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EFFECT OF A PROSTAGLANDIN ANTAGONIST, N-0164, ON cAMP GENERATION AND HYDROLYSIS IN THE RAT UTERUS*

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Abstract—N-0164 (sodium-*p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenyl propyl] phenylphosphonate) (20–100 μM), an antagonist of the contractile effect of prostaglandins, reversed the prostaglandin E_2 (PGE_2) inhibition of isoproterenol-induced cAMP accumulation in rat uterus. N-0164, at the same concentrations, was a potent cAMP-phosphodiesterase inhibitor in broken cell preparations and potentiated the cAMP response to isoproterenol in intact tissue. The potency of N-0164 to inhibit cAMP-phosphodiesterase and to reverse the effect of PGE_2 on the cAMP response to isoproterenol were comparable (EC_{50}: 50 and 60 μM respectively). In the presence of 10 mM theophylline, N-0164 did not affect the inhibitory effect of PGE_2. Furthermore, N-0164 produced similar proportional increases in the cAMP response to isoproterenol in the presence and absence of PGE_2. These results suggest that the apparent reversal by N-0164 of the PGE_2 effect on the cAMP response to isoproterenol is not due to its prostaglandin antagonistic action but to inhibition of cAMP-phosphodiesterase. N-0164, at concentrations lower than those inhibiting cAMP-phosphodiesterase, selectively inhibited the PGE_2-induced contractions of the rat uterus (EC_{50}, 4 μM), while at higher concentrations it also diminished carbachol-induced contractions. These results indicate that in the rat uterus N-0164 has at least two effects, prostaglandin antagonism and cAMP-phosphodiesterase inhibition, and suggest that the contractile effect of PGE_2 is independent of the effect of PGE_2 on the isoproterenol-induced rise in cAMP.

Prostaglandins of the E and F series have been shown previously to produce a dose-dependent inhibition of the catecholamine-induced increase of cAMP in rat uterus [1–3], which was thought to be related to the contractile effect of these prostaglandins [1]. If this were the case, a specific antagonist of the contractile effect of prostaglandins such as N-0164,‡ a phenylphosphonate derivative of phloretin phosphate, would be expected to antagonize the inhibitory effects of these prostaglandins on catecholamine-induced cAMP accumulation in the rat uterus. The results of this study show that N-0164 is both a potent inhibitor of cAMP-phosphodiesterase and a selective inhibitor of the contractile effect of PGE_2 in rat uterus, and they suggest that the effect of PGE_2 on the cAMP response to β-adrenergic stimulation is not related to its contractile activity.

METHODS

Virgin female rats weighing 180–200 g were pretreated with diethylstilbestrol (60 μg/100 g) and decapitated the following day. The uterus was excised and dissected free of fat and connective tissue in ice-cold saline.

Cyclic AMP was determined in uterine segments preincubated at 37° for 60 min in Krebs–Ringer bicarbonate (KRB) medium, containing 10 mM glucose and equilibrated with a gas mixture of 95% O_2 and 5% CO_2 to maintain pH at 7.5. The uterine segments were then incubated with the test drugs in fresh medium under the same conditions. The tissues were exposed to isoproterenol and PGE_2 for 5 min and to N-0164 for 20 min. At the end of the incubation, the tissue was immediately frozen in Freon 12 and homogenized in ice-cold 5% trichloroacetic acid. After centrifugation, the trichloroacetic acid in the supernatant fraction was extracted with water-saturated ether, and cAMP was determined in aliquots of the supernatant fraction by the protein kinase binding assay of Gilman [4]. Protein content was measured in the precipitate by the method of Lowry *et al.* [5]. Protein kinase and the heat stable inhibitor were prepared from beef skeletal muscle according to the method of Gilman [4]. Reagent blanks and appropriate standards were carried through the entire procedure. Determinations were made in triplicate, and the results are expressed in pmoles cAMP/mg protein. At least four experiments of each type were performed.

