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# Prostaglandins and muscarinic agonists induce cyclic AMP attenuation by two distinct mechanisms in the pregnant-rat myometrium

## Interaction between cyclic AMP and $Ca^{2+}$ signals

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In pregnant-rat myometrium (day 21 of gestation), isoprenaline-induced cyclic AMP accumulation, resulting from receptor-mediated activation of adenylate cyclase, was negatively regulated by prostaglandins [$PGE_2$, $PGF_{2\alpha}$; $EC_{50}$ (concn. giving 50% of maximal response) = 2 nM] and by the muscarinic agonist carbachol ($EC_{50}$ = 2 $\mu$M). PG-induced inhibition was prevented by pertussis-toxin treatment, supporting the idea that it was mediated by the inhibitory G-protein $G_i$ through the inhibitory pathway of the adenylate cyclase. Both isoprenaline-induced stimulation and PG-evoked inhibition of cyclic AMP were insensitive to $Ca^{2+}$ depletion. By contrast, carbachol-evoked attenuation of cyclic AMP accumulation was dependent on $Ca^{2+}$ and was insensitive to pertussis toxin. The inhibitory effect of carbachol was mimicked by ionomycin. Indirect evidence was thus provided for the enhancement of cyclic AMP degradation by a $Ca^{2+}$-dependent phosphodiesterase activity in the muscarinic-mediated effect. The attenuation of cyclic AMP elicited by carbachol coincided with carbachol-stimulated inositol phosphate ($InsP_3$, $InsP_2$ and $InsP$) generation, which displayed an almost identical $EC_{50}$ (3 $\mu$M) and was similarly unaffected by pertussis toxin. Both carbachol effects were reproduced by oxotremorine, whereas pilocarpine (a partial muscarinic agonist) failed to induce any decrease in cyclic AMP accumulation and concurrently was unable to stimulate the generation of inositol phosphates. These data support our proposal for a carbachol-mediated enhancement of a $Ca^{2+}$-dependent phosphodiesterase activity, compatible with the rises in $Ca^{2+}$ associated with muscarinic-induced increased generation of inositol phosphates. They further illustrate that a cross-talk between the two major transmembrane signalling systems contributed to an ultimate decrease in cyclic AMP in the pregnant-rat myometrium near term.

## INTRODUCTION

In the myometrium, as in many other smooth-muscle preparations, $Ca^{2+}$ and cyclic AMP, the two major intracellular second messengers, exert opposite effects at the level of contractility. A rise in intracellular $Ca^{2+}$ is stