radioactivity of these extracts was averaged to determine a single correction factor to account for procedural losses of testosterone in all serum samples.

Standard testosterone (Sigma Chemical Co.) was pipetted from a stock solution of 10 ng/ml and at least two sets (0.0, 0.025, 0.05, 0.1, 0.25, 0.50, 1.00 and 1.50 ng) were included in each assay. Standard testosterone and serum extracts were dried under nitrogen. Tube walls were rinsed once with minimal amounts of analytical grade diethyl ether and dried under nitrogen.

Gelatin (0.1 percent, Knox Gelatin), in 0.01 M phosphate-buffered saline, was used to dilute the antibody to 1:3000.

Antibody (0.2 ml) was added to each tube, vortexed for 10 sec and allowed to incubate at room temperature for 30 min. Two hundred microliters of 0.1 percent gelatin (Knox Gelatin, Inc., Johnstown, N. Y.) in 0.01 M phosphate-buffered saline, containing 30,000 dpm of ^3H -1,2,6,7-testosterone (New England Nuclear; 91 c/mM), was added to each tube. The contents of the tubes were mixed for 5 sec and incubated for 12 to 18 hr at 5 C.

To separate bound and free testosterone, 1.0 ml of 0.025 percent dextran 150 (Pharmacia, Uppsala, Sweden) and 0.25 percent carbon decolorizing neutral norit (Fisher Scientific Co.) in glass distilled water was added to each tube. Contents were mixed, incubated in an ice bath for 10 min and then centrifuged at 2,500 g for 10 min at 5 C. A 0.5 ml-aliquot of the supernatant fluid was diluted with a liquid scintillation fluid (Bray, 1960) for quantification of radioactivity in a liquid scintillation spectrometer (Nuclear Chicago Corp., Mark I).

For comparison among assays, standard sera with high and low testosterone, and extracts from blank extraction tubes, were assayed with each set of unknown serum samples.

Estimations of serum unknowns from both bulls and rabbits determined by this method do not differ significantly from estimations performed on the same samples purified on Sephadex LH-20 columns ($r = 0.985$ and 0.932 , respectively). Neither do values obtained by this method differ from determinations made on the same bull samples by gas liquid chromatography ($r = 0.812$) and competitive protein binding ($r = 0.957$).

RESULTS

Interactions of PGE₁ with acetylcholine,
PGF_{2α} and epinephrine

Testicular contractions in vivo were typically rhythmic (Figure 2). Acetylcholine stimulated capsular tonus (Figure 2A) with a threshold of about $2 \times 10^{-8}M$. In the presence of $10^{-8}M$ PGE₁, however, the capsular tonus increased to a greater extent while amplitude of contraction decreased (Figure 2B). Higher concentrations of PGE₁ ($10^{-7}M$) reduced or abolished spontaneous motility and stimulation due to acetylcholine (Figures 2C and 4B). Two of the five preparations in vivo failed to respond to acetylcholine in the absence of PGE₁ while the others contracted markedly. This type of variability was not seen in response to epinephrine.

PGE₁ at $10^{-7}M$ final concentration also abolished the response to high doses of PGF_{2α} in vivo (Figure 2D). It prevented PGF_{2α}-induced stimulation of the testicular capsule in vitro in a non-competitive manner at concentrations of PGE₁ greater than $7 \times 10^{-8}M$ (Figure 3).

Figure 3C depicts the control LDR curve for epinephrine in vitro; the LDR curve for norepinephrine was also determined but did not differ significantly from that for epinephrine (data not shown). The presence of 1.4×10^{-8} or $1.4 \times 10^{-9}M$ PGE₁ (Figures 5A and B, respectively) markedly potentiated the

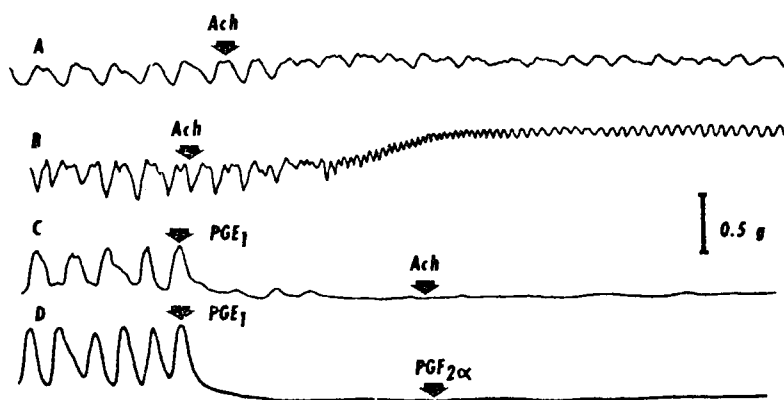
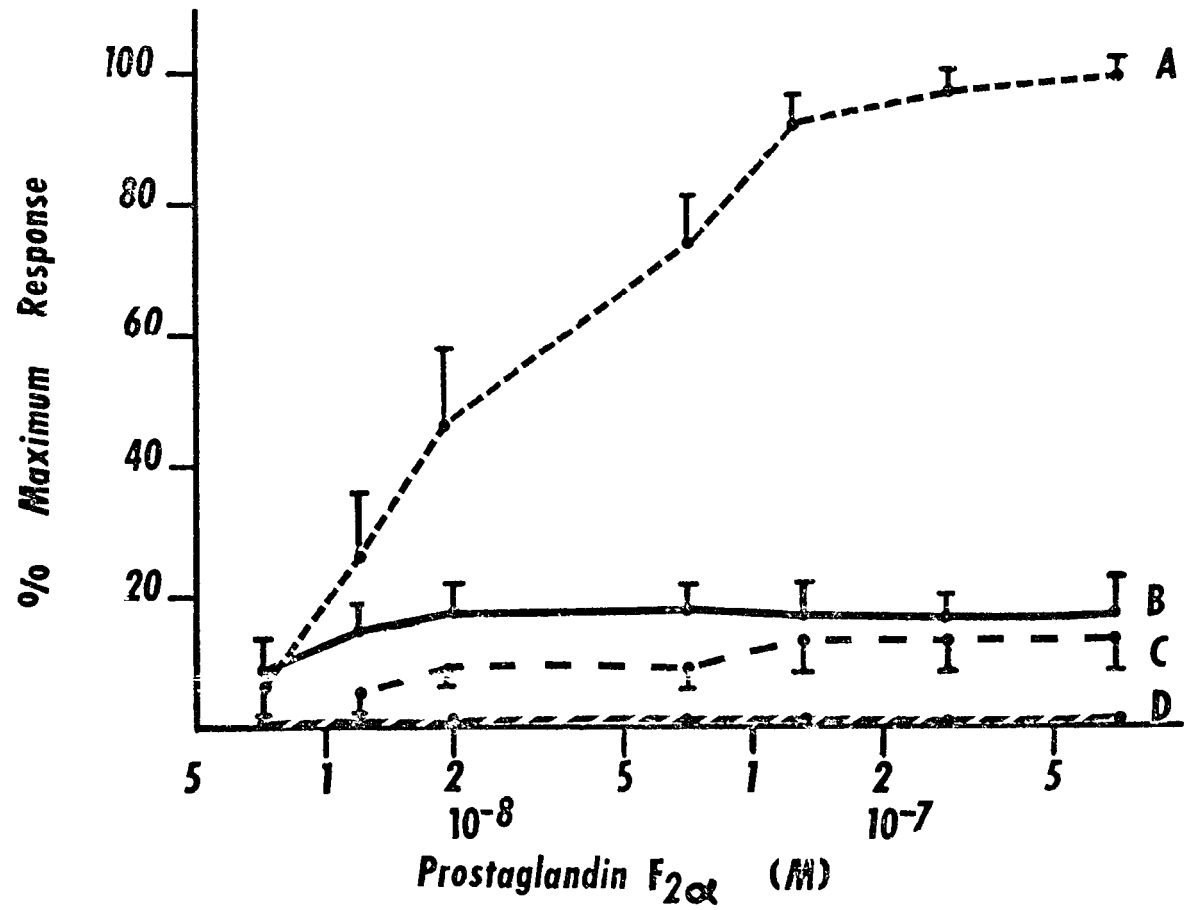


Figure 2. A. Response of testicular capsule *in vivo* to 1.3×10^{-6} M acetylcholine (final concentration). B. Response of the same capsule *in vivo* to 1.3×10^{-6} M acetylcholine in the presence of 1.4×10^{-8} M PGE_1 . C. Absence of capsular response to 1.3×10^{-6} M acetylcholine in the presence of 7×10^{-7} M PGE_1 . D. Absence of capsular response to 7×10^{-7} M $PGF_{2\alpha}$ in the presence of 7×10^{-7} M PGE_1 .

Figure 3. A. LDR curve for $\text{PGF}_{2\alpha}$ -induced increases in tonus of the testicular capsule in vitro.
B. $\text{PGF}_{2\alpha}$ LDR curve in the presence of $7 \times 10^{-8} \text{M PGE}_1$. C. $\text{PGF}_{2\alpha}$ LDR curve in the presence of $1.1 \times 10^{-7} \text{M PGE}_1$. D. Abolition of testicular response in vitro to $\text{PGF}_{2\alpha}$ in the presence of $1.4 \times 10^{-7} \text{M PGE}_1$. Vertical bars equal the S.E.M.



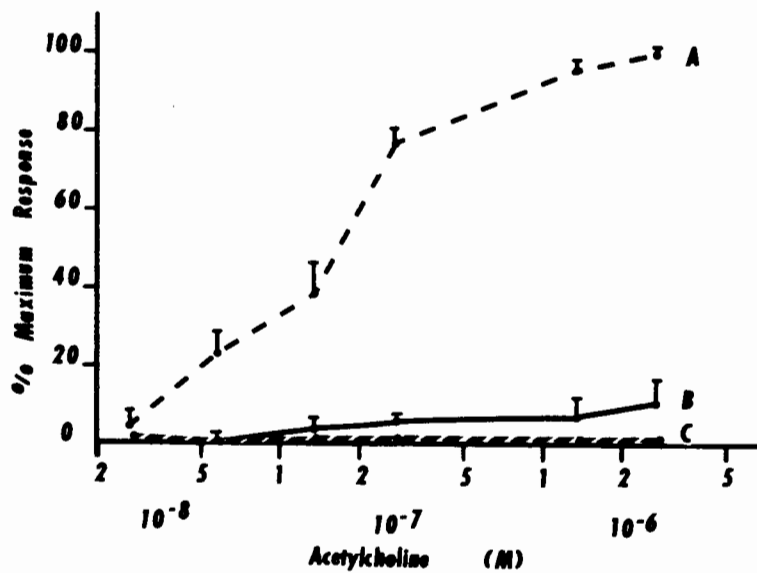
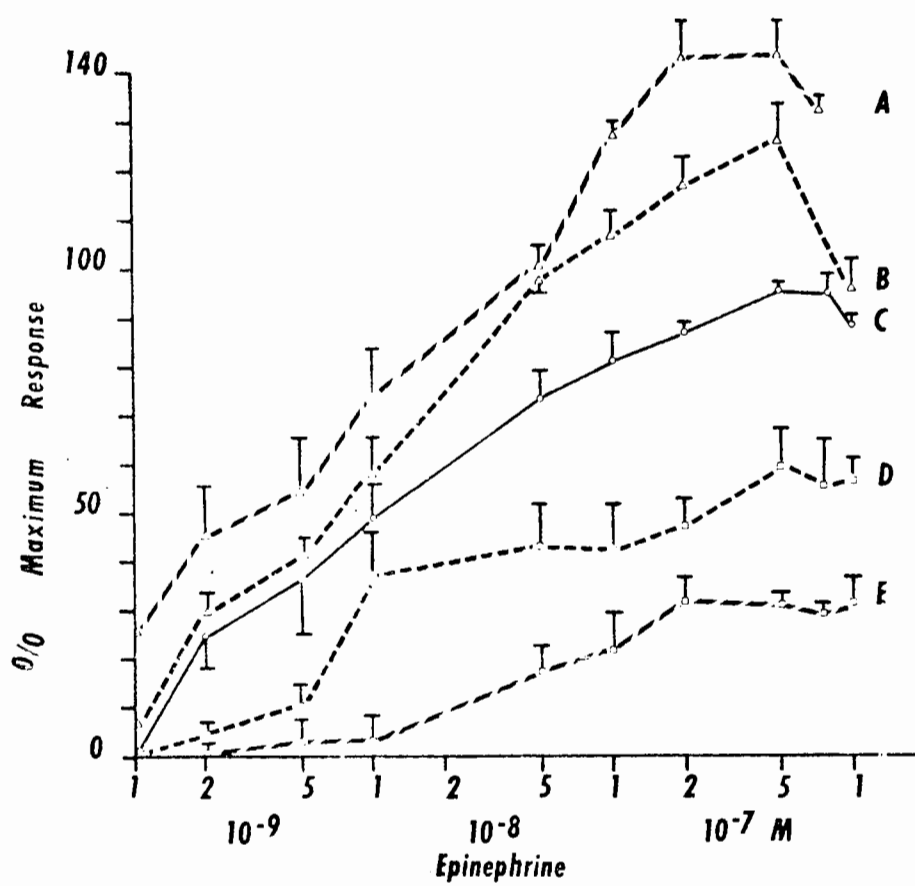