For the enzyme assays, the uterine segments were homogenized in ice-cold 50 mM Tris–HCl (pH 7.5), in an all-glass homogenizer. Phosphodiesterase activity was measured in the 500 *g* supernatant fraction from tissue homogenates by the two-step method of Thompson and Appleman [6]; cAMP, labeled with [^{3}H]cAMP, is first hydrolyzed by phosphodiesterase to 5′-AMP, which is then converted quantitatively to adenosine by an excess of 5′-nucleotidase from snake venom; adenosine is separated by ion-exchange column chromatography and

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‡ Sodium-*p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenyl propyl] phenylphosphate.

radioactivity counted in a liquid scintillation spectrometer. The assay mixture, in a total volume of 400 μl/assay, consisted of 0.4 μM cAMP labeled with about 200,000 cpm [^{3}H]cAMP (30–50 Ci/mmole), 5 mM $MgCl_2$, 3.75 mM mercaptoethanol, 50 mM Tris–HCl (pH 7.5), 100 μl of enzyme preparation (100–200 μg protein), and the test drugs when indicated. Blank values were obtained by substituting the same volume of Tris–HCl for the enzyme preparation; similar blank values were obtained using a heat-denatured enzyme. Under the conditions of the experiment, the enzyme activity was linear with time and protein concentration. Determinations were made in triplicate. The enzyme activity was calculated from the specific activity of the substrate, corrected for recovery of adenosine [7], and expressed in pmoles cAMP hydrolyzed · (mg protein)$^{-1}$ · 5 min^{-1}.

Adenylate cyclase activity was assayed in whole tissue homogenates by the method of Krishna *et al.* [8] in which ATP labeled with ^{32}P is enzymatically and stoichiometrically converted to cAMP in the presence of Mg^{2+}. An ATP regenerating system (phosphoenolpyruvate and pyruvate kinase) was added to the assay mixture to maintain a constant substrate concentration, and an excess of unlabeled cAMP to minimize the breakdown of the [^{32}P]-cAMP by phosphodiesterase and for the determination of recovery. The assay mixture (300 μl/test tube) consisted of 1 mM ATP, labeled with 3 to 3.5 μCi [α-^{32}P]ATP (2–20 Ci/mmole), 3 mM $MgSO_4$, 3.3 mM phosphoenolpyruvate, 15 units/ml pyruvate kinase, 5 mM cAMP, 50 mM Tris–HCl (pH 7.5), 150 μl of tissue homogenate (400–500 μg protein) and the test drugs as indicated. A reagent blank using heat-denatured enzyme was processed with each experiment. Determinations were made in duplicate. The enzyme activity, which was linear with time and protein concentration under the assay conditions, was calculated from the specific activity of the substrate, corrected for recovery and expressed in pmoles cAMP formed · (mg protein)$^{-1}$ · 5 min^{-1}.

Segments of uterine horn were suspended under 1.0 *g* tension in Krebs–Ringer bicarbonate medium containing 10 mM glucose maintained at 37° and at pH 7.5 with a mixture of 95% O_2 and 5% CO_2 constantly bubbled through the medium. Tension was measured isometrically with a Statham force-displacement transducer and recorded on a Grass polygraph. The tissue was allowed to equilibrate until a stable response to an agonist was obtained. Uterine segments contracting spontaneously were discarded. Contractions were induced by PGE_2 or carbachol at concentrations of the agonists producing 50–60 per cent of maximum contraction. N-0164 was added to the bath 15 min before the agonist. The decrease in tension induced by N-0164 is expressed as the percentage of the control PGE_2 or carbachol-induced contractions.

Statistics. Statistical significance of the results, at the 95 per cent confidence limit, was evaluated by Student's *t*-test.

Material. [^{3}H]cAMP was obtained from the New England Nuclear Corp. (Boston, MA); [^{32}P]ATP from ICN Pharmaceuticals, Inc. (Cleveland, OH); *dl*-isoproterenol–HCl and snake venom 5′-nucleotidase, from the Sigma Chemical Co. (St. Louis, MO); cAMP and theophylline from CalBiochem (San Diego, CA); and ATP, pyruvate kinase and phosphoenolpyruvate from Boehringer-Mannheim (Indianapolis, IN). PGE_2 was a gift from The Upjohn Co. (Kalamazoo, MI); N-0164 was supplied by the Nelson Research & Development Corp. (Irvine, CA); and resin AG 1-X2 (chloride form) and Dowex AG 50 W-X4 (hydrogen form) by Bio-Rad Laboratories (Richmond, CA). All other chemicals were reagent grade.

