MOLECULAR PHARMACOLOGY, 21:654–663

The Relationship between the Carbachol Stimulatory Effect on Cyclic GMP Content and Activation by Fatty Acid Hydroperoxides of a Soluble Guanylate Cyclase in the Guinea Pig Myometrium

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Received February 6, 1981; Accepted November 30, 1981

SUMMARY

In estrogen-dominated rat and guinea pig myometrium, guanylate cyclase activities were found in both soluble and particulate fractions. In both species the soluble form was activated by sodium nitroprusside, whereas activity of the particulate fraction was enhanced in the presence of unsaturated fatty acids and their hydroperoxy derivatives. The latter activation appears to result from direct lipid-protein interaction rather than peroxide or free radical generation. In addition, cytosolic activity of guinea pig myometrium was stimulated by micromolar concentrations of arachidonic and linoleic acids; their hydroperoxides were more effective, and formation of peroxide species by endogenous lipoxygenase(s) was required for the fatty acid effect. In marked contrast, the soluble form of rat myometrium appeared insensitive to such a fatty acid-promoted oxidative activation. Sodium nitroprusside provoked a Ca^{2+}-independent increase in intracellular cyclic GMP in both rat and guinea pig intact myometrium, but fatty acids and their hydroperoxides exerted a stimulatory effect, requiring Ca^{2+}, only in guinea pig myometrial tissue. Also in rat myometrium, stimulation of muscarinic receptors or Ca^{2+} influxes as well as high K^+ did not lead to increases in cyclic GMP, whereas these stimulants enhanced cyclic GMP accumulation in guinea pig tissue. Cyclic GMP stimulations caused by fatty acids and other Ca^{2+}-requiring effectors were markedly suppressed by incubating guinea pig myometrium with eicosatetraynoic acid, which prevents fatty acid peroxidation, and were similarly unaffected by indomethacin, a selective inhibitor of the cyclooxygenase pathway. Inhibitors of phospholipase activity (viz., mepacrine, bromophenacylbromide, and compound 874 CB) reduced cyclic GMP responses to carbachol, A 23187, and high K^+. It is thus suggested that cyclic GMP stimulations promoted by the latter effectors are mediated by a phospholipase A_2-induced activation and by the formation of hydroperoxy derivatives of the liberated fatty acids which are potential activators of cytosolic guanylate cyclase of guinea pig myometrium. Insensitivity of soluble guanylate cyclase of rat myometrium to activation by fatty acid hydroperoxides may provide an explanation for the lack of observable cholinergic cyclic GMP responses.

INTRODUCTION

In most intact cells, cyclic GMP accumulation may be modulated through apparently Ca^{2+}-dependent and Ca^{2+}-independent mechanisms (1, 2). Thus, cellular increases in cyclic GMP induced by cholinergic agonists, generally observed to occur with high K^+ and the ionophore A 23187, exhibit a stringent requirement for Ca^{2+} (3, 4). By contrast, accumulation of cyclic GMP promoted by SNP[1] and other NO-forming agents does not require Ca^{2+} (4, 5). In our previous studies with estrogen-dominated rat myometrium, it was found that cyclic GMP levels were markedly enhanced in the presence of NH_2OH and SNP but that this rat tissue was insensitive, in terms of cyclic GMP accumulation, to the different Ca^{2+}-requiring effectors (6). The latter casual observation prompted us to investigate possible mechanisms underlying regulation of soluble and particulate guanylate cyclase activities of rat myometrium (7). A comparative study was then undertaken with guinea pig myometrium, which is herein demonstrated to exhibit large increases

This work was supported by Grants LA 272 and ATP 4162 from the Centre National de la Recherche Scientifique, by Grant 79.7.0782 from the Délégation à la Recherche Scientifique et Technique, and by a contribution from the Fondation pour la Recherche Médicale Française.

[1] The abbreviations used are: SNP, sodium nitroprusside; MIX, 3-isobutyl 1-methylxanthine; TYA, 5,8,11,14-eicosatetraynoic acid; DTT, dithiothreitol; BHA, butylated hydroxyanisole; NDGA, nordihydroguaiaretic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

0026-895X/82/030654-10$02.00/0

in intracellular cyclic GMP content in response to carbachol, high K^+, and the Ca^{2+} ionophore as well as to SNP. Although it has been possible to demonstrate direct activation *in vitro* of the soluble form of guanylate cyclases from many tissues by SNP and other nitro compounds (5, 8, 9), it is generally considered that the stimulatory effect of carbachol on cyclic GMP is an event distal to hormone-receptor interaction and presumably involves an intermediate role for substances generated within the cell upon hormonal stimulation (1, 2). Polyunsaturated fatty acids were among the first-defined naturally occurring compounds shown to activate guanylate cyclase. Fatty acid activation, in some cases, involved direct hydrophobic interactions (1, 7, 10), while in other instances activation occurred as a result of fatty acid oxidative metabolism (11, 12). Furthermore, it has recently been pointed out that unsaturated fatty acids may play a key role in mediating Ca^{2+} and carbachol stimulatory effects on cyclic GMP levels in rat ductus deferens (13) and renal cortex (14).

Accordingly, in the present study, particular attention was given to the effect of fatty acids and their hydroperoxides both at the level of guanylate cyclase activation in cell-free soluble and particulate preparations and at the level of cyclic GMP in intact myometrium. The analysis was further extended to the effect of various agents that might interfere both with the release of fatty acids from endogenous phospholipids and with fatty acid metabolic peroxidation. The reported data provide some evidence that, in guinea pig myometrium, effects of carbachol as well as high K^+ and A 23187 on cyclic GMP could be mediated via the generation of endogenous fatty acids and their subsequent transformation to hydroperoxy derivatives whose ability to activate the soluble form of guanylate cyclase is further demonstrated. In rat tissue, the lack of detectable fatty acid-promoted oxidative stimulations may readily explain the concomitant failure of carbachol to alter cyclic GMP levels.

EXPERIMENTAL PROCEDURES

Chemicals. β-Estradiol-3 benzoate, SNP, butylated hydroxyanisole, thiourea, nordihydroguaiaretic acid, quinacrine dihydrochloride (mepacrine), and *p*-bromophenacyl bromide were obtained from Sigma Chemical Company (St. Louis, Mo.); MIX from Aldrich Chemical Company (Milwaukee, Wisc.), and soybean lipoxygenase (~250,000 units/mg) from Serva. Creatine phosphate and creatine kinase were obtained from Boehringer (Mannheim, Federal Republic of Germany), carbamoylcholine hydrochloride (carbachol) from Merck (Darmstadt, Federal Republic of Germany), and atropine from Calbiochem (San Diego, Calif.). Ionophore A 23187 was supplied by Lilly Laboratories (Indianapolis, Ind.). ^{125}I-labeled succinyl cyclic GMP tyrosine methyl ester and the antibody for radioimmunoassay of cyclic GMP were provided by Institut Pasteur Production (Paris, France). Indomethacin was kindly supplied by Merck Sharp & Dohme (Rahway, N. J.), and TYA by Hoffmann-La Roche (Nutley, N. J.). Compound 874 CB, 2,3-dibromo-(4′-cyclo hexyl-3′-chloro)phenyl-4-oxobutyric acid, was a generous gift from Clin Midy (Montpellier, France) to Dr. B. Vargaftig.