Figure 4. A. LDR curve for acetylcholine stimulation of testicular tonus in vivo. B. Acetylcholine's LDR curve in the presence of 7×10^{-7} M PGE₁ in vivo. C. Lack of stimulation by acetylcholine for the rinsed, inactive rabbit testis in vitro.

Figure 5. A. LDR curve for epinephrine-induced increases in tonus in vitro in the presence of 1.4×10^{-9} M PGE_1 . B. Epinephrine LDR curve in the presence of 1.4×10^{-8} M PGE_1 . C. Epinephrine LDR curve with no PGE_1 in medium. D. Inhibitory effect of 1.4×10^{-7} M PGE_1 on epinephrine LDR curve. E. Effect of 7×10^{-7} M PGE_1 on epinephrine LDR curve.



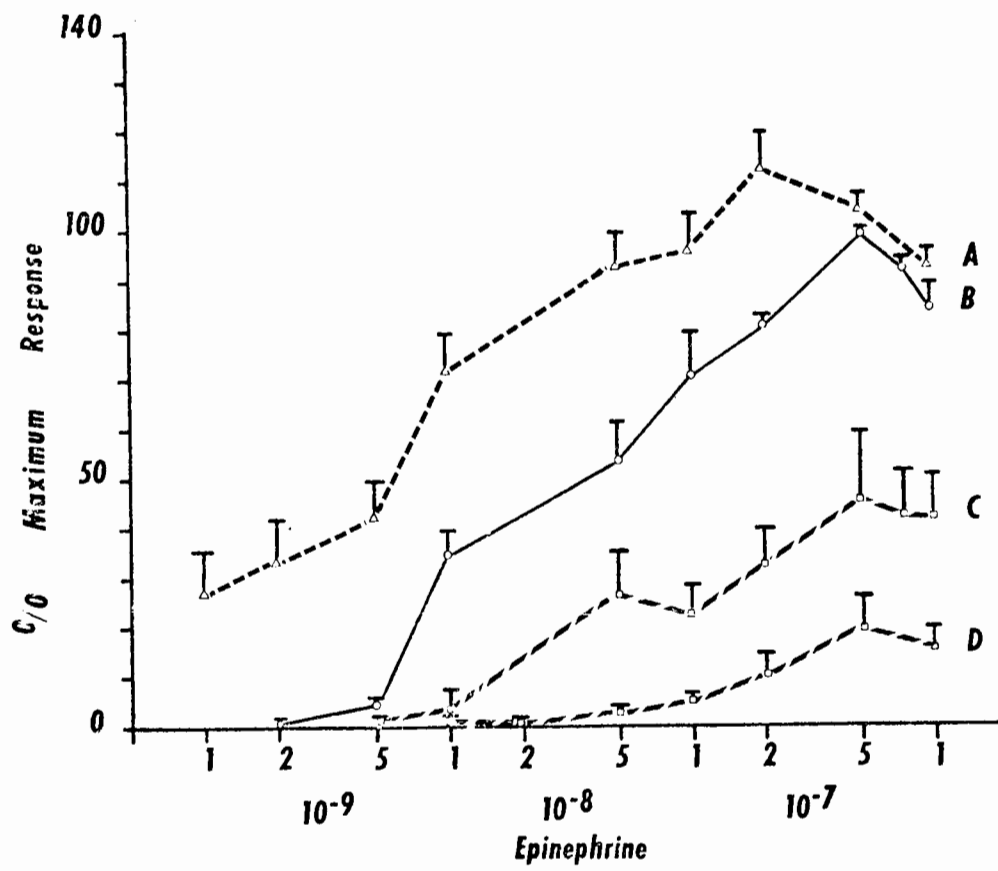
epinephrine-induced tonus increase of in vitro testicular capsules. Higher concentrations of PGE_1 , however, noncompetitively inhibited epinephrine-induced tonus increases at all concentrations tested and increased the threshold for stimulation (Figures 5D and E). At 7×10^{-7} PGE_1 , the maximum average stimulation by epinephrine was reduced to less than 30 percent of the control value. However, $\text{PGF}_{2\alpha}$ (10^{-7} to 10^{-6}M) did not reduce epinephrine stimulation (data not shown).

PGE_1 at $1.4 \times 10^{-8}\text{M}$ potentiated the response of the capsule in vivo to epinephrine as well (Figure 6A). As with the in vitro preparation, higher concentrations of PGE_1 inhibited the testis and increased the amount of epinephrine required to stimulate the capsule (Figures 6C and D). At $7 \times 10^{-7}\text{M}$, PGE_1 reduced the average maximum tonus increase induced by epinephrine to about 20 percent of the control value; in the presence of this concentration of PGE_1 , two of the five preparations studied gave no response to epinephrine.

Epinephrine's efficacy in stimulating the capsule in vitro exceeded that of either PGE_1 or acetylcholine. Higher concentrations of PGE_1 became progressively less stimulatory on isolated, rinsed preparations, while acetylcholine alone had no effect on such units in contrast to its stimulation of the testis in vivo (Figure 7C).

According to the LDR curve for epinephrine in vitro (Figure 5C), higher doses became slightly less stimulatory.

Figure 6. A. Potentiation of the in vivo testicular tonus increase to epinephrine by 1.4×10^{-8} M PGE₁. B. Epinephrine LDR curve in vivo in the absence of PGE₁. C. and D. Inhibitory effects of 1.4×10^{-7} and 7×10^{-7} M PGE₁ on the epinephrine LDR curve in vivo.



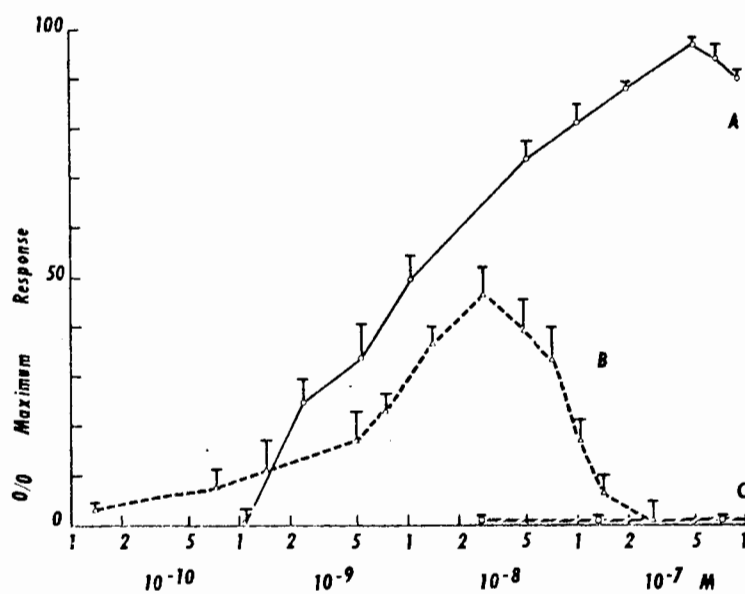


Figure 7. A. Epinephrine LDR curve for the in vitro testicular capsule. B. PGE₁ LDR curve in vitro expressed as percent of the maximum response to epinephrine. C. Acetylcholine LDR curve in vitro based on the maximum responses to epinephrine.

Theophylline (0.1 mM), a phosphodiesterase inhibitor, reduced epinephrine stimulation to 60 percent of the control value (compare Figures 8B and C). However, the beta-adrenergic-blocking agent, propranolol, not only restored, but also increased, the response of the capsule to a given dose of epinephrine in the presence of theophylline (Figure 8A).

Effects of prostaglandin inhibitors
and reserpine on testicular contractility

The testicular contractions of 20 rabbits were measured in vivo for two hours after the preparations were set up. During this time, the Tyrode's solution was changed twice. Initial contractile force equaled a pen deflection of 0.324 g. After the first change of medium, the force was 0.322 g, and it was equivalent to 0.329 g after the second change. Neither of these latter values represented a statistical change in force ($p > 0.50$ in both cases, according to a t-test).

Injecting rabbits with indomethacin (15 mg/kg) twice prior to measuring in vivo testicular contractility resulted in an average decrease in the initial amplitude of contraction, even though mean testicular weights for this group were greater than in the control group (see Table 1). Contractions were not abolished in nine of the 11 animals used, but were in two. In these two rabbits, the testes set up initial contractions that decreased markedly or disappeared when fresh medium was substituted

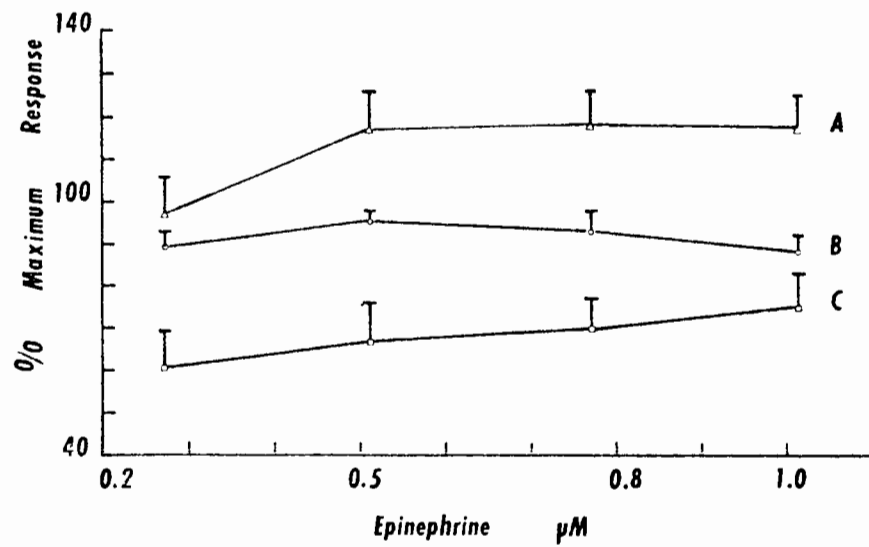


Figure 3. A. Partial epinephrine LDR curve in the presence of 0.1 mM theophylline and 1.2 μM propranolol. B. Partial epinephrine LDR curve with no other treatment. C. Inhibition of epinephrine LDR curve in the presence of theophylline (0.1 mM final concentration).

for the original (Figure 9A). This low level of contractility could be increased by administering $\text{PGF}_{2\alpha}$ at a concentration of $7 \times 10^{-8}\text{M}$ into the Tyrode's solution (Figure 9B). In addition, three of the 11 animals pretreated with indomethacin had scrotal sacs filled with serous fluid, a condition known as hydrocoel.