RESULTS

PGE_2 (2.8 μM) diminished the isoproterenol (5 μM)-induced cAMP rise by 56 per cent (from 160.4 ± 16.2 to 70.5 ± 2.4 pmoles cAMP/mg protein, $P < 0.005$) without altering the control level of cAMP of the uterus (Fig. 1). With increasing concentrations of N-0164, which did not change the control cAMP level, the inhibitory effect of PGE_2 was gradually diminished (EC_{50}. 60 μM), suggesting that N-0164 antagonized the effect of PGE_2. At the highest concentration of N-0164 tested (100 μM), the cAMP response to isoproterenol in the presence of PGE_2 was even higher than that of isoproterenol alone (+38 per cent, $P < 0.01$, Fig. 1), suggesting another mechanism of action of N-0164, such as an interaction with one of the enzymes metabolizing cAMP. Furthermore, N-0164 (20–100 μM) markedly increased, in a dose-dependent manner, the cAMP response to isoproterenol (a 4-fold increase with 100 μM N-0164, $P < 0.02$, Fig. 2). This potentiation of the cAMP response to a β-adrenergic agonist by N-0164, without a significant change of the basal level of cAMP, indicated the possibility of an inhibitory action on cAMP-phosphodiesterase.

Indeed, the rate of cAMP hydrolysis was strongly inhibited by N-0164 in a dose-dependent manner, with an EC_{50} value of 50 μM (Fig. 3). When compared to theophylline, N-0164 appears to have been about a five times stronger inhibitor of phosphodiesterase, the EC_{50} values being 250 and 50 μM respectively (Fig. 3). On the other hand, N-0164 (5–100 μM) did not alter the activity of basal or isoproterenol-stimulated adenylate cyclase. Furthermore, in the presence of a cAMP-phosphodiesterase inhibitor, N-0164 did not alter the inhibitory effect of PGE_2. When uterine pieces were incubated in KRB containing 10 mM theophylline, PGE_2, 2.8 μM, inhibited the isoproterenol (5 μM)-induced rise in cAMP by 42.0 ± 6.4 and 44.4 ± 9.1 per cent in the absence or presence of 50 μM N-0164 respectively.

The effects of N-0164 on the isoproterenol-induced rise in uterine cAMP content in the absence and presence of PGE_2, 2.8 μM, are compared in Fig. 4. The effect of N-0164 is expressed as per cent increase over the cAMP response to isoproterenol alone or isoproterenol plus PGE_2. In both instances, N-0164, at all concentrations tested, produced identical proportional increases in the cAMP response, indicating that its effect was independent of the effect of PGE_2. The results thus indicate that the reversal of the PGE_2 effect on isoproterenol-induced increase in cAMP content by N-0164 is not due to an antagonism

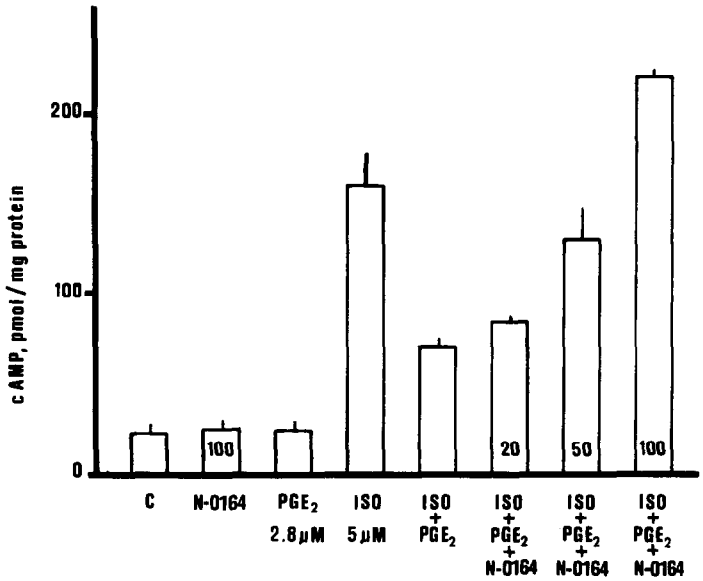

Fig. 1. Effect of PGE_2 on isoproterenol-induced rise in cAMP in the absence and presence of N-0164 in rat uterus. Bars represent means ±S.E.M. of triplicate determinations from one representative experiment. Numbers inside the bars indicate the concentration of N-0164 (μM).

of PGE_2 but to the inhibitory effect of N-0164 on cAMP phosphodiesterase.