Fatty acids. Arachidonic $C_{20:4}$, linoleic $C_{18:2}$, γ-linolenic $C_{18:3}$, and oleic $C_{18:1}$ acids were from NuChekPrep Inc. Solutions of the ammonium salt were prepared by dissolving 1 mg/ml in NH_4OH for neutralization with agitation under a stream of N_2.

15-Hydroperoxyarachidonic and 13-hydroperoxylinoleic acids were obtained by catalytic oxygenation of the corresponding fatty acid using soybean lipoxygenase (15). The reaction was carried out at room temperature in 2.0 ml of 50 mM Tris-HCl buffer (pH 8.0) with 600 μM fatty acid and 75 μl of the lipoxygenase solution (5000 units). Measurement of absorbance at 238 nm indicated that oxygenation was complete within 5–10 min. The reaction mixture was then extracted with 6 ml of cold ether and evaporated to dryness under a stream of N_2; the residue was redissolved in 1 mM NH_4OH. In some experiments, aliquots of the oxygenation reaction mixtures containing the hydroperoxy derivatives were used directly, omitting the ether extraction step. Hydroperoxyarachidonic and linoleic acids were also subjected to reduction by sodium borohydride to the corresponding hydroxy derivatives before ether extraction and evaporation to dryness. The oxidized and reduced products were further characterized by thin-layer chromatography as previously described (16).

Animals. Young female Wistar rats (4 weeks old) and Hartley guinea pigs (5 weeks old) were treated with 30 and 100 μg of estradiol, respectively, for 2 days and used on the following day.

Tissue preparation. Animals were killed by decapitation. Their uteri were immediately removed and immersed in Krebs-Ringer bicarbonate buffer (pH 7.4) at 4°. The myometrium was separated by stripping away the endometrium as previously described for rat tissue (16), and the procedure was similarly applied to guinea pig uteri.

Incubation experiments for assay of cyclic GMP levels. Myometrial preparations obtained from about 20 rat or 6 guinea pig uteri were cut into two or three segments which were pooled and divided into equal parts. About 50 mg of myometrial strips were added to 2 ml of Krebs buffer and allowed to equilibrate for 30 min at 37° (gas phase, 95% O_2–5% CO_2) under constant agitation. Incubations were further continued for 5 min with or without the addition of MIX (final concentration 138 μM) and were followed by an additional incubation for 3 min in the absence or presence of the different agents to be tested. Reactions were then stopped by immersing the tissue strips in 2 ml of cold 7% trichloroacetic acid with an immediate homogenization of the samples and centrifugation for 30 min at 30,000 × *g*. Cyclic GMP was estimated in the trichloroacetic acid-soluble extract as previously described (6) according to the radioimmunoassay after the succinylation step proposed by Cailla *et al.* (17). The centrifuged pellets were dissolved in 1 N NaOH for protein determination (18). Cyclic GMP levels were expressed as picomoles per milligram of protein.

Extraction of tissue and assay of guanylate cyclase activity. The procedure was essentially as previously described (7). Myometrial strips from either rat or guinea pig uteri were homogenized with a Turrax ultrahomogenizer in 10 ml of cold buffer consisting of 0.25 M sucrose,

5 mM Tris-HCl (pH 7.5), and 1 mM EDTA and centrifuged at 700 × *g* for 5 min to eliminate cell debris. The sediment was washed by resuspension in the buffer (one-third of the volume of the homogenate) and centrifugation at 700 × *g*. The pooled 700 × *g* supernatant fractions constitute the "crude extract" which was further centrifuged at 105,000 × *g* for 1 hr in order to obtain the particulate and soluble cell fractions. The pellet was resuspended in a volume of buffer equal to that of the original homogenate. Guanylate cyclase activity was assayed by a modification of the method of Katsuki *et al.* (8). The standard reaction mixture contained 50 mM Tris-HCl (pH 7.5), 0.5 mM MIX, 11.2 mM creatine phosphate, 33 μg of creatine phosphokinase (0.8 unit), 1 mM GTP, and 2 mM $MnCl_2$ in a final volume of 150 μl. Various agents to be tested were included as indicated. The reaction was initiated by the addition of 10–20 μl of the supernatant or particulate fraction (15–30 μg of protein), and incubation was carried out at 37° for 10 min unless otherwise indicated. Reactions were terminated by the addition of 1.35 ml of 50 mM sodium acetate buffer (pH 4.0) and heated for 3 min at 90°. Cyclic GMP formed was determined in triplicate without purification by the radioimmunoassay as outlined above. Over this range of protein concentration, cyclic GMP formation was proportional to the amount of protein added and linear with respect to incubation time up to 20 min. We ensured that under such conditions enzyme activity, when measured in the presence of the different agents, was still linear with respect to time and protein concentrations. It was systematically checked that each specific agent, whenever used at the indicated concentration, had no effect on the final cyclic GMP radioimmunoassay.

RESULTS

Regulation of cyclic GMP levels in rat and guinea pig myometrium. Results of Table 1 show that the cyclic GMP concentration in estrogen-dominated guinea pig myometrium was 3- to 4-fold higher than that in rat tissue. In both species, cyclic GMP levels were increased in the presence of MIX, a phosphodiesterase inhibitor, as well as in the presence of SNP, whose stimulatory effect could as well be expressed in the absence or presence of Ca^{2+}, in agreement with reports for other tissues (4, 5). In contrast, rat and guinea pig myometrium differed markedly in their responsiveness to cholinergic stimulation. It was noted in initial studies (6), such as those depicted in Fig. 1, that carbachol was unable to alter the cyclic GMP content of rat myometrium over a wide range of concentrations (1–100 μM). The lack of a carbachol effect in terms of cyclic GMP accumulation in rat myometrium could not be due to an absence or alteration of the muscarinic receptors, since the cholinergic effector was able to cause a normal contractile response that was inhibited by atropine (50%, 90%, and 100% inhibitions of the contractile response elicited by 15 μM carbachol were obtained with 6, 10, and 15 nM atropine, respectively).