Injecting male rabbits with 5,8,11,14-eicosatetraynoic acid (an acetylenic inhibitor of prostaglandin synthesis) at 24 and one hour prior to the experiment, did not statistically reduce the amplitude of contraction (see Table 1). In only one of the six animals tested was there an obvious effect--a dramatic reduction in frequency of contraction (Figure 9C).

To ascertain whether the reduction in amplitude of testicular contraction after indomethacin treatment was a direct or indirect effect, a solution of indomethacin in ethanol (10 mg/ml) was made up. Aliquots of this solution were added to the bathing media of preparations in vivo and in vitro. Indomethacin at a final concentration of $7 \times 10^{-5}\text{M}$ inhibited spontaneous contractions in vitro (Figure 10A) and also reduced the tonus increase caused by $7 \times 10^{-8}\text{M}$ $\text{PGF}_{2\alpha}$ (Figure 10B). No such effect of indomethacin was noted in vivo, however. Even at concentrations of $1.4 \times 10^{-4}\text{M}$ of indomethacin, only a slight reduction of amplitude was observed in vivo (Figure 10C).

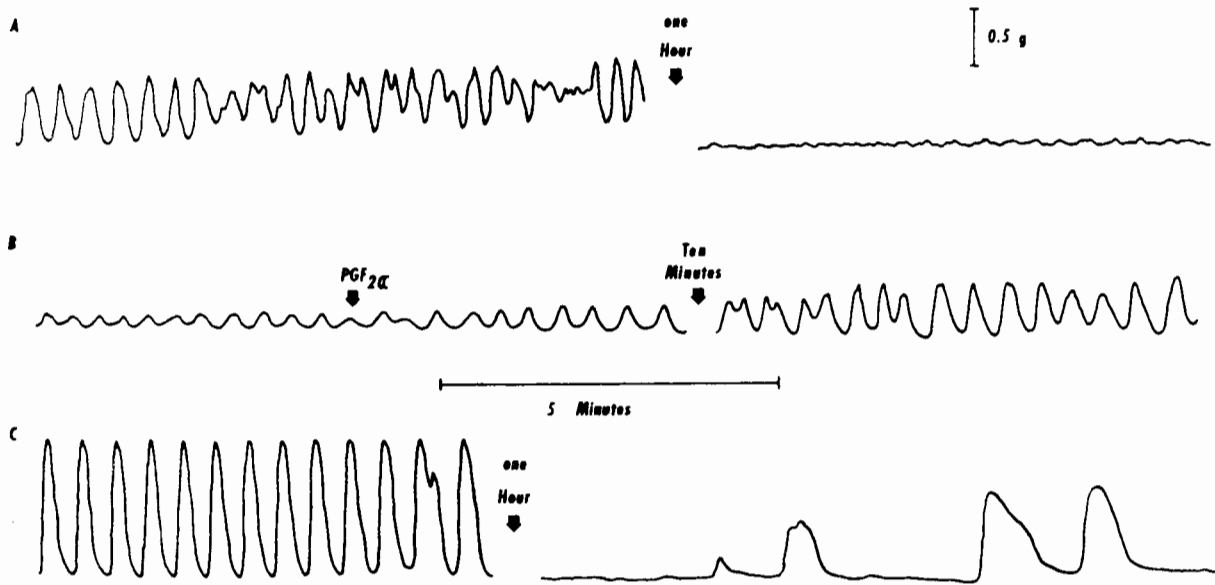
Catecholamines affect the testicular contractions pronouncedly. When one mg of epinephrine was injected iv through the marginal ear vein, testicular tonus increased to the point that contractions were temporarily effaced (Figure 11A). Although catecholamines of

Table 1. Effect of treatments on the amplitude of testicular contractions in vivo (means compared statistically with a t-test).*

	Number of animals	Mean amplitude of contraction (cm)	P value	Mean weight of testes (g)
1. None	20	1.62 ± 0.157	-	2.192 ± 0.130
2. Indomethacin (15 mg/kg ip at 12 and 1 hours prior to experiment)	11	0.98 ± 0.195	< 0.05	2.459 ± 0.138
3. Tetraynoic acid (25 mg/kg ip at 12 and 1 hours prior to experiment)	6	1.36 ± 0.370	> 0.20	1.93 ± 0.126
4. Reserpine (5 mg/kg ip at 18 hours prior to experiment)	5	1.675 ± 0.382	> 0.50	2.15 ± 0.331

*Values given as means ± standard error of the mean. None of the means for testicular weights differed statistically from the control group.

Figure 9. A. Abolition of testicular contractility in vivo one hour after injection with indomethacin (15 mg/kg ip). B. Stimulation of testicular contractions in an indomethacin-pretreated (15 mg/kg) rabbit in vivo by prostaglandin $F_{2\alpha}$ ($7 \times 10^{-8}M$) C. Decreased testicular contractility in vivo one hour after injection with 5,8,11,14-eicosatetraynoic acid (25 mg/kg ip).



neural origin are potential regulators of testicular contractility, injecting the rabbits with reserpine (5 mg/kg) at 18 hours prior to the experiment did not reduce the amplitude of contraction (Table 1). In addition, dibenamine (an agent that blocks catecholamine receptors) at concentrations of $9.6 \times 10^{-6}M$ did not reduce contractility even in reserpine-pretreated animals (Figure 11B). Dibenamine was potent in abolishing catecholamine stimulation of inactive testes in vitro, however (Figure 11C).

Another potential regular of testicular motility is acetylcholine. This compound stimulates the tonus of testes in vivo and increases frequency of contraction (Figure 11D). Adding atropine (a blocker of acetylcholine receptors) to the medium in vivo did not reduce contractility at low doses; at concentrations greater than $10^{-4}M$, it stimulated testicular tonus increases (Figure 11E). Variability of total PG content of testes from each treatment group (i.e., control, indomethacin, tetraynoic acid or reserpine) was so large that no statistical differences were observable among groups (Table 2).

Electrical stimulation and intratesticular pressure recordings

Small rhythmical fluctuations in pressure occurred in the intact testis (Figure 12A). Neither an increase nor a decrease in pressure followed electrical stimulation of the perivascular nerve and spermatic cord (Figure 12B). Pressure changes did

Figure 10. A. Decreased rate of spontaneous testicular contractions in vitro after addition of indomethacin ($7 \times 10^{-5}\text{M}$) to the bathing medium. B. Inhibition of prostaglandin $F_{2\alpha}$ - induced tonus increase of rabbit testis in vitro by indomethacin ($7 \times 10^{-5}\text{M}$). C. Lack of effect of indomethacin ($7 \times 10^{-5}\text{M}$) directly added to medium on testicular contractions in vivo.

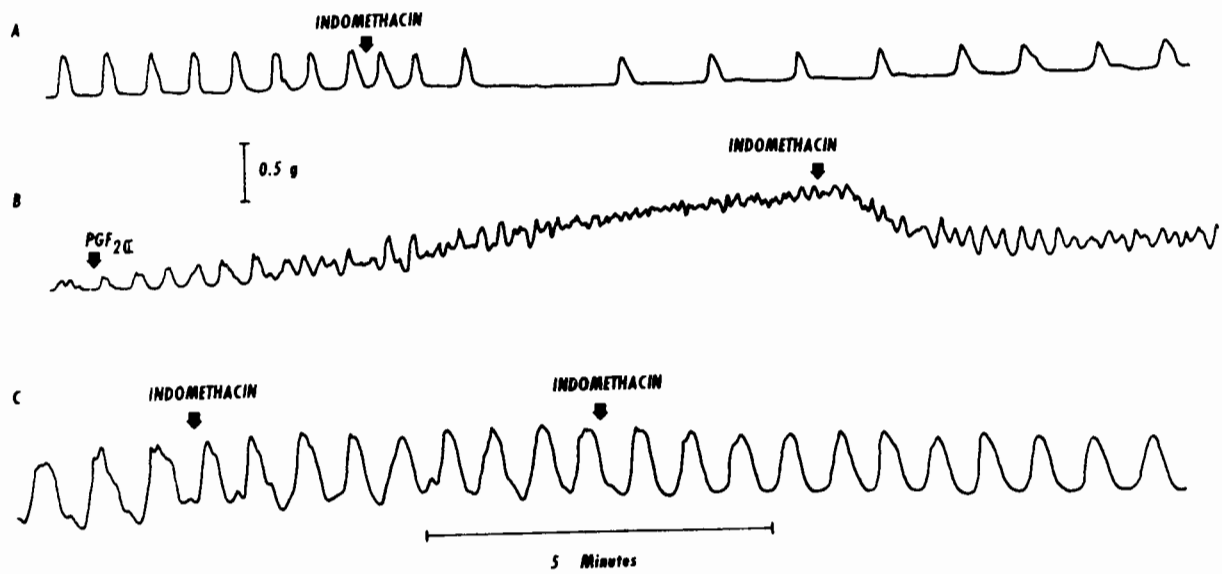


Figure 11. A. Testicular tonus increases recorded in vivo following two injections of epinephrine (1 mg) into the marginal ear vein. B. Lack of inhibitory effect of dibenamine (9.6×10^{-6} M) in vivo on testicular contractions of a rabbit pretreated with reserpine (5 mg/kg). C. Inhibitory effect of dibenamine (4.8×10^{-6} M) on tonus increase and contractility induced in vitro. D. Stimulation of testicular contractility in vivo by acetylcholine (1.35×10^{-6} M). E. Stimulation of testicular contractility in vivo by atropine (2.1×10^{-4} M).