Figure 5 shows that N-0164 was a more potent inhibitor of the contractile response of the isolated rat uterus to PGE_2 than to carbachol; while the response to PGE_2 was completely prevented by 10 μM N-0164, the carbachol effect was decreased by only 25 per cent. The concentration of N-0164 required to inhibit the contractile response to PGE_2 was lower (1–10 μM; EC_{50}, 4 μM) than that needed to abolish the PGE_2 effect on isoproterenol-induced rise in cAMP (EC_{50}, 60 μM).

DISCUSSION

N-0164 has been shown to be a potent, relatively specific, inhibitor of the contractile effect of prostaglandins E_2 and $F_{2\alpha}$ on preparations of gerbil colon, guinea pig ileum and rat fundus as well as of the effect of prostaglandin G_2 on platelet aggregation, exhibiting some of the characteristics of a competitive antagonist [9, 10]. N-0164 therefore appeared to be a useful tool to evalute whether the decreased cAMP response to β-adrenergic stimulation produced by PGE_2 could be related to the contractile effect of PGE_2 in rat uterus, as suggested previously [1].

The results of the study show that N-0164 diminished and abolished both the contractile and the cAMP-lowering effects of PGE_2. As an inhibitor of the contractile response to PGE_2 in uterine muscle,

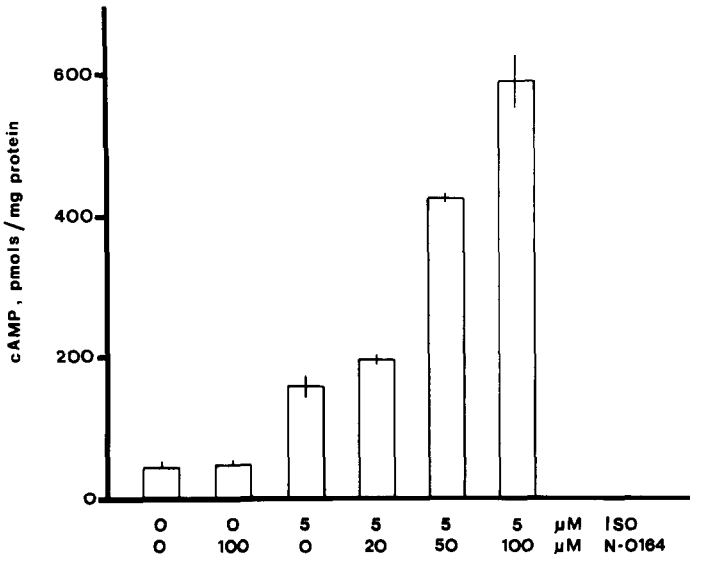

Fig. 2. Effect of N-0164 on isoproterenol-induced rise in cAMP in rat uterus. Bars represent means ±S.E.M. of triplicate determinations from one representative experiment.

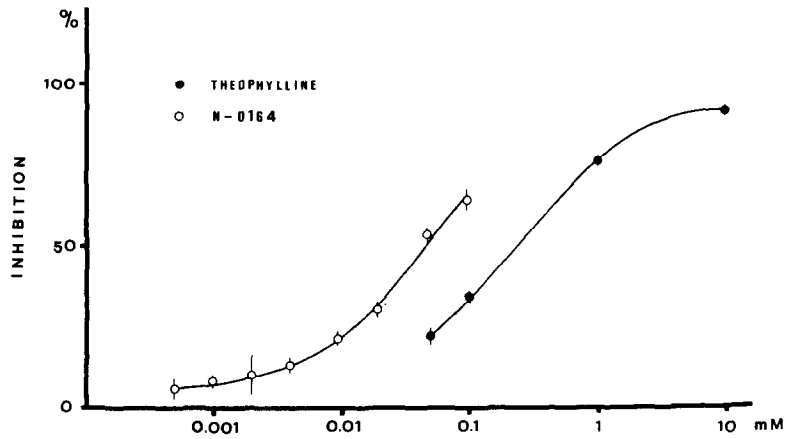

Fig. 3. Effect of N-0164 on cyclic AMP hydrolysis in uterine tissue homogenates. Phosphodiesterase activity was determined at a 0.4 μM substrate concentration. Points are mean values ±S.E.M. from four experiments.