The stimulatory effect of carbachol on the cyclic GMP content of guinea pig myometrium was dose-dependent (6.02 ± 0.7, 9.1 ± 0.8, 12.2 ± 1.5, and 12.5 ± 2 pmoles of cyclic GMP per milligram of protein at 1, 10, 50, and 100 μM carbachol, respectively), potentiated by MIX, and abolished in the presence of atropine in the same range of concentrations that prevented the contractile effect of the cholinergic agent: inhibition of the cyclic GMP response due to 20 μM carbachol averaged 70 and 100% at

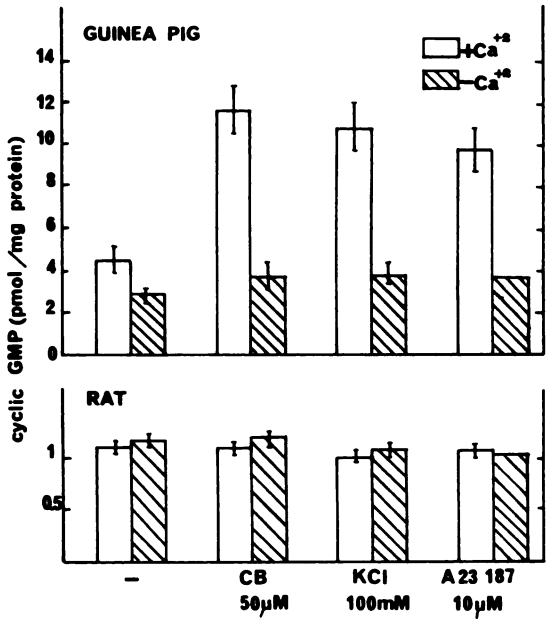

FIG. 1. *Effects of carbachol, KCl, and ionophore A23187 on cyclic GMP levels in rat and guinea pig myometrium*: role of Ca^{2+}

Myometrial strips were incubated for 30 min in Krebs buffer in the presence or absence of Ca^{2+} (2.4 mM) with or without 4 mM EGTA. The tissues were then transferred to a fresh corresponding medium containing MIX (138 μM) and incubated for 5 min before the addition of carbachol (*CB*) or compound A 23187 at the indicated concentrations. For incubations with 100 mM KCl, K^+ concentrations in the buffer were increased from 6 to 100 mM with a concomitant decrease in Na^+ concentration in order to maintain total monovalent cation concentration at 150 mM. Incubations were continued for 3 min and cyclic GMP was assayed after tissue extraction as described in the text. Means ± standard errors of four to six different experiments are shown.

TABLE 1

Effect of MIX and SNP on cyclic GMP levels in rat and guinea pig myometrium: role of Ca^{2+}

Myometrial strips were preincubated for 30 min in 2 ml of Krebs buffer in the presence or absence of Ca^{2+} with or without 4 mM EGTA. The tissues were then transferred to a fresh corresponding medium and further incubated for 5 min with or without the addition of MIX (138 μM). Incubations were continued for 3 min in the absence or presence of SNP (1 mM). Tissue extraction and cyclic GMP determinations were carried out as described in the text. Values represent the mean ± standard error of three different experiments.

Addition	Rat		Guinea pig	
	$+Ca^{2+}$ (2.4 mM)	$-Ca^{2+}$, +EGTA (4 mM)	$+Ca^{2+}$ (2.4 mM)	$-Ca^{2+}$, +EGTA (4 mM)
	pmoles cyclic GMP/mg protein			
None	0.39 ± 0.1	0.47 ± 0.1	1.6 ± 0.3	1.02 ± 0.2
MIX, 138 μM	1.8 ± 0.36	2.02 ± 0.15	4.4 ± 0.48	2.8 ± 0.4
SNP, 1 mM	2.12 ± 0.21	3.0 ± 0.4	6.5 ± 0.7	—
MIX, 138 μM, + SNP, 1 mM	16.5 ± 2.2	ND[a]	33.5 ± 3.2	31.0 ± 2.1

[a] ND, Not determined.

10 and 20 nM atropine, respectively. Similarly, KCl was without effect on rat tissue; nevertheless it increased the cyclic GMP content of guinea pig myometrium (Fig. 1). Both carbachol and KCl stimulations were not induced in a Ca^{2+}-deprived medium, in agreement with previous observations in different systems (3, 4). Levels of cyclic GMP were also enhanced by the ionophore A 23187 in guinea pig myometrium but not in rat myometrium. Of interest also were the observations that in rat myometrium the basal turnover of cyclic GMP as well as the increase in cyclic GMP due to the phosphodiesterase inhibitor were not effectively changed by omission of Ca^{2+}. In contrast, levels of cyclic GMP both in the absence and presence of MIX were consistently lower in guinea pig myometrial strips incubated in the absence of Ca^{2+} as compared with the corresponding incubation in the presence of Ca^{2+} (Table 1). The Ca^{2+} concentration (2.4 mM) present in the normal physiological Krebs-Ringer buffer was routinely chosen for the above-reported experiments. It was also observed that carbachol, KCl, and A 23187 similarly failed to affect cyclic GMP levels in rat myometrium when incubated in media containing different Ca^{2+} concentrations varying from 0 to 2.4 mM (data not shown). These findings indicated that the Ca^{2+}-dependent mechanisms through which cyclic GMP accumulation may occur in guinea pig myometrium (as well as in most investigated tissues stimulated by carbachol, K^+, or A 23187) do not operate significantly in rat myometrium under our experimental conditions.

Modulation of soluble guanylate cyclase activities of rat and guinea pig myometrium by SNP. In both species, the Mn^{2+}-dependent guanylate cyclase activities were associated with both soluble and particulate fractions of myometrial homogenates (1,167 and 2,037 pmoles of cyclic GMP per minute per gram of tissue, respectively, for the soluble and 105,000 × *g* particulate activity of guinea pig myometrium and 870 and 1,513 pmoles of cyclic GMP per minute per gram of rat tissue). In the presence of Triton X100, there was marked stimulation of the particulate activity (10- to 13-fold), whereas the soluble activity was only slightly (60%) enhanced. As reported for most guanylate cyclases of different sources (1), including rat myometrium (7), both soluble and particulate guanylate cyclases of guinea pig myometrium preferred Mn^{2+} as the sole cation for their activity. Indeed, as illustrated in Table 2 for the soluble forms, in the presence of 5 mM Mg GTP no more than 20% of the activity was obtained with a maximal effective concentration of 1 mM Mn GTP. Table 2 demonstrates that SNP increased the activity of guanylate cyclase in the crude soluble fraction from both guinea pig and rat myometrium. SNP stimulation was dose-related and was more pronounced in the presence of DTT. These findings, which substantiate our previous observations with rat myometrium (7) and which have been emphasized for other systems (9), could imply that SNP stimulation may occur via oxidative mechanisms, presumably at the level of —SH groups critically involved in the expression of full enzyme activity. The cation requirement for the soluble guanylate cyclase activity of both rat and guinea pig myometrium changed with SNP-activated enzyme, so that Mg^{2+} became as effective as Mn^{2+} as the sole cation (Table 2).

TABLE 2

Activation of soluble guanylate cyclase activities of rat and guinea pig myometrium by SNP

Guanylate cyclase activity was assayed in 105,000 × *g* soluble fractions of rat and guinea pig myometrial extracts in the presence of 1 mM $MnCl_2$ and 1 mM GTP, or 5 mM $MgCl_2$ and 5 mM GTP, with or without the addition of different concentrations of SNP. DTT (1 mM) was absent or present during the assay. Incubations were carried out for 10 min at 37° as described under Experimental Procedures. Values represent the mean ± standard error of three or four experiments.