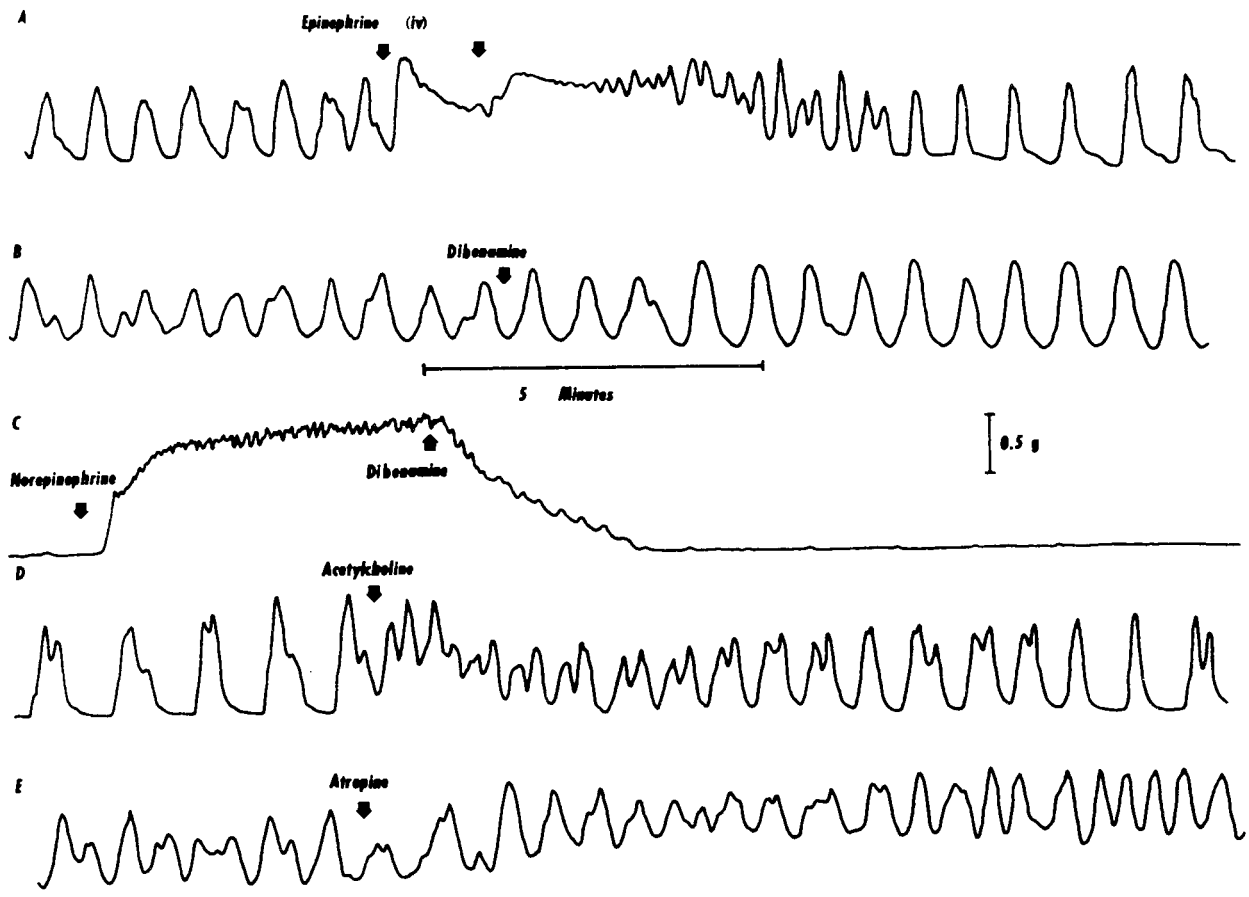


Table 2. Bioassay data showing average prostaglandin $F_{2\alpha}$ equivalents (μg per g of testis weight) recovered by extraction and chromatography procedure.*

	PGE_1	PGE_2	$\text{PGF}_{1\alpha}$	$\text{PGF}_{2\alpha}$
Control (n = 4)	0.834 ± 0.458	0.564 ± 0.299	0.21 ± 0.15	0.058 ± 0.018
Indomethacin (n = 5)	1.162 ± 0.692	1.33 ± 0.49	0.683 ± 0.353	0.826 ± 0.894
Reserpine (n = 4)	1.51 ± 0.657	1.493 ± 0.937	0.393 ± 0.148	2.28 ± 2.07
Tetraynoic acid (n = 4)	0.43 ± 0.15	0.278 ± 0.098	0.29 ± 0.135	0.078 ± 0.039

*No value was significantly different from the controls at a level of 0.05. Values are given as means \pm standard error of the mean.

follow iv infusion of pharmacological agents, however. Both norepinephrine and $\text{PGF}_{2\alpha}$ increased intratesticular pressure, whereas isoproterenol and PGE_1 decreased it (Figure 12C-F). The inhibition of pressure fluctuations that took place after iv infusion of 0.2 mg isoproterenol lasted for a minimum of 30 minutes. During this time norepinephrine failed to increase intratesticular pressure (data not shown).

Miscellaneous pharmacological effects

To ascertain whether any interaction occurred between PGs and alpha-adrenergic-blocking agents, dibenamine ($1.9 \times 10^{-6}\text{M}$) was added to testicular preparations in vitro after they had been stimulated to contract by $\text{PGF}_{2\alpha}$. Dibenamine did not reduce PG-induced tonus increases or amplitude of contraction (Figure 13A). The effect of a PG precursor, arachidonic acid, on spontaneous contractions in vitro was found to be inhibition (Figure 13B). The inhibitor of PG synthesis, aspirin, had no obvious effect on testicular contractions in vitro at concentrations $4 \times 10^{-5}\text{M}$ (Figure 13C). Prostaglandin A_2 , like PGFs, was stimulatory in vitro at all concentrations tested (Figure 14).

Adenosine (0.1 mM) inhibited tonus increases caused by PGE_1 (Figure 15A). At a ten-fold higher concentration, adenosine abolished testicular contractility that had been brought about by PGE_1 (data not shown). Caffeine, a cyclic nucleotide-phosphodiesterase inhibitor, also reduced tonus and frequency of testicular contractions (Figure 15B). Cyclic 3',5'-guanosine

Figure 12. A. Pressure fluctuation recorded from rabbit testis in vivo. B. Intratesticular pressure during electrical stimulation (1.0 V, 50 Hz, 20 ms duration) of spermatic cord. C. Intratesticular pressure fluctuation inhibited by iv isoproterenol infusion (0.2 mg). D. Intratesticular pressure increase following iv norepinephrine infusion (0.2 mg). E. Intratesticular pressure following iv $\text{PGF}_{2\alpha}$ infusion (0.2 mg). F. Intratesticular pressure following iv PGE_1 infusion (0.2 mg).

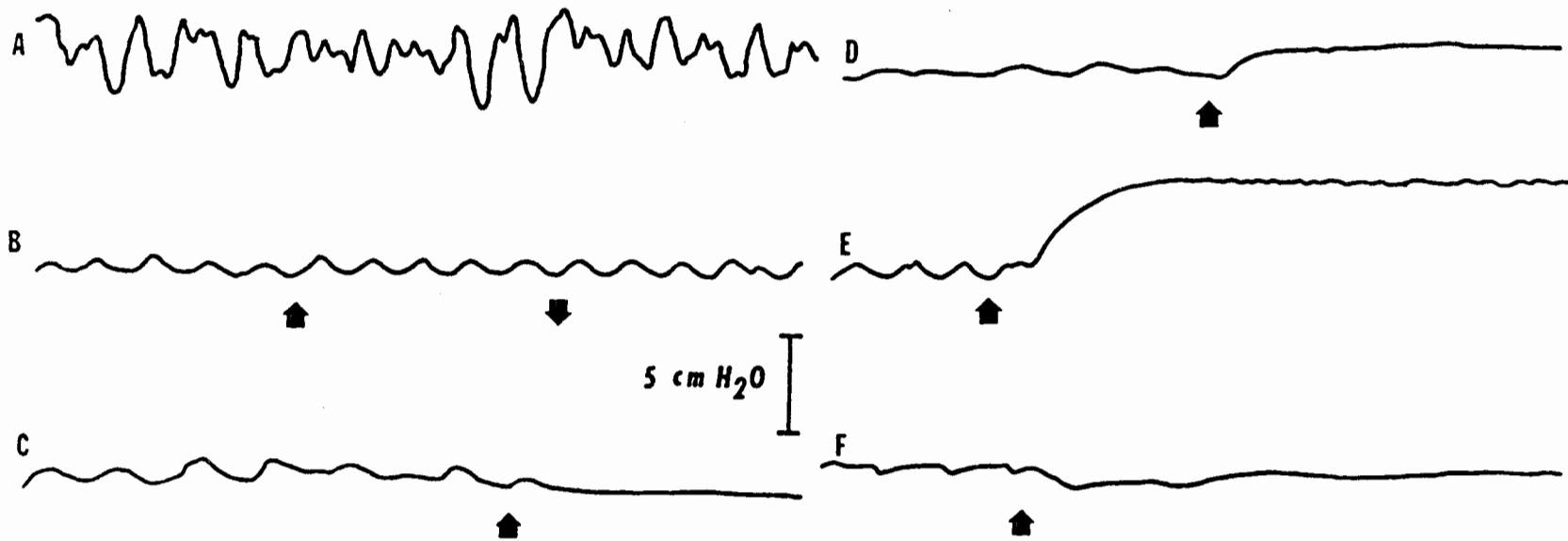
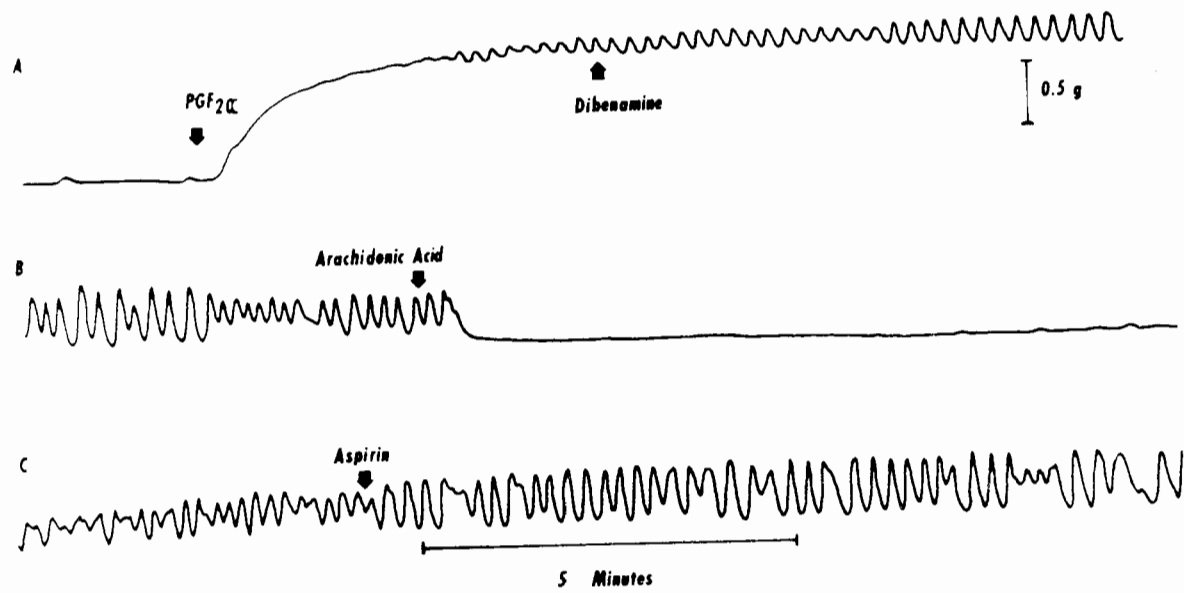


Figure 13. A. Lack of a dibenamine-induced inhibition of testicular tonus increase on contractility in vitro caused by prostaglandin $F_{2\alpha}$ ($7 \times 10^{-7}M$).
B. Inhibitory effect of arachidonic acid ($1.65 \times 10^{-4}M$) on spontaneous testicular contractions in vitro. C. Lack of effect of aspirin ($4.2 \times 10^{-5}M$) on spontaneous contractions in vitro.