N-0164 exhibited a certain specificity of action. N-0164 was a more potent inhibitor of the PGE_2-induced contraction than of the carbachol-induced contraction, and its potency to inhibit the PGE_2 contractile effect in uterine muscle (EC_{50}, 4 μM) was comparable to that found in other smooth muscle preparations [9]. In contrast, the potency of N-0164 to abolish PGE_2 effect on the isoproterenol-induced cAMP response was considerably less (EC_{50}, 60 μM). Furthermore, at the highest concentration of N-0164 tested, the PGE_2 inhibitory effect on the isoproterenol-induced cAMP response was not only abolished but the cAMP response to isoproterenol was even increased, suggesting another mechanism of action than PGE_2 antagonism. Inhibition of cAMP-phosphodiesterase was indicated by the potentiation of the isoproterenol-induced cAMP response in the presence of N-0164 and by the observation that, when cAMP-phosphodiesterase was inhibited by theophylline, N-0164 did not affect the inhibitory effect of PGE_2. The potency of N-0164 to inhibit cAMP phosphodiesterase (EC_{50}, 50 μM) is comparable to that reversing the cAMP-lowering effect of PGE_2 (EC_{50}, 60 μM) and about ten times higher than that inhibiting the contractile effect of PGE_2 (4 μM). In addition, the effect of N-0164 on the cAMP response to isoproterenol was identical in the presence and absence of PGE_2. Thus, the reversal of the PGE_2 effect on catecholamine-induced rise in cAMP by N-0164 was probably due to its cAMP-phosphodiesterase inhibitory property and not to a PGE_2 antagonistic effect. The results therefore indicate that the PGE_2 inhibition of the isoproterenol-induced increase in cAMP and the PGE_2-induced contraction in the rat uterus are not related.

It is possible that the inhibition of cAMP-phosphodiesterase by N-0164 accounted for the decreased

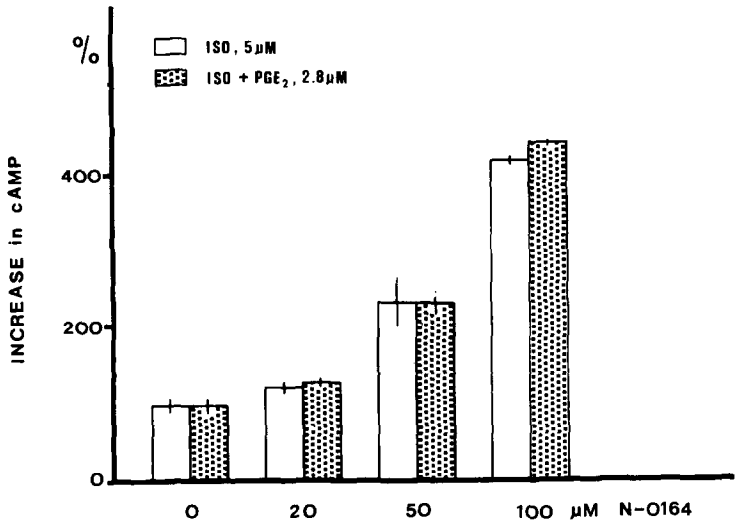

Fig. 4. Effect of N-0164 on isoproterenol-induced increase in cAMP in the absence and presence of PGE_2. The effect of N-0164 is expressed as percentage of the cAMP response to isoproterenol alone (133.7 ± 16.2 pmoles cAMP/mg protein) or isoproterenol + PGE_2 (43.8 ± 2.4 pmoles cAMP/mg protein) respectively. Bars are means ± S.E.M. of triplicate determinations from one representative experiment.