Addition	Guanylate cyclase activity			
	Rat		Guinea Pig	
	−DTT	+DTT	−DTT	+DTT
	pmoles cyclic GMP/min/mg protein			
Mn GTP				
Control	24 ± 5	25 ± 4	50 ± 6	28 ± 6
SNP				
1 μM	28 ± 11	52 ± 10	ND[a]	96 ± 10
10 μM	68 ± 11	150 ± 23	ND	243 ± 30
100 μM	72 ± 15	160 ± 15	74 ± 3	296 ± 27
1000 μM	25 ± 14	160 ± 20	ND	295 ± 32
Mg GTP				
Control		6 ± 2		11 ± 2
SNP, 100 μM		130 ± 9		235 ± 15

[a] ND, Not determined.

Stimulation of particulate guanylate cyclase activity of rat and guinea pig myometrium by various fatty acids. The particulate activity of both guinea pig and rat myometrium extracts was similarly increased in the presence of series of unsaturated (Fig. 2) and saturated (Table 3) fatty acids. Activation was dose-related, being almost undetectable below 20 μM and reaching a maximum at 80–100 μM. Increasing the fatty acid concentrations resulted in a progressive loss of the stimulatory effect. The effectiveness of all of the tested compounds was quite similar for the particulate activity in myometrium of both species. There was no appreciable alteration in the stimulating activity by varying the hydrocarbon chain length of the saturated fatty acid (C_{12}–C_{20}) nor by increasing the number of double bonds in the long-chain unsaturated fatty acids. The hydroperoxy derivatives of arachidonic, linoleic, and γ-linolenic acids also served as effective enhancers of the particulate enzyme activity (Fig. 2). Even though maximal responses attained with the hydroperoxides were similar to or even higher than those attained with the fatty acids under similar conditions and did not decline with increasing concentrations, it clearly emerged that, for both species, at low concentrations (20–100 μM) fatty acids appeared more effective than the hydroperoxides. This observation was taken as indirect evidence that the effects of fatty acid could not be attributed to their conversion to hydroperoxides during guanylate cyclase assay incubation. Furthermore, the presence of a series of reagents such as DTT (1 mM), which inhibits both the lipoxygenase and cyclooxygenase reactions (19); indomethacin (15 μM), a well-known inhibitor of the cyclooxygenase (20); or BHA, at a concentration (2–5 mM) previously shown to scavenge hydroxyl radicals (2, 21), did not appreciably alter basal activity nor affect the ability of either the fatty acids or their

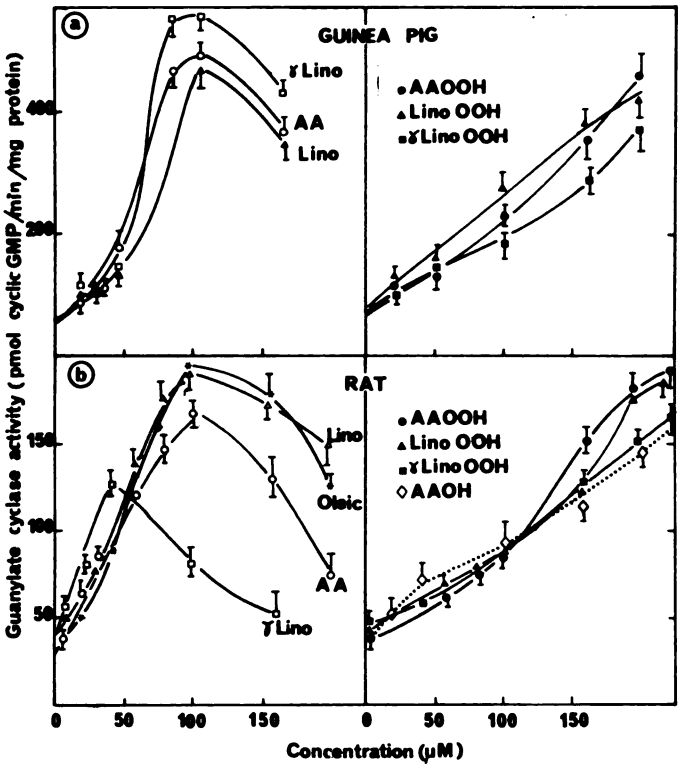

FIG. 2. *Dose-dependent stimulatory effects of various fatty acids and their hydroperoxides on particulate guanylate cyclase activities of rat and guinea pig myometrial extracts.*

Guanylate cyclase in the 105,000 × *g* particulate fraction was assayed in the presence of 1 mM GTP and 2 mM $MnCl_2$, 10 min at 37° as described under Experimental Procedures, with the addition of the indicated concentrations of arachidonic acid (*AA*), oleic acid, linoleic acid (*lino*), and γ-linolenic acid (*γlino*) and hydroperoxides of arachidonic acid (*AAOOH*), linoleic acid (*LinoOOH*), and γ-linolenic acid (*γLinoOOH*) or hydroxyarachidonic acid (*AAOH*). Results represent the mean ± standard error of three to five different experiments.

hydroperoxides to stimulate particulate guanylate cyclase of rat (7) and guinea pig myometrium (guanylate cyclase activities were as follows: with 100 μM arachidonic acid, 498 ± 38; with arachidonic acid + DTT, 470 ± 51; with arachidonic acid + indomethacin, 554 ± 49; and with arachidonic acid + BHA, 420 ± 45 pmoles of cyclic GMP per minute per milligram of protein). Of significance also was the observation that the reduced derivative, 15-hydroxyarachidonic acid, was as effective as the corresponding hydroperoxy compound in stimulating particulate guanylate cyclase activity (Fig. 2). These data taken together strongly imply that neither oxidative products of fatty acids nor the generation of reactive radicals were involved in the fatty acid stimulatory effect. Most probably activation might result from an interaction of the hydrocarbon chain of the lipid components with hydrophobic regions of the particulate guanylate cyclase or associated regulatory units. Nevertheless, our data do not establish, at least under our experimental conditions, a close correlation between the degree of guanylate cyclase activation and the energy of such a hydrophobic interaction since the latter may be assumed to be dependent on the fluidity (i.e., chain length and degree of unsaturation) of the hydrocarbon chain of the fatty acid.

Oxidative stimulation of the soluble guanylate cyclase activity of guinea pig myometrium by fatty acid hydroperoxides: lack of effect on the soluble rat preparation. Data of Fig. 3 illustrate the stimulatory effect of arachidonic acid and the hydroperoxides of arachidonic acid and linoleic acids on soluble guanylate cyclase activity of guinea pig myometrium. The dose-related stimulatory pattern was clearly different from that obtained with the particulate enzyme (Fig. 2). Activation of the soluble form was achieved at lower concentrations of the fatty acid (5–20 μM); both hydroperoxides were consistently more effective than the nonoxidized fatty acids, maximal activation being similar in magnitude with both series of compounds. In addition, and in contrast to the particulate enzyme, saturated fatty acids (lauric, myristic, and arachidic) were totally ineffective in stimulating soluble guanylate cyclase activity (Table 3). It was also of significance to note the relatively weak activating potencies of hydroxyarachidonic acid and of methyl arachidonate, which could not be substrates for lipoxygenases. Thus it was tempting to speculate that the fatty acid hydroper-

TABLE 3

Effect of unsaturated fatty acids on particulate and soluble guanylate cyclase activities of rat and guinea pig myometrium

Guanylate cyclase activity was assayed in 105,000 × *g* particulate and soluble fractions of guinea pig and rat myometrium in the presence of 1 mM GTP and 2 mM $MnCl_2$, 10 min at 37° as described under Experimental Procedures with the addition of the indicated concentrations of the different fatty acids. Results represent the mean ± standard error of three different experiments.