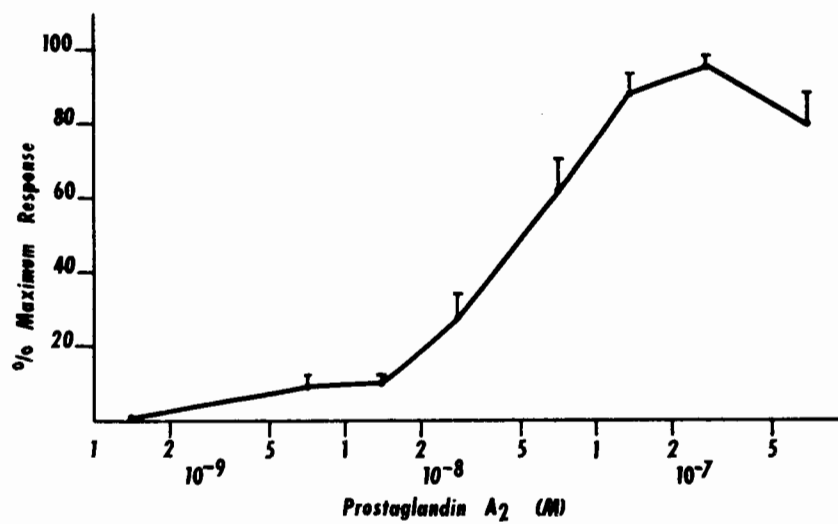
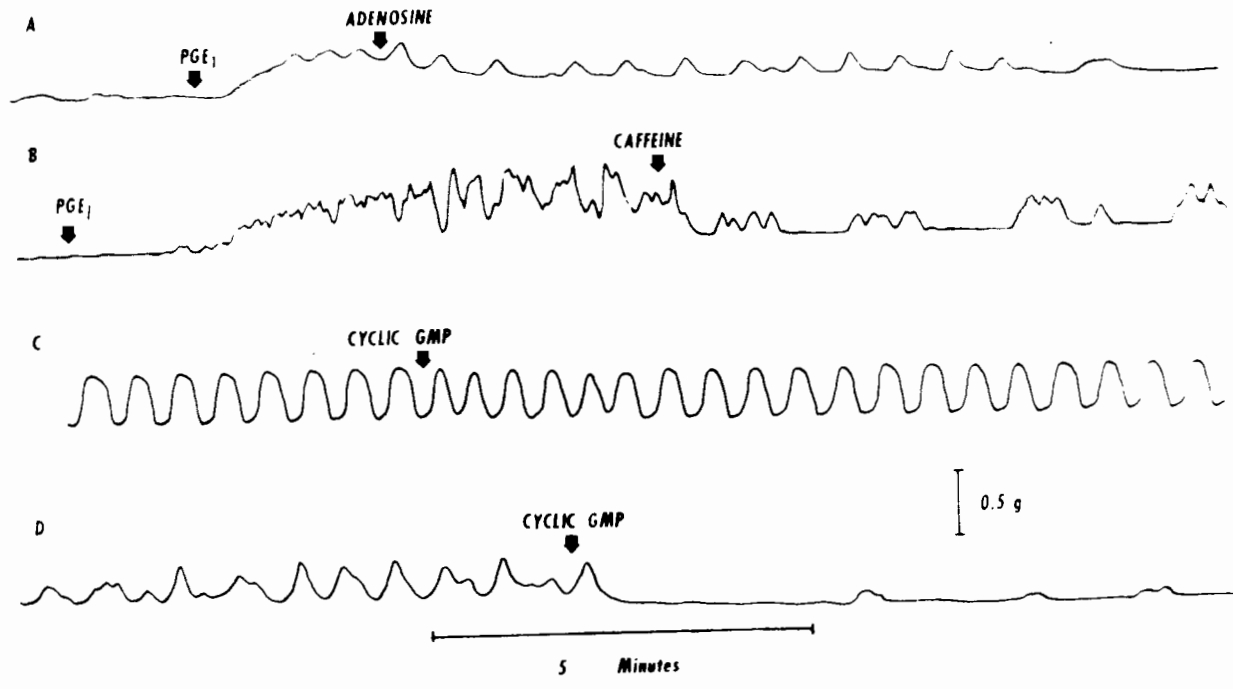


Figure 14. LDR curve for prostaglandin A₂ stimulation of testicular tonus in vitro.

Figure 15. A. Stimulation of testicular tonus in vitro by prostaglandin E_1 ($7.2 \times 10^{-8}M$) followed by inhibition after addition of adenosine ($10^{-4}M$). B. Stimulation of tonus in vitro by prostaglandin E_1 ($7.2 \times 10^{-9}M$) with subsequent inhibition by caffeine ($3.5 \times 10^{-5}M$). C. Lack of effect of cyclic GMP ($10^{-4}M$) on spontaneous testicular contractions in vitro. D. Inhibitory effect of cyclic GMP ($10^{-3}M$) on testicular contractions in vivo.



monophosphate slightly inhibited spontaneous testicular contractions in vitro at a concentration of 0.1 mM (Figure 15C). This cyclic nucleotide also inhibited contractions in vivo, particularly at 1 mM concentrations (Figure 15D).

Testosterone progressively inhibited testicular tonus increases in vitro that were caused by $\text{PGF}_{2\alpha}$ (Figure 16A-C). Increasing the concentration of $\text{PGF}_{2\alpha}$ did not overcome this inhibition.

Testosterone radioimmunoassay

Taking sequential blood samples by cardiac puncture resulted in a decreased plasma testosterone level in several animals (Table 3). This decrease obscured any differences that may have been due to treatment. In addition, temporarily high levels of testosterone in a few individuals caused total variability to be very large, further confounding the measurement of plasma testosterone.

Exposure of male rabbits to females

Testes of 11 male rabbits that had been exposed to females for two weeks contracted autorhythmically in vivo and in vitro. The mean amplitude of testicular contraction in vitro was 0.155 ± 0.059 g. No statistical difference was found between this value and the amplitude of contraction of 12 testes in vitro from isolated animals, which equaled 0.261 ± 0.077 g ($p > 0.20$). The

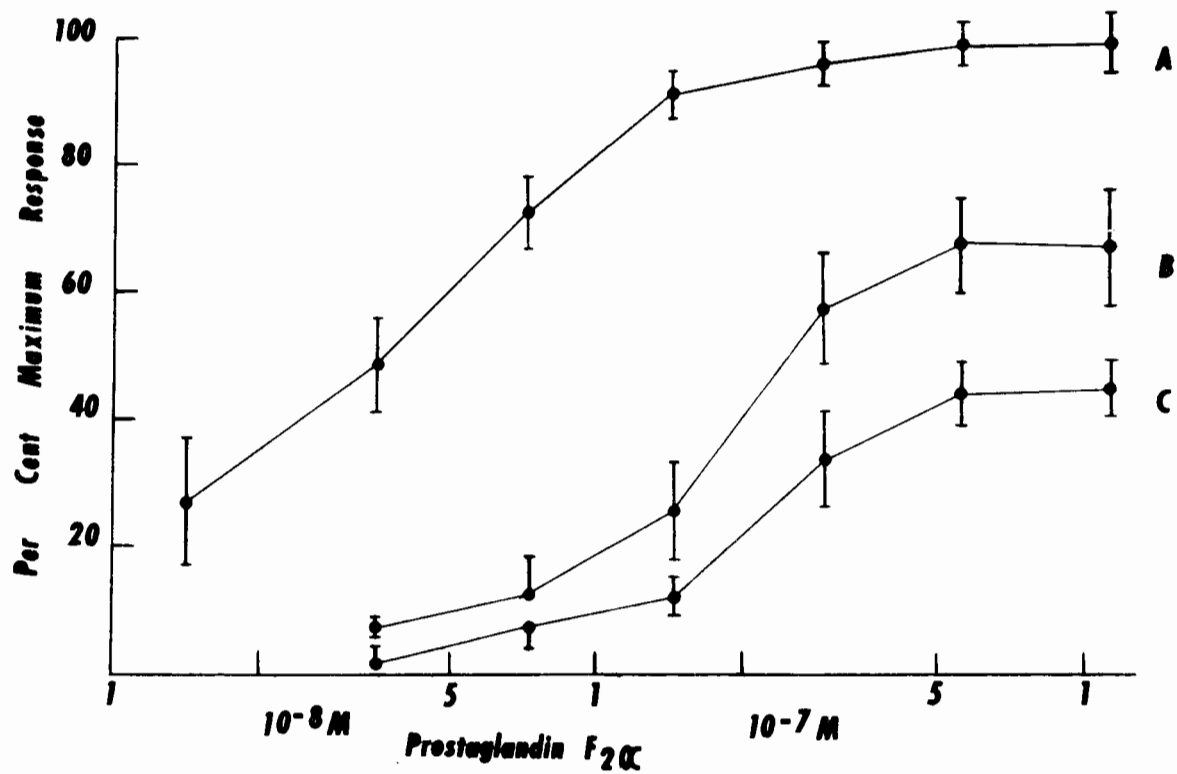


Figure 16. A. LDR curve for prostaglandin $F_{2\alpha}$ -induced tonus increase in vitro. B. Inhibition of prostaglandin $F_{2\alpha}$ LDR curve in vitro by testosterone (2.18×10^{-5} M). C. Inhibition of prostaglandin $F_{2\alpha}$ LDR curve in vitro by testosterone (4.36×10^{-5} M).

Table 3. Serum testosterone levels (ng/ml) obtained from male rabbits injected with indomethacin, tetraynoic acid, reserpine, prostaglandin E₁, or prostaglandin E₁ plus indomethacin. Values are given as means \pm standard error of the mean.

	Time, post-injection (hours)			
	0	1	4	12
Control (n = 6)	4.51 \pm 1.83	2.41 \pm 0.43	1.48 \pm 0.68*	2.71 \pm 0.86
Indomethacin (n = 5)	1.81 \pm 0.50	1.01 \pm 0.83	5.28 \pm 4.59	1.15 \pm 0.29
Tetraynoic acid (n = 5)	12.70 \pm 5.20	2.84 \pm 1.09	5.49 \pm 3.67	2.40 \pm 1.27
PGE ₁ (n = 4)	8.08 \pm 2.82	3.27 \pm 0.94	1.07 \pm 0.14*	4.84 \pm 2.09
PGE ₁ + Indomethacin (n = 5)	4.95 \pm 1.19	3.75 \pm 1.30	8.27 \pm 5.27	9.44 \pm 2.84
Reserpine (n = 4)	7.25 \pm 2.75	4.28 \pm 2.01	1.14 \pm 0.62	0.87 \pm 0.10

*Asterisk indicates significant difference from the initial value (0 hours after injection) at the 0.05 level.

range of contractile amplitude ran from 0 to 1.0 g. Testicular weights were 2.104 ± 0.167 g for the exposed males and 2.267 ± 0.133 g for the isolated animals.

DISCUSSION

The general potentiating effect of PGs at very low concentrations on contractions of smooth muscle induced by agents such as epinephrine, acetylcholine, and calcium was noticed by Clegg, Hall and Pickles (1966) and Eagling, Lovell and Pickles (1971). This phenomenon was attributed to increased calcium entry into the cell (Clegg, Hall and Pickles, 1966). Indeed, not only do PGs depend on calcium for their stimulation of the rabbit testicular capsule, but a reciprocal dependency exists, since high calcium concentrations (4-12 mM) have no effect on capsular tonus in vitro unless a stimulating agent such as a PG, epinephrine or histamine is present (Hargrove, Seeley and Ellis, 1973). Our results demonstrate that PGE₁ at low concentrations potentiates testicular capsular contractions induced by other agonists in vivo and in vitro. Since PGs are synthesized in rat and rabbit testes (Carpenter, Manning and Wiseman, 1971; Ellis, Johnson and Hargrove, 1972; Carpenter, 1974), such potentiation may occur naturally.

The bimodal effect of PGE₁, which leads to inhibition at higher doses, correlates well with the observation of Clegg (1966), who showed that low concentrations of PGE₁ potentiate, while high doses inhibit, the response of guinea pig seminal vesicles to catecholamines. In addition, it agrees with the bronchodilator and vasodilator properties of PGEs (Smith, 1973) that are not

observed with PGFs. Andersson et al. (1973) recently demonstrated that the relaxing effect of PGE₂ on the sphincter of Oddi coincided with a 50 percent increase in tissue cyclic AMP content. However, the gall bladder contracted in response to PGE₂ and showed a decrease in cyclic AMP. Increased cyclic AMP correlates with an inhibition of smooth muscle motility in several organs (Triner et al., 1971). PGE₁ does stimulate testicular cyclic AMP formation (Kuehl et al., 1972). These facts suggest that the bimodal action of PGE₁ in altering testicular capsular responses to epinephrine and acetylcholine may be due to increased calcium loading at low concentrations, followed by cyclic AMP accumulation at higher concentrations.