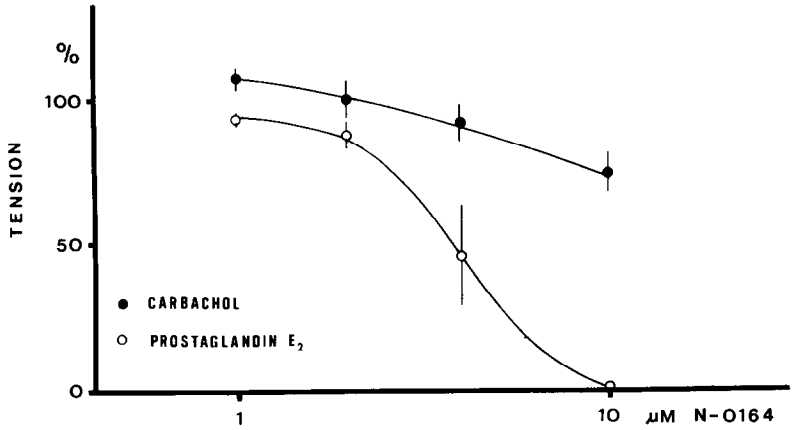

Fig. 5. Effect of N-0164 on PGE_2- and carbachol-induced contractions of rat uterine strip. Points are means ± S.E.M. of four experiments.

contractile response to carbachol observed in the presence of N-0164, since the concentrations of N-0164 needed to inhibit phosphodiesterase and the carbachol-induced contractile response are in the same range (10 μM N-0164 will inhibit both effects by about 25 per cent). Other non-specific effects of N-0164, such as inhibition of the contractile response to acetylcholine, angiotensin, serotonin or potassium ions [9], might be also accounted for by the inhibitory effect of N-0164 on cAMP-phosphodiesterase. These effects of N-0164 imply an intracellular action of N-0164. In platelets, however, the disparity between the effects of N-0164 on thromboxane synthesis in intact and broken cell preparations and between the effects of N-0164 on thromboxane synthesis and aggregation suggested that N-0164 does not penetrate the platelet cell membrane [10, 11]. However, the good correlation between inhibition of cAMP-phosphodiesterase, antagonism of the carbachol-induced contractions, and potentiation of the cAMP response to isoproterenol by N-0164 indicates that N-0164 does have an effect on cAMP-phosphodiesterase in the intact cell.

The fact that N-0164 has these two actions, prostaglandin antagonism and cAMP-phosphodiesterase inhibition, is not unique to this compound. A wide variety of compounds that either antagonize prostaglandin action or inhibit its synthesis—indomethacin, aspirin and flufenamic acid—have been shown to also inhibit cAMP-phosphodiesterase [12, 13]. In addition to their effects on cAMP-phosphodiesterase, both N-0164 and these compounds modify the activities of other enzymes, most of which are membrane-bound, such as prostaglandin cyclooxygenase, prostaglandin dehydrogenase, thromboxane synthetase, adenylate cyclase, guanylate cyclase and protein kinases [9, 11, 12, 14]. It is possible then that such compounds alter the activities of these enzymes through a common mechanism, such as an interaction with calmodulin, a calcium-dependent modulator protein [15]. It is known that all these enzymes are calcium,dependent and that most of them are regulated by calmodulin [15].

The recent findings of Gorman *et al.* [16] are of interest in relation to our present findings. Using a calcium antagonist, these investigators have been able to dissociate the inhibition of prostacyclin-induced cAMP accumulation by thromboxane A_2 from platelet functions induced by thromboxane A_2, the ADP and serotonin release reaction and aggregation. Since thromboxane A_2 has been shown to mobilize calcium ions, they postulated that both effects of thromboxane A_2, the aggregation-release reaction and the lowering of cAMP, would be regulated by the increase in calcium ions, which would initiate platelet aggregation and secretion, and inhibit adenylate cyclase. This hypothesis could also serve to explain the two effects of PGE_2 on uterus. It has been demonstrated that PGE_2 inhibits ATP-dependent calcium binding and enhances calcium release from sarcoplasmic reticulum in uterus, effects associated with the contractile properties of PGE_2 [17]. The resulting increased availability of calcium ions could thus account for the PGE_2 inhibition of isoproterenol-induced increase in cAMP, since calcium can inhibit uterine adenylate cyclase.*

The results of this study show that N-0164 is a potent inhibitor of cAMP-phosphodiesterase and that it selectively inhibits the contractile effect of PGE_2 in rat uterus. Reversal of the PGE_2 inhibition of isoproterenol-induced increase in cAMP by N-0164 is due to its inhibitory effect on cAMP-phosphodiesterase and not to an antagonism of PGE_2, suggesting that the PGE_2-inhibitory action of the cAMP response to isoproterenol is not related to the contractile effect of PGE_2 in rat uterus.

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* Unpublished observation.

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