Myometrial fraction	None	Addition: Lauric (μM)			Myristic (μM)			Arachidic (μM)		
		50	100	150	50	100	150	50	100	150
		pmoles cyclic GMP/min/mg protein								
Particulate										
Guinea pig	52 ± 4	198 ± 20	600 ± 40	507 ± 42	186 ± 16	549 ± 42	422 ± 35	214 ± 15	647 ± 30	437 ± 35
Rat	30 ± 3	107 ± 9	136 ± 8	134 ± 10	142 ± 9	133 ± 10	137 ± 15	122 ± 9	118 ± 8	134 ± 12
	None	Addition: Lauric (μM)			Myristic (μM)			Arachidic (μM)		
		20	40	100	20	40	100	20	40	100
		pmoles cyclic GMP/min/mg protein								
Soluble										
Guinea pig	50 ± 6	62 ± 5	64 ± 7	69 ± 9	53 ± 4	42 ± 5	50.3 ± 6	43 ± 5	48 ± 5	53.5 ± 6
Rat	25 ± 2	27.5 ± 2	29.4 ± 3	27.5 ± 5	29.8 ± 3	30.5 ± 2.5	24.8 ± 4	28.5 ± 3	28.9 ± 4	24.6 ± 4

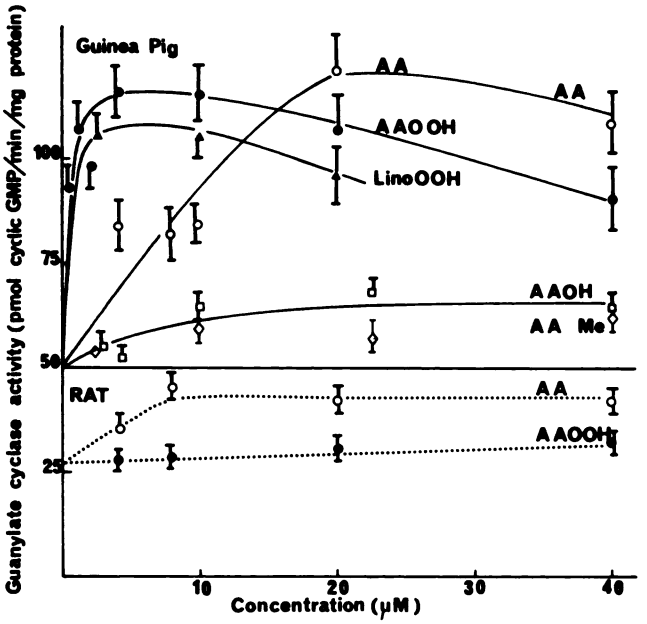

FIG. 3. *Effects of various concentrations of fatty acids and fatty acid hydroperoxides on soluble guanylate cyclase activity of rat and guinea pig myometrium*

Guanylate cyclase activity in 105,000 × *g* soluble fractions of rat (·····) and guinea pig (——) myometrium was assayed in the presence of 1 mM GTP and 2 mM $MnCl_2$, 10 min at 37° as described in the text, with the addition of the indicated concentrations of arachidonic acid (*AA*), hydroperoxyarachidonic acid (*AAOOH*), hydroperoxylinoleic acid (*LinoOOH*), and hydroxyarachidonic acid (*AAOH*) or methylarachidonate (*AA Me*). Values represent the mean ± of three to five different experiments.

oxide, rather than the fatty acid itself, might constitute the reactive species involved in the stimulation of the soluble guanylate cyclase activity of guinea pig myometrium. This would tend to imply that in the arachidonic acid effect depicted in Fig. 3 there was a transformation of the fatty acid to a hydroperoxide derivative through the action of a lipoxygenase(s) present in the crude soluble myometrial fraction. To test this view, experiments were carried out in the presence of two lipoxygenase inhibitors, viz., TYA (22) and NDGA (23). As shown in Fig. 4, both TYA (20 μM) and NDGA (10 μM) markedly suppressed (60–85%) the ability of arachidonic and linoleic acids to stimulate soluble guanylate cyclase activity of guinea pig myometrium. Under similar conditions, activation by hydroperoxylinoleic acid was not appreciably affected. Indomethacin failed to counteract the fatty acid stimulatory effect, whereas DTT and BHA were potent inhibitors of the activation normally induced by both the fatty acids and their hydroperoxides. Inhibition in the presence of DTT averaged 60–80% and in the presence of BHA 60–70%. DTT not only prevented activation when present at the onset of the reaction but was also able to reverse the activation after it occurred in the presence of arachidonic acid. Thus when 1 mM DTT was added to the assay mixture 5 min after exposure to arachidonic acid, guanylate cyclase activity estimated during 5 min of further incubation was reduced to 52 ± 7 versus 100 ± 12 pmoles of cyclic GMP per minute per milligram of protein for the activated system in the absence of the reducing agent. All of these data provide evidence that an oxidative process was involved in the stimulation of the crude soluble guanylate cyclase of the guinea pig myometrium by either added or locally generated (via lipoxygenase(s) fatty acid hydroperoxides.

Nevertheless, a more striking observation concerns the failure of fatty acids and fatty acid hydroperoxides to stimulate appreciably soluble guanylate cyclase activity of the rat myometrium. As illustrated in Fig. 3, arachidonic acid (5–40 μM) increased by no more than 60% the guanylate cyclase activity in soluble rat myometrial extract, and hydroperoxy arachidonic acid was almost inactive as opposed to the 3- to 4-fold stimulations reached in the case of guinea pig preparations. Mixing guinea pig and rat myometrial preparations did not improve the stimulatory effects inasmuch as activation by arachidonic and hydroperoxyarachidonic acid of the mixed fractions essentially accounted for the guinea pig guanylate cyclase activity [during a 10-min incubation with 20 μM hydroperoxy arachidonic acid, values for cyclic GMP formation were as follows: with the rat preparation (15 μg of protein), 3.7 ± 0.4; with the guinea pig preparation (14 μg of protein), 20.5 ± 3; and with the mixed myometrial fractions, 26 ± 3 pmoles]. This precluded the presence in the rat preparation of an inhibitor or the absence of a limiting factor that could account for the lack of stimulability noted with the fatty acids. The observations rather indicated that under our experimental conditions guanylate cyclase of rat myometrium was insensitive to these effectors. It is interesting to note that the extent, although small (60%) of activation of the soluble rat myometrial enzyme by arachidonic acid was almost identical with the stimulation caused by 0.1% Triton X-100 with the