Propranolol, a beta-adrenergic-blocking agent, markedly increased the response of the testicular capsule to high doses of epinephrine, while theophylline (a phosphodiesterase inhibitor) reduced the capsular response. Rikimaru and Suzuki, 1972, showed that both alpha- and beta-adrenergic receptors exist in the capsule, and that epinephrine becomes inhibitory in the presence of alpha-adrenergic-blocking agents. The inhibitory action of theophylline in rabbit aorta and epinephrine in rat uteri coincides with a rise in smooth muscle cyclic AMP and can be duplicated by exogenously-added dibutyryl cyclic AMP (Triner et al., 1971). The increased testicular capsular stimulation by epinephrine seen in the presence of the beta blocker, propranolol, may therefore be due to a decrease in cyclic AMP. The beta-adrenergic agonist, isoproterenol, inhibits the testicular capsule

in vitro and in vivo (Davis and Langford, 1970; Seeley et al., 1973).

All the agents tested (except acetylcholine) gave similar responses in vitro and in vivo. Acetylcholine will cause capsular tonus increases in vitro if the preparation is spontaneously contracting (Davis and Langford, 1970). Substances released by the testis into the bathing medium initiate contractions and appear to be necessary for the response to acetylcholine in vitro. We have previously shown that several prostaglandin-like compounds are responsible for much of this spontaneous activity (Hargrove et al., 1973). It is therefore plausible that presence of these compounds, as well as nerve activity, could account for the observed difference between the testicular capsular response to acetylcholine in vitro and in vivo.

The fact that PGE_1 suppresses increases in tonus and contractility brought about by $\text{PGF}_{2\alpha}$ and other agonists demonstrates that this inhibition is a general phenomenon in the testicular capsule. At least one PG-induced effect can be totally blocked by a second type of prostaglandin, a result of special interest, since PGs of both the E and F type are generally produced together in tissues (Carpenter, Manning and Wiseman, 1971; Sih and Takeguchi, 1973), although interactions between them have rarely been considered. In this regard, rat and rabbit testes normally produce more $\text{PGF}_{2\alpha}$ than PGE_2 . Known mechanisms could shift this balance or increase the total PG content. For instance, epinephrine,

norepinephrine or serotonin can activate PG synthetase and furnish reducing equivalents that increase F-type PGs at the expense of E-type (Sih and Takeguchi, 1973); divalent metal ions such as Cu^{++} can have the same effect (Maddox, 1973). Either physiological or pathological changes of function could thereby modify testicular capsular motility, possibly resulting in altered sperm transport or testicular hemodynamics.

Testicular capsules from some sources, but not all, do receive autonomic innervation. The canine testis responds to stimulation of the perivascular nerve with a rise in intratesticular pressure equivalent to 50 mm of water (Rikimaru et al., 1972). Other capsules--such as those of the bull (Shioda and Nishida, 1966), the rat (Bell and McLean, 1972), the rhesus monkey and man (H. G. Baumgarten, personal communication to Norma Hodson, 1970)--contain nerve fibers that may go to the vascular system, but any connection with the smooth muscle of the tunica albuginea has not been demonstrated. Although Cross (1959) noted increased contractility of rabbit testes in vivo after stimulating the sympathetic centers of the hypothalamus, this effect may have been mediated by adrenal catecholamine release (Cross and Silver, 1962). In the human testis, only vascular nerves were found (Baumgarten et al., 1968), a report that was confirmed by Shirai, Suzuki and Rikimaru, (1973). Some contradiction exists between this latter report and an earlier pharmacological investigation that suggested the presence of such nerves. In this regard, Rikimaru and Shirai (1972) found that bretylium and tetrodotoxin interfered with the

response of testicular muscle to field stimulation (50 Hz, 3V/cm); bretylium interferes with norepinephrine release from sympathetic nerves.

Rikimaru and Suzuki (1972) also found that bretylium (but not hexamethonium or atropine) reduced contractions of rabbit testes that were elicited by field stimulation. The ganglionic stimulant, dimethylpiperazinium, also contracted the preparation; this contraction was reduced by bretylium, but recovered after washing the bretylium out of the bath and adding tyramine, a sympathomimetic agent. Though these results suggest the presence of sympathetic nerves, neither atropine nor bretylium reduced spontaneous contractions in vitro according to these investigators. The findings that neither atropine, dibenamine nor reserpine altered testicular contractions in vivo (Figure 11E; Table 1), plus the absence of tonus increases in the rabbit testis in vivo after electrical stimulation, cause one to conclude that nerves are not important factors in regulation of these contractions.

Other alternatives include locally-produced, smooth muscle-active compounds such as PGs, genuine autorhythmicity caused by electrogenic pumps, or sensitive response to adrenal catecholamines. This latter possibility is not likely, since dibenamine did not affect the contractions in vivo (Figure 11B). PGs, however, are produced by the testis (Ellis, 1972; Carpenter, 1974) and are released by the rabbit testis into the medium in vitro to initiate motility (Hargrove et al., 1973). The less labile contractions

in vivo are responsive to PGs, but only small amounts are actually released into the bathing fluid (Seeley et al., 1974). It is of interest, then, that injecting rabbits with the PG synthetase inhibitor, indomethacin, significantly reduced testicular contractions (Figure 9; Table 1). Although another PG synthetase inhibitor, 5,8,11,14-eicosatetraynoic acid, was without this effect, Willis, Davison and Ramwell (1974) point out that this compound does not distribute readily in the body fluids, and therefore is often without effect on PG synthesis. Indomethacin added to the bathing medium had no direct effect in vivo (Figure 10C), although it did inhibit tonus and contractility both of spontaneous origin and caused by $\text{PGF}_{2\alpha}$ (Figures 10A and B). In addition, $\text{PGF}_{2\alpha}$ restored contractility in testes from indomethacin-pretreated animals (Figure 9B). The lack of a direct effect in vivo may indicate that indomethacin inhibits by preventing PG biosynthesis, rather than via its immediate action as a phosphodiesterase inhibitor that would cause cyclic AMP accumulation. Such an effect of this compound on phosphodiesterase has been demonstrated in bovine heart muscle by Stefanovich (1974). Due to the large variability of the PG estimates following extraction, thin-layer chromatography and bioassay, an effect of indomethacin on testicular PGs could not be confirmed (Table 2). At present, it seems that rabbit testicular contractions in vivo are either due to prostaglandin production, or to spontaneous muscular processes, involving neither neurogenic factors nor prostaglandins. The information available, however, is more suggestive than conclusive.

The inhibitory action of PGE_1 on testicular contractions appears to be due to the accumulation of adenosine 3',5'-monophosphate (cyclic AMP). Beta-adrenergic agonists, such as isoproterenol, also inhibit capsular contractions; this effect is abolished by beta-adrenergic-blocking agents such as propranolol (Seeley, 1973; Seeley et al., 1974). Agents that cause accumulation of cyclic AMP by blocking the hydrolytic enzyme, cyclic nucleotide phosphodiesterase, inhibit contractions. Theophylline causes inhibition in this manner both in vitro and in vivo (Seeley et al., 1974), and caffeine has similar effects in vitro (Figure 15B). Both cyclic AMP and its dibutyryl derivative decrease tonus and augment the inhibitory effect of PGE_1 (Seeley et al., 1974). In the present study, inhibitory effects were also noted in response to both adenosine and guanosine 3'5'-monophosphate (cyclic GMP), as depicted in Figure 15A, C and D. An inhibitory activity may therefore be a general property of nucleotides and cyclic nucleotides when added to testicular preparations at pharmacological doses. Lower levels of cyclic GMP within the cell may not affect muscle in the same way, since Kuehl et al. (1974) found increased cyclic GMP in rat uteri after estradiol treatment; these investigators noted that estradiol enhances uterine contractility. On the other hand, Mitznegg, Hach and Heim (1971) failed to observe any change in uterine responsiveness to oxytocin in the presence of 10^{-3}M cyclic GMP.

Arachidonic acid, a PG precursor, inhibited spontaneous testicular activity in vitro at concentrations of 10^{-3} M (Figure 13B). Whether this effect was related to increases in cyclic AMP was not investigated. PGA_2 increased testicular activity and tonus at all concentrations, in this respect paralleling $\text{PGF}_{1\alpha}$ and $\text{F}_{2\alpha}$ (Johnson, Hargrove and Ellis, 1971; Seeley et al., 1974).

Testosterone inhibits spontaneous testicular contractions in vitro but not in vivo, and similarly depresses $\text{PGF}_{2\alpha}$ -induced tonus increases and contractility (Seeley et al., 1972 and 1973). In the present study, this inhibitory effect of the androgen on testicular activity caused by $\text{PGF}_{2\alpha}$ was found to be noncompetitive, in that high concentrations of $\text{PGF}_{2\alpha}$ would not overcome the inhibition by testosterone (Figure 16). Any other relationships between steroids and PGs could not be ascertained, since the radioimmunoassay data were confounded by the stress of cardiac puncture (Table 3). In addition, human chorionic gonadotropin produced neither inhibition nor stimulation of testicular contractility (Seeley, 1973), even though it increases plasma testosterone in rabbits (Saginer and Horton, 1968) and seminal PGs in men (Sturde and Bohm, 1971). Finally, exposing male rabbits to females for two weeks did not significantly affect testicular contractions as measured by the presently-used technique ($p > 0.2$).

SUMMARY AND CONCLUSIONS

The bimodal, stimulating and inhibiting effect of PGE₁ on testicular contractility alters the reactivity of smooth muscle from this preparation to other agents, including PGF_{2α}, acetylcholine and epinephrine. At low concentrations of PGE₁ (10⁻¹⁰ to 10⁻⁹ M), increased stimulation is seen in response to acetylcholine and epinephrine, whereas at higher doses (> 10⁻⁸ M PGE₁), the response to all three agents is depressed. The suggested modes of action for PGE₁ include increased Ca⁺⁺ influx at low concentrations, but cyclic AMP accumulation within the cells at high concentrations. Since prostaglandins do occur in rabbit testes, similar effects may occur in the live animal.

Both acetylcholine and norepinephrine, which are found respectively in the parasympathetic and sympathetic nervous systems, stimulated testicular contractions in vivo. Agents that block receptors to these neurotransmitters, however, did not reduce in vivo testicular contractility. Nor did reserpine, a sympatholytic drug, reduce contractility. In addition, attempts at stimulating the spermatic nerve did not increase testicular tonus or motility. These results imply that nerves do not regulate normal testicular contractions. The reduction of contractile amplitude and occasional abolition of contractions following indomethacin injections ip, support the idea of local control by prostaglandins or other agents. The fact that tetraynoic acid failed to significantly reduce the amplitude of testicular

contractions may be attributed to the poor distribution this compound achieves in bodily fluids.

Nucleotides such as adenosine, and cyclic nucleotides, including cyclic GMP, inhibited testicular contractions. Testosterone also depressed testicular contractility, especially that caused by $\text{PGF}_{2\alpha}$. The stimulation of testicular contractions by $\text{PGF}_{2\alpha}$, however, was not altered by alpha-adrenergic blocking agents such as dibenamine. Since dibenamine abolished the stimulation of testicular tonus increases and contractility by catecholamines, one assumes that different receptors are present for the PGs and the catecholamines.

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APPENDIX

Indomethacin Induces Rat Uterine ContractionsIn Vitro and Alters Reactivity to Calcium and Acetylcholine

J. L. Hargrove, D. Nesbitt, M. J. Gaspar, L. C. Ellis

Abstract

The initial contractions of uteri in vitro from castrated, estrogen-treated rats, were markedly diminished following replacement with fresh bathing medium. Indomethacin and aspirin (10^{-5} to 10^{-4} M) strongly stimulated such quiescent preparations and reduced their subsequent responsiveness to Ca^{++} . Reintroducing the initial bathing medium (which contained prostaglandin-like material), or adding prostaglandin $\text{F}_{2\alpha}$ to the fresh medium, initiated uterine contractions and restored responsiveness to calcium ion. Injections of indomethacin into castrated, estrogen-treated rats reduced initial in vitro uterine motility, abolished production of prostaglandin-like compounds, and prevented either indomethacin, aspirin or Ca^{++} from stimulating uterine contractions. Uterine responsiveness to acetylcholine in vitro was significantly reduced in rats pretreated with indomethacin.