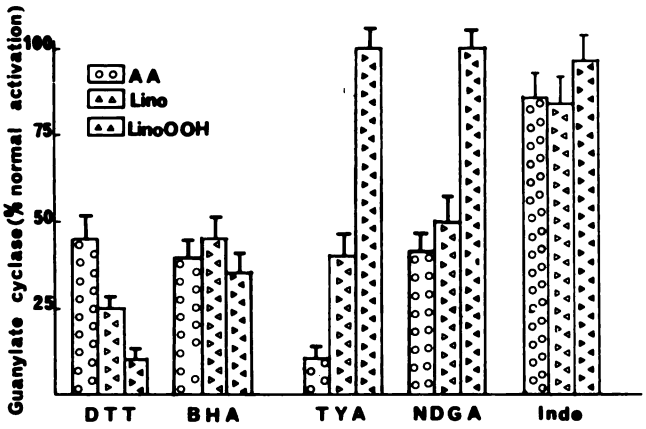

FIG. 4. *Inhibitory effects of DTT, butylated hydroxyanisole, and lipoxygenase inhibitors on fatty acid activation of soluble guanylate cyclase of guinea pig myometrium*

Aliquots of the 105,000 × *g* soluble fraction of guinea pig myometrial extract were introduced in the guanylate cyclase assay mixture (described under Experimental procedures) and incubated at 37° for 2 min with 1 mM DTT, 2 mM BHA, 20 μM TYA, and 10 μM NDGA or 15 μM indomethacin (*Indo*) before the addition of 20 μM arachidonic acid (*AA*), 20 μM linoleic acid (*Lino*), or 10 μM hydroperoxylinoleic acid (*LinoOOH*). Incubations were further continued for 10 min. TYA was prepared as an ammonium salt solution. BHA and NDGA were added in ethanolic solutions; the final concentration of ethanol was 0.3% with the same amount of ethanol being added to the control. Results are expressed as percentage of the normally evoked stimulation for each specific agent in the absence of inhibitors. Values represent the mean ± standard error of three or four different experiments.

soluble fraction of both rat and guinea pig myometrium. Such an activation was also comparable to that found with the guinea pig soluble fraction in the presence of hydroxyarachidonic acid or methylarachidonate, which could not participate in the oxidative-promoted activation described above.

Effect of fatty acids and fatty acid hydroperoxides on cyclic GMP content of rat and guinea pig myometrium. In the case of guinea pig myometrium, both arachidonic and linoleic acids as well as their corresponding hydroperoxides induced a marked elevation of tissue cyclic GMP which was dependent on the dose of the fatty acid. Stimulation by the fatty acid hydroperoxide occurred at lower concentrations as compared with the corresponding fatty acid (50% stimulation at 10–15 μM hydroperoxylinoleic and hydroperoxyarachidonic acids and 30–40 μM linoleic and arachidonic acids). Maximal responses were similar in magnitude in all cases. Saturated fatty acids (lauric, myristic, and arachidic) were totally ineffective. Figure 5 also demonstrates that the stimulatory effect of linoleic acid as well as that of hydroperoxy linoleic and hydroperoxyarachidonic acids occurred via a Ca^{2+}-dependent process, as stimulations were totally abolished when incubations of myometrial strips were carried out in a Ca^{2+}-deprived medium containing 4 mM EGTA. Thus, as far as the stringent requirement for Ca^{2+} was concerned, the fatty acid-induced elevation of guinea pig myometrium cyclic GMP was comparable to the stimulations by carbachol and high K^+ outlined in Fig. 1. Again, a marked difference was noted with intact rat myometrium inasmuch as arachidonic and linoleic acids as well as their hydroperoxy derivatives, in both the absence and presence of Ca^{2+}, were totally ineffective in raising intracellular cyclic GMP levels. Although the results obtained with a single Ca^{2+} concentration of 2.4 mM are depicted, lack of fatty acid stimulations was also confirmed with different Ca^{2+} concentrations varying from 0 to 2.4 mM. Furthermore, increasing concentrations of the lipid compound up to 1–3 mM still resulted in a negative response (data not shown). Therefore it was interesting to draw a parallel between failure of the fatty acids and their hydroperoxides to alter the intracellular cyclic GMP content and the absence of effect noted with carbachol, high K^+, and ionophore A 23187 on cyclic GMP levels in the myometrium (Fig. 1).

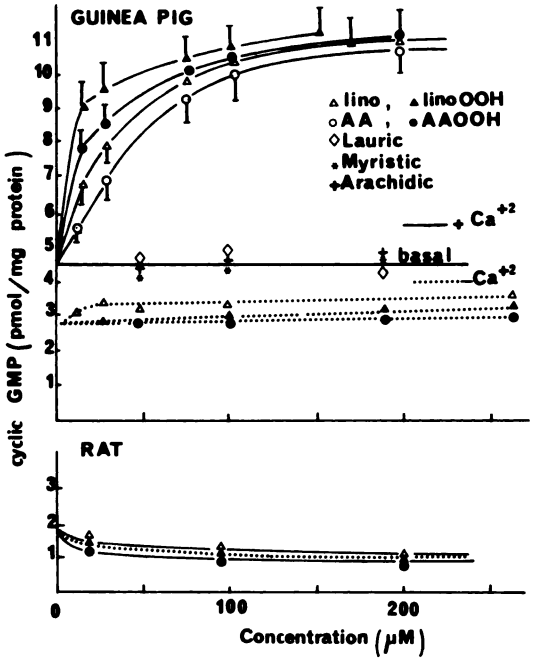

FIG. 5. *Effects of various concentrations of fatty acids and fatty acid hydroperoxides on cyclic GMP levels of rat and guinea pig myometrium: role of* Ca^{2+}

Guinea pig myometrial strips were incubated in the presence (——) or absence (·····) of Ca^{2+} as described in the legend to Fig. 1. Incubations with the indicated concentrations of the saturated fatty acids as well as arachidonic acid (*AA*), linoleic acid (*Lino*), hydroperoxyarachidonic acid (*AAOOH*), and hydroperoxylinoleic acid (*linoOOH*) were carried out for 3 min before tissue extraction and cyclic GMP determinations. Values represent the mean ± standard error of four different experiments. Bars represent standard error; where no bars are shown, the error was within the size of the symbol.

Effect of phospholipase A_2 *and lipoxygenase inhibitors on cyclic GMP accumulation induced by* Ca^{2+}*-requiring effectors in guinea pig myometrium.* In order to investigate the relationship between the stimulatory effects on cyclic GMP levels of fatty acids and other Ca^{2+}-requiring effectors, guinea pig myometrial strips were treated with mepacrine, which has been shown in different tissue preparations to inhibit phospholipase A_2 activity and thus limit the availability of free endogenous fatty acids (24). Results of Fig. 6 show that in myometrial strips treated with mepacrine (75 μM for 20 min), there was a marked inhibition (about 75–80%) of the rise in cyclic GMP normally induced by carbachol, high K^+, and A 23187. By contrast, mepacrine failed to alter cyclic GMP accumulation caused by arachidonic and linoleic acids. The latter observation precluded any unspecific effect of the drug at the level of the Ca^{2+}-dependent process involved in the fatty acid-promoted activation of cyclic GMP accumulation. Nevertheless, mepacrine was previously shown to exert some activities at the membrane level which might be unrelated to its mere ability to inhibit phospholipase A_2 (25, 26). Thus, it was of interest to note that two other phospholipase A_2 inhibitors, *p*-bromophenacylbromide (27, 28) and compound 874 CB (29), both at 50 μM, provoked 90% and 93% inhibition of the carbachol response, respectively (cyclic GMP levels after 3 min of incubation with 50 μM carbachol were 12.9 ± 1.2; with *p*-bromophenacyl bromide + carbachol, 7.3 ± 1; and with compound 874 CB, 6.4 ± 1.3 pmoles of cyclic GMP per milligram of protein). Neither inhibitor altered fatty acid or SNP stimulations or affected the contractile uterine responses normally evoked by the cholinergic effector (experiment not shown). All of these data could thus be taken, with good reason, as indirect evidence that the effects of carbachol as well as KCl and A 23187 on cyclic GMP accumulation might be initiated by a phospholipase activation with a possible intermediately effective role for the liberated fatty acids.

As illustrated in Fig. 6, prior treatment of myometrial strips (5 min) with 20 μM TYA markedly reduced the stimulatory effects of both arachidonic and linoleic acids. The increases in cyclic GMP induced by the fatty acids were no more than 25–30% of the responses observed in the absence of inhibitor. The stimulatory effect of hydro-

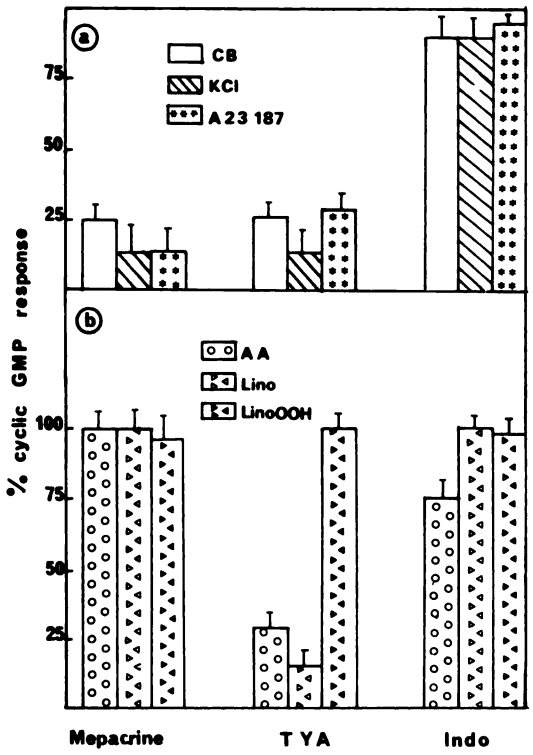

FIG. 6. *Comparison of the effects of mepacrine, TYA, and indomethacin on the elevation of cyclic GMP levels in guinea pig myometrium promoted by carbachol, KCl,* A 23187, *and fatty acids*

Guinea pig myometrial strips were incubated in the absence or presence of 75 μM mepacrine for 20 min, 20 μM TYA for 5 min, or 15 μM indomethacin (*Indo*) for 10 min before the subsequent addition of 138 μM MIX. Incubations were continued for 3 min with and without the addition of 100 μM carbachol (*CB*), 100 mM KCl, 10 μM A 23187, 100 μM arachidonic acid (AA), linoleic acid (*Lino*), or hydroperoxylinoleic acid (*LinoOOH*). Results are expressed as percentage of the normally evoked stimulation for each specific agent in the absence of inhibitors. Values represent the mean ± standard error of three different experiments, each carried in duplicate.

peroxylinoleic acid was fully expressed in the presence of TYA. Indomethacin, employed at concentrations (15 μM) previously shown to inhibit the synthesis of prostaglandins in intact myometrium (16, 30), failed to inhibit linoleic acid-induced cyclic GMP accumulation. In the presence of indomethacin, the effect of arachidonic acid was slightly reduced, but inhibition did not exceed 25% and was thus much less pronounced that that obtained with TYA. These observations favor the interpretation that arachidonic and linoleic acids exert their stimulatory effects on cyclic GMP accumulation through their conversion, via endogenous lipoxygenases, to hydroperoxy derivatives. In the case of arachidonic acid, there might also be a participation of endogenous endoperoxides formed through the cyclooxygenase pathway which nevertheless seems to be of minor importance compared with the lipoxygenase route. It also appears (Fig. 6) that treatment of guinea pig myometrial strips with TYA resulted in a significant reduction of the cyclic GMP response to carbachol. TYA also severely suppressed stimulation of cyclic GMP accumulation caused by high K^+ and ionophore A 23187. In the presence of TYA, there was more than 80% inhibition of the normal carbachol, high K^+, and ionophore responses while no appreciable inhibitory effect of indomethacin could be demonstrated. The data imply that fatty acid hydroperoxides may be considered as important modulators of cyclic GMP formation in intact myometrium and that generation of these hydroperoxylipids constitutes an obligatory intermediate step for the expression of cholinergic stimulations in terms of cyclic GMP accumulation.

Finally, it was tempting to associate the stimulatory action of cholinergic effectors and fatty acids on cyclic GMP accumulation in guinea pig myometrium with the sensitivity of the soluble guanylate cyclase to activation by fatty acid hydroperoxides. In rat tissue, the absence of effects of fatty acids and cholinergic agents on intracellular cyclic GMP was concomitantly observed with the insensitivity of the soluble guanylate cyclase to lipid peroxide activation. Further support for the insensitivity of the soluble guanylate cyclase of rat myometrium to activation by fatty acids was provided by the demonstration that the enzyme prepared from rat myometrial strips which had been pretreated with mepacrine and TYA (as described in the legend to Fig. 6) and with both inhibitors present in the subsequent homogenizing buffer exhibited no alteration in its basal activity and still remained insensitive to the addition of fatty acid (basal guanylate cyclase activities were 31.5 ± 4 and 35 ± 5 for treated and untreated tissue; activities in the presence of 20 μM hydroperoxy arachidonic acid were 41 ± 5 and 44 ± 3 pmoles of cyclic GMP per minute per milligram of protein for treated and untreated tissue, respectively). This eliminated the hypothesis that the sensitivity of the soluble guanylate cyclase of rat myometrium to fatty acid could have been masked because of a possible activation of the enzyme by endogenous lipid material during its preparation.