Prostaglandins are now thought to be major intrinsic regulators of uterine contractility.¹ Prostaglandin production may increase following activation of the uterus by estradiol,² and prostaglandin $\text{F}_{2\alpha}$ levels are dependent on the stage of the menstrual cycle in the human fallopian tube.³ Two established inhibitors of prostaglandin synthesis, aspirin and indomethacin,⁴ have been reported to abolish uterine contractions in vitro.^{5, 6} They also prolong parturition in the rat⁷ and the monkey,⁸ presumably by blocking endometrial prostaglandin synthesis. Salicylates, however, elicit an apparently

contradictory result--premature parturition in mice.⁹ Informal reports suggest that aspirin also may precipitate abortion in women during early pregnancy.¹⁰

Prostaglandin production often contributes to spontaneous contractions that smooth muscles develop in vitro. Adding fresh bathing medium may remove these prostaglandins, abolish or reduce initial motility, and alter the response of a smooth muscle preparation to calcium ion and other stimulatory agents.¹¹ Indomethacin affects not only prostaglandin synthesis but also calcium balance.¹² We, therefore, investigated the effect of changing the Tyrode's solution prior to administering indomethacin, aspirin, Ca^{++} or prostaglandin $\text{F}_{2\alpha}$ to rat uterine preparations in vitro. We also studied the effects of indomethacin injections prior to sacrifice on rat uterine contractility, prostaglandin production, and responsiveness to acetylcholine.

Materials and Methods

Two groups of six ovariectomized female rats received 10 $\mu\text{g}/\text{kg}$ of estradiol benzoate in 0.2 cc of corn oil daily for five days. One of the groups also received 10 mg/kg of indomethacin in corn oil ip during the final two days and one hour prior to sacrifice. The uteri were excised and suspended in all-glass, water-jacketed muscle warmers containing 20 ml of oxygenated Tyrode's solution (37°C), immediately after the rats had been sacrificed by decapitation. Contractions were recorded isometrically against a tension that was equivalent to less than 0.5 g. The uteri were incubated for 30 minutes to allow recovery from thermal or operative shock, and fresh medium was added prior to treatment of inactive preparations with pharmacological agents. Indomethacin, aspirin, and $\text{PGF}_{2\alpha}$ were dissolved in ethyl alcohol and added to the bathing media in aliquots of 5-100 μl . CaCl_2 (5 mM) was dissolved in 20 ml of Tyrode's solution and put directly into the muscle warmer.

Two additional groups of four rats were similarly ovariectomized and treated with estradiol (10 $\mu\text{g}/\text{day}$ for five days); one such group received indomethacin (10 mg/kg) on the final two days and one hour before sacrifice. Uteri from these animals were suspended in Tyrode's solution as stated above and used to generate log dose-response curves in response to acetylcholine (Sigma).

Prostaglandin Extraction and Chromatography

Initial bathing media (20 ml volumes) were collected following the 30-minute incubation period and stored at -20° C. Media from four preparations of each treatment group (estradiol alone, or indomethacin plus estradiol) from above were pooled, acidified to pH 3.0 with HCl, and partitioned three times against equal volumes of distilled ethyl acetate. The organic solvent was collected and dried at 40° C in rotary evaporators. Residues were taken up with three 5-ml volumes of ethyl acetate, transferred to 15-ml capacity test tubes, and evaporated under nitrogen gas. Lipidic material was dissolved in 50 μ l of ethanol and spotted on chromatographic plates coated with a slurry (0.25 mm thick) of 30 g silica gel H (Merck) and 6 g silver nitrate. Plates containing authentic prostaglandin were developed with phosphomolybdic acid for confirmation of the R_f values reported by Green and Samuelsson.¹³ The silica gel coat was scraped from the plates on which the extracts had been spotted in zones corresponding to these R_f values, transferred to centrifuge tubes and eluted three times with 5-ml aliquots of methanol to recover any prostaglandin-like material. This solvent was then evaporated under nitrogen gas at 40° C. To remove salts from the residue, the material was redissolved in 5-ml portions of phosphate buffer (pH, 3.0) and partitioned three times against equal volumes of ethyl acetate. After evaporating the organic solvent, the residues were taken up in Tyrode's solution

for bioassay on the rabbit testicular capsule as previously reported.¹⁴ To estimate the percent recovery of prostaglandins, 0.5 μg of PGF_1 and 1.0 μg of $\text{PGF}_{2\alpha}$ were added to Tyrode's solution; this fluid was extracted and chromatographed in the same manner as the uterine bath fluid prior to bioassay.

Results

Uteri from estrogen-treated rats contracted autorhythmically in vitro at a greater amplitude than those treated with estrogen and indomethacin (Table 4). The contractions were markedly diminished or totally abolished by a change of the bathing medium, but were restored if the original medium was reintroduced (Figure 17A and B). Extraction of the original media for acidic lipids followed by chromatography and bioassay demonstrated that media from estrogen-treated preparations contained prostaglandin-like material in zones corresponding to authentic prostaglandins $F_{2\alpha}$, $F_{1\alpha}$, and E_1 , but not E_2 . However, indomethacin-pretreated preparations were less motile than untreated units, and produced no detectable stimulatory material (Table 4).

The contractile responses of quiescent preparations to aspirin or indomethacin proved to be dose-dependent, with a brief stimulation followed by tachyphylaxis seen at 10^{-5} to 10^{-4} M final concentrations (Figure 17C and D). At concentrations greater than 10^{-4} M, both aspirin and indomethacin initiated contractions in previously inactive preparations (Figure 18A and B): this motility persisted for at least 30 minutes in vitro. Replacing the media with fresh Tyrode's solution abolished this contractility (data not shown). However, when actively-contracting uteri were treated with doses of these compounds from 3.5×10^{-5} to 1.4×10^{-4} M, neither marked stimulation nor inhibition was observed (Figure 18C and D). Inactive

Table 4. Initial amplitude of contraction and prostaglandin-like material recovered from the bathing media of uteri from estradiol-treated (10 μ g per day) and estradiol plus indomethacin-treated (10 mg per kg) rats.

	Estradiol (n = 6)	Estradiol and indomethacin (n = 6)	P
Initial Contractions (g displacement)	1.65 \pm 0.37	0.98 \pm 0.31	< 0.05
Prostaglandin-like material* (ng per 20 ml bath fluid)			
PGF _{2α}	320	0	-
PGF _{1α}	150	0	-
PFE ₂	0	0	-
PGE ₁	30	0	-

*Corrected for recovery of 78 percent. Values are given as means \pm standard error of the mean.

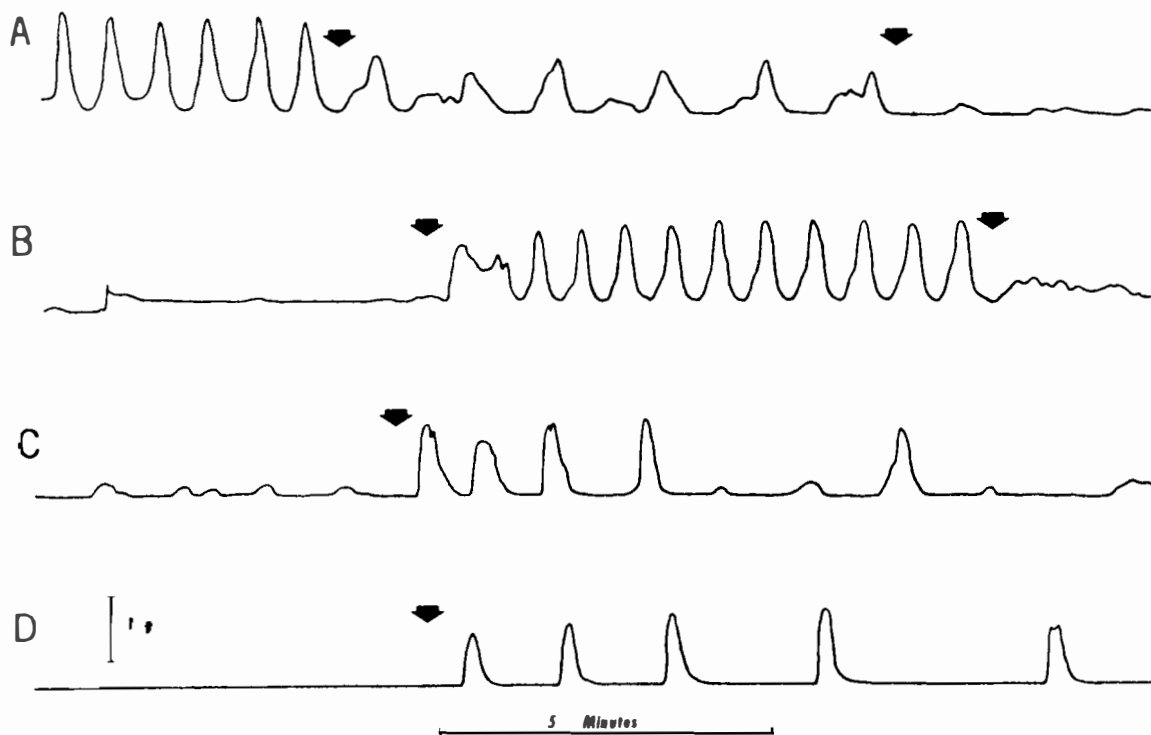


Figure 17. A. Reduction of rat uterine contractility in vitro following two changes of the bathing medium (indicated by arrows). B. Restoration of contractility after initial medium from an active preparation was added back to quiescent preparation (first arrow), with reduction in activity after one rinse (second arrow). C. Contractions induced by addition (at arrow) of $28 \mu\text{M}$ indomethacin, followed by tachyphylaxis. D. Contractions in response to 0.10 mM aspirin (arrow) followed by tachyphylaxis. Vertical bar is equivalent to a pen deflection caused by 1 g ; preparation volume equals 20 ml .

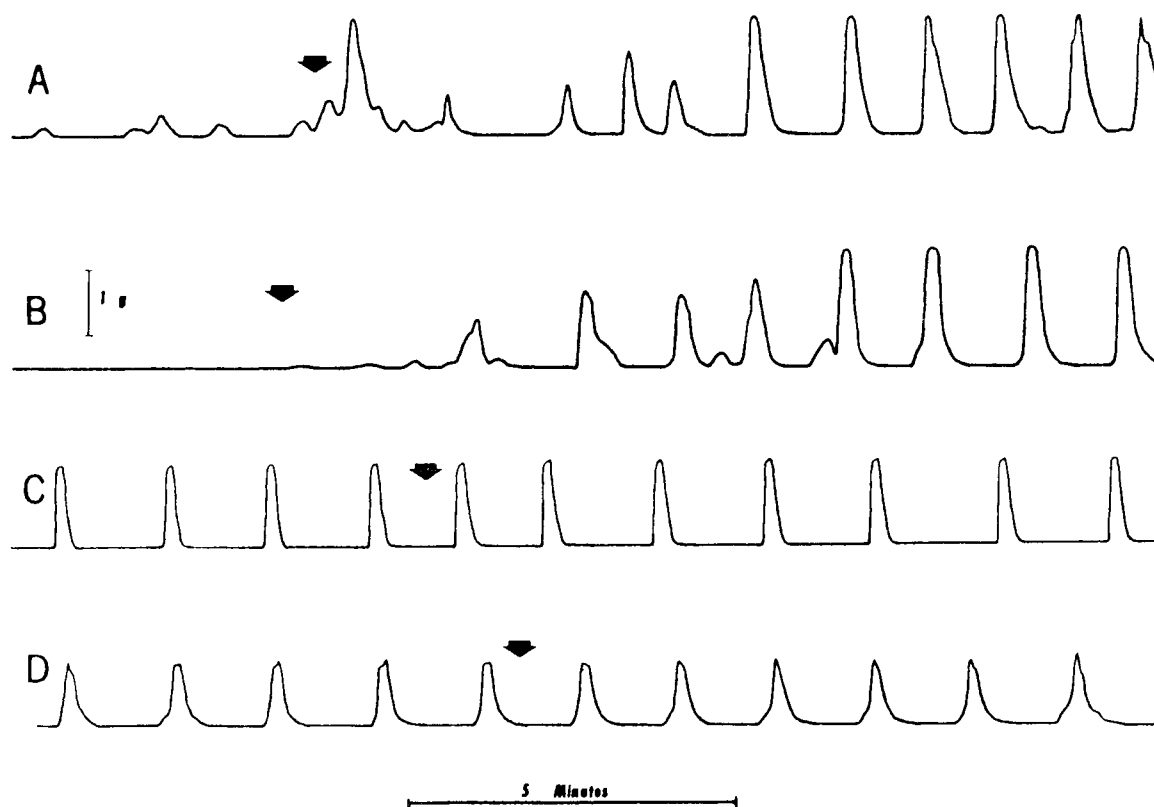


Figure 18. A. Stimulation of inactive rat uterus by 0.14 mM indomethacin (added at arrow). B. Stimulation of inactive rat uterus by 0.28 mM aspirin (added at arrow). C. Lack of inhibition of rat uterine contractility following addition (at arrow) of 0.035 mM indomethacin. D. Lack of inhibition of actively contracting rat uterus after addition (at arrow) of 0.056 mM aspirin.

preparations from indomethacin-pretreated animals responded neither to aspirin nor indomethacin with contractions (Table 5).

Uteri from estradiol-pretreated rats responded to Ca^{++} with increased contractility; this phenomenon was particularly evident in quiescent, rinsed preparations (Figure 19A). However, uteri from indomethacin-pretreated rats reacted little to the addition of Ca^{++} to the medium (Figure 19B; Table 5). In every case, however, $7 \times 10^{-7}\text{M}$ $\text{PGF}_{2\alpha}$ completely restored uterine responsiveness to Ca^{++} (Figure 19C). Ethanol (10-100 μl) did not markedly affect either spontaneously active or inactive, rinsed preparations (Figure 19D and E).

Pretreatment of spayed female rats with indomethacin (10 mg/kg) prior to sacrifice significantly reduced the responsiveness of uterine preparations to acetylcholine ($p > 0.05$; Figure 20A and C) when compared to the response of uteri from control animals. Addition of prostaglandin $\text{F}_{2\alpha}$ to the media at threshold levels ($7 \times 10^{-9}\text{M}$) failed to increase significantly the response to acetylcholine of uteri from animals pretreated with indomethacin ($p > 0.20$; Figure 20B).

Comment

Nonsteroidal, antiinflammatory drugs may inhibit smooth muscle contractility. Northover¹² has attributed this effect to an alteration by indomethacin of Ca^{++} binding by subcellular membranes. Other workers have found prostaglandin output to decrease following indomethacin or aspirin treatment of rat uterine and other preparations.^{5,6} In addition, cyclic AMP production increases in rat gastric mucosa in

Table 5. Contraction frequency (10-minute interval) of uteri from estradiol-treated (10 μ g per day) rats versus those from estradiol plus indomethacin-treated (10 mg per kg) rats in response to stimulation by indomethacin, aspirin and CaCl_2 .

	Estradiol (n = 6)	Estradiol and indomethacin (n = 6)	P
Aspirin (2.8×10^{-4} M)†	10.67 \pm 0.88	0.5 \pm 0.21	< .001
Indomethacin (1.4×10^{-4} M)†	8.75 \pm 0.63	0.0	< .001
CaCl_2 (7 mM)	9.2 \pm 0.80	0.83 \pm 0.41	< .001
CaCl_2 (7 mM) + PGF_2 (5×10^{-7} M)	14.1 \pm 0.53	12.0 \pm 0.23	> .20

† 0.05 mg/ml

Values are given as means \pm standard error of the mean.

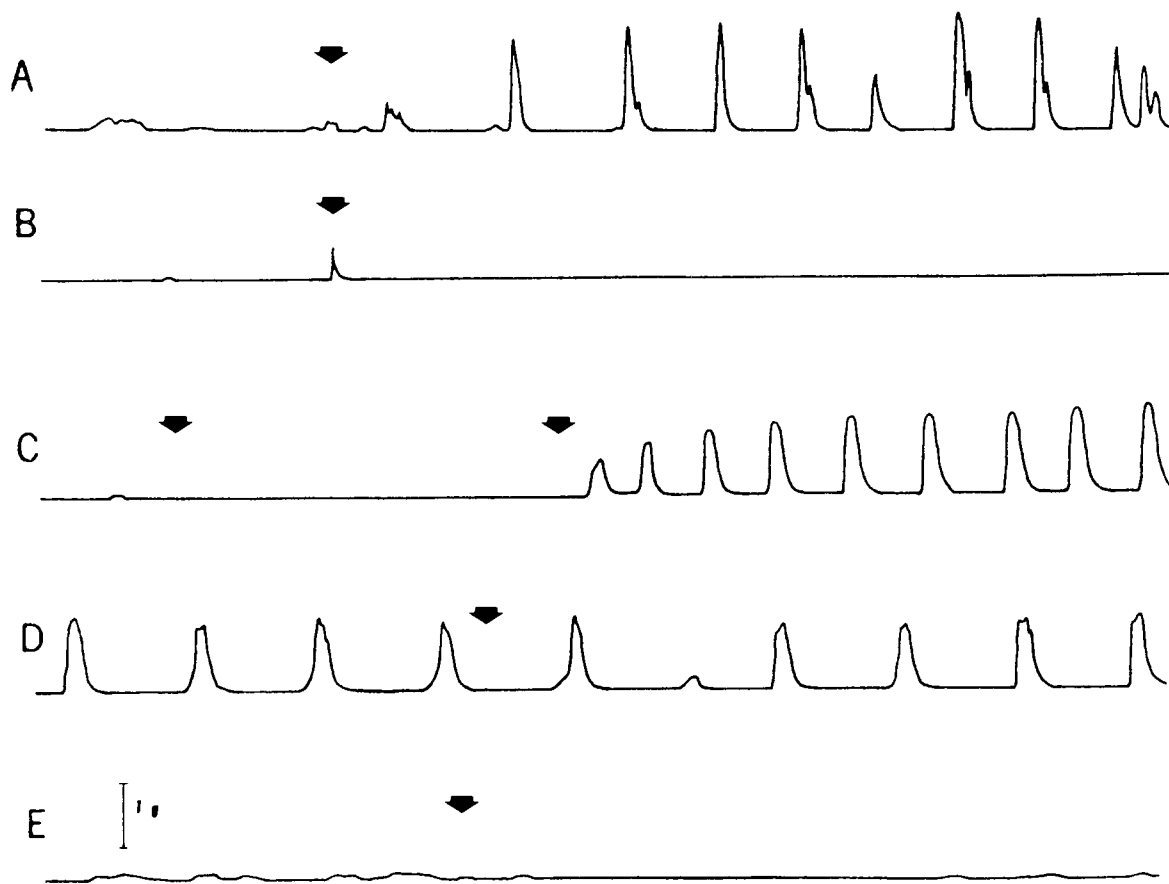


Figure 19. A. Stimulation of rat uterine contractility by 7 mM CaCl_2 (added at arrow). B. Lack of effect of 7 mM CaCl_2 (arrow) on quiescent uterus from indomethacin-pretreated rat. C. Lack of effect of 7 mM CaCl_2 (first arrow) followed by contractions induced by addition of 71 nM prostaglandin $\text{F}_{2\alpha}$ (second arrow). D. Slight inhibition of actively-contracting rat uterus caused by addition of 50 μ l of ethanol (42 mM final concentration) at arrow. E. Lack of stimulation of quiescent rat uterus by 50 μ l of ethanol (added at arrow).

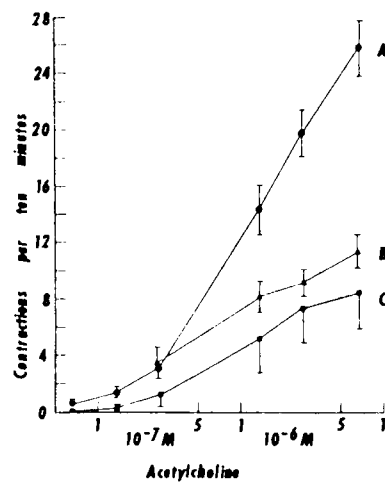


Figure 20. A. Log dose-response (LDR) curve for the acetylcholine-caused contraction rate in uteri from estradiol-pretreated rats. B. LDR curve for acetylcholine stimulation of uteri from estradiol- and indomethacin-pretreated rats, in the presence of 7 nM prostaglandin $F_{2\alpha}$. C. LDR curve depicting the response of uteri from estradiol- and indomethacin-pretreated rats to acetylcholine. Vertical bars equal \pm the standard error of the mean.

the presence of aspirin.¹⁵ We here report an additional, stimulatory effect of these drugs on smooth muscle contractility, in consonance with aspirin's ability to bring about premature parturition in mice.⁹ One plausible cause of increased motility would be a displacement of Ca^{++} from subcellular membranes, leading to temporarily high levels of unbound, intracellular Ca^{++} . Reduced motility would be expected following an efflux of this ion from smooth muscle cells. Our observations that uteri from indomethacin-pretreated rats are initially quiescent in vitro, and that these uteri respond to either acetylcholine or Ca^{++} at a low level, accord with such a train of events.

In a previous report, Vane and Williams⁶ did not find any change in uterine sensitivity to acetylcholine after indomethacin treatment in vitro. These workers, however, added the indomethacin directly to the uterine bathing medium for brief periods (30-90 sec), whereas we injected rats with this compound ip for two days prior to the experiment. In addition, their failure to observe increased contractility could be due to their use of preparations that were already motile as a consequence of prostaglandins present in the bathing medium. By first rinsing the uteri with fresh Tyrode's solution, we reduced contractility, thereby increasing the sensitivity of the preparation to aspirin or indomethacin. Our present results support the conclusion drawn from previous studies^{5, 6} concerning the contribution of prostaglandins to smooth muscle motility in vitro, since uteri from rats pretreated with indomethacin produced no prostaglandin-like material and were inactive. In addition,

prostaglandin $F_{2\alpha}$ always restored uterine contractions and responsiveness to Ca^{++} . Similarly to Vane and Williams,⁶ we failed to detect any prostaglandin E_2 in the media from actively-contracting preparations, although other PGs were present. However, we found no direct inhibition of active uteri by either aspirin or indomethacin. We conclude, therefore, that reports of immediately-reduced motility may be attributable to rinsing the preparations, thereby removing any prostaglandins that may already have been present. Alternatively, indomethacin or aspirin may directly inhibit uterine contractions, irregardless of the presence of prostaglandins, at the lower concentrations used by other researchers.^{5, 6}

The evidence presented here suggests that, in addition to exerting inhibitory effects on prostaglandin synthesis, indomethacin and aspirin directly affect smooth muscle. Observed activation of uterine musculature by high doses of antiinflammatory compounds may explain the premature induction of labor by indomethacin or aspirin,^{9,10} while their reported delay of parturition^{7, 8} may be due to their effects on prostaglandins, cyclic AMP, or Ca^{++} balance. It now seems apparent, in any case, that the actions of indomethacin and aspirin may not be totally explained by their inhibiting prostaglandin synthesis.

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