DISCUSSION

Cellular accumulation of cyclic GMP has generally been shown to occur in association with muscarinic high K^+ and ionophore A 23187 stimulations (1). We have demonstrated in earlier studies (6) and in this work that these effectors do not lead to any increase in cyclic GMP of the rat myometrium. These agents were nevertheless presently shown to enhance markedly the cyclic GMP levels of guinea pig myometrium. Evidence was further obtained that unsaturated fatty acids, and particularly their corresponding hydroperoxides, also constitute a class of potential activators of the cyclic GMP system, inducing cyclic GMP elevations in guinea pig myometrium similar to the previously described effects in the intact platelets (1) and renal cortical slices (14). Several lines of evidence indicate that the stimulatory effects of fatty acids on cyclic GMP share some features in common with cholinergic effects as well as those of high K^+ or A23187: (a) there were no demonstrable fatty acid- or fatty acid hydroperoxide-induced cyclic GMP elevations in the rat myometrium; (b) fatty acid and fatty acid hydroperoxide effects on cyclic GMP, as well as stimulations induced by carbachol, KCl, and ionophore A 23187, showed a stringent requirement for Ca^{2+}; and (c) eicosatetraynoic acid, a lipoxygenase inhibitor, prevented cyclic GMP elevations caused by both the fatty acids and the other Ca^{2+}-requiring effectors, while all responses

were similarly unaffected in the presence of a cyclooxygenase inhibitor, indomethacin. Finally, the observation that agents which are known to inhibit the activity of phospholipase A_2 [such as mepacrine (24), bromophenacyl bromide (27, 28), and compound 874 CB (29)] caused a substantial decrease in the carbachol-induced cyclic GMP response was consistent with the interpretation that the cholinergic effect was mediated through a phospholipase A_2-induced activation with the liberation of free fatty acids. These in turn have to be converted by endogenous lipoxygenase(s) to hydroperoxy derivatives in order to provoke cyclic GMP accumulation in guinea pig myometrium. It further appeared that the same cascade of events may be involved as well in the cyclic GMP stimulations caused by high K^+ and ionophore A 23187, as both were inhibited in the presence of mepacrine and eicosatetraynoic acid. A similar mechanism has recently been postulated for the Ca^{2+}- and hormone-induced cyclic GMP elevations in rat ductus deferens (13). Briggs and De Rubertis (14) have also demonstrated a close relationship between Ca^{2+}-induced cyclic GMP accumulation and arachidonate release and peroxidation in renal cortical slices.

There is considerable evidence that membrane-bound phospholipases A_2 show an absolute requirement for Ca^{2+} (31) and that in many intact cells the ionophore A 23187, which is known to promote Ca^{2+} transport through cellular membranes, produces an increase in phospholipase A_2 activity (28, 31, 32). Moreover, Ca^{2+}-dependent stimulation of phospholipase A_2 activity by a variety of agents, including thrombin, bradykinin, and ACTH, has recently been described in some cellular systems (28, 32). Thus, although no direct measurement of phospholipase A_2 was carried out in the present study, it was conceivable that a similar activation could also be promoted in guinea pig myometrium by carbachol (33) and the calcium ionophore. Nevertheless, the Ca^{2+} dependence for cyclic GMP responses induced by carbachol, high K^+, and A 23187 could not be explained exclusively by an effect of this cation on phospholipase activity and the release of arachidonic or other fatty acids. Indeed, the fatty acid as well as the fatty acid hydroperoxide stimulations in terms of cyclic GMP also require the presence of Ca^{2+} in the medium, suggesting an additional role for Ca^{2+} beyond the fatty acid release and peroxidation steps. Our previous studies (16, 30) have shown the occurrence in rat myometrium of local synthesis of prostaglandin material from both exogenous and endogenous arachidonic acid, a result consistent with a normal expression of phospholipase A_2 activity linked to the oxidative metabolism of arachidonic acid at least via the cyclooxygenase route. The latter observation, added to the fact that not only carbachol but also fatty acids and their hydroperoxides similarly failed to stimulate cyclic GMP levels in rat myometrium, would tend to imply that the absence of Ca^{2+}-mediated stimulatory effects on cyclic GMP levels in that particular tissue was most probably due to a defective, yet undertermined, step beyond the putative phospholipase and lipoxygenase reactions.

A comparison between the respective modulations of guanylate cyclase activities in rat and guinea pig myometrial extracts suggested interesting relationships between enzyme activation and cyclic GMP accumulation in the intact cell. It appeared that, for both species, the stimulatory effect of SNP on the soluble guanylate cyclase form, which presumably occurred via the oxidative-reductive NO pathway (2, 8, 9), may readily account for the SNP-induced enhancement of cyclic GMP levels demonstrated in the intact tissue. We have previously proposed (7) and herein confirmed that the particulate guanylate cyclase of both rat and guinea pig myometrium is susceptible to activation by fatty acids as well as their hydroperoxy and hydroxy derivatives. The latter fatty acid activation process did not require an oxidative metabolism via the cyclooxygenase or lipoxygenase pathway and appeared to be the result of hydrophobic interactions, similar to the activation which could also be promoted by Triton X-100. Simulations involving lipid-protein interactions have already been reported for membrane-associated guanylate cyclase activities of different cellular systems (1, 2). Nonetheless, the implication of such a mechanism in the modulation of cyclic GMP levels in intact tissue has not yet been established. Our present findings are not compatible with its involvement in regulation of the cyclic GMP content of rat and guinea pig myometrium, at least under the foregoing experimental conditions. Indeed, fatty acids which activate the particulate guanylate cyclase of rat myometrium were totally ineffective in raising cyclic GMP concentrations in intact tissue. Moreover, saturated fatty acids which enhance activity of particulate, but not soluble, guanylate cyclase of guinea-pig myometrium were similarly without effect on the cyclic GMP content of intact tissue. On the other hand, several lines of evidence seem to indicate that the arachidonic and linoleic acid-induced elevations of cyclic GMP levels demonstrated in guinea pig myometrium involved a distinct fatty acid-promoted activation process at the level of the soluble guanylate cyclase fraction.

As shown in the present study, unsaturated fatty acids also caused the stimulation of the soluble guanylate cyclase activity in disrupted guinea pig myometrial tissue. Hydroperoxy derivatives of fatty acids were consistently more effective while the hydroxy compounds were inactive. Fatty acid-induced stimulations were abolished in the presence of lipoxygenase inhibitors but not cyclooxygenase inhibitors and were prevented by reducing agents and free radical scavengers. Thus our combined observations favor the interpretation that fatty acid activation of the crude soluble guanylate cyclase fraction was a result of oxidation (11, 12) via the generation of fatty acid hydroperoxides and/or other reactive oxygen species. This mechanism appears operational in intact guinea pig myometrium, where it mediates, via endogenous fatty acids and ultimately fatty acid peroxides, the cyclic GMP responses evoked by the cholinergic agonist and ionophore A 23187. The insensitivity of the soluble guanylate cyclase of rat myometrium to activation by the lipid peroxide-promoted process could thus explain the failure of carbachol and other Ca^{2+}-requiring effectors to enhance the cyclic GMP level in intact tissue. Studies with a purified enzyme preparation are necessary to clarify such an apparent species variation in the cyclic GMP-forming system.

ACKNOWLEDGMENTS

We wish to thank Mrs. J. Robichon and Mrs. G. Thomas for excellent technical assistance, and Mrs. J. Jalicot and Mrs. M. G. Guyon for preparing the manuscript.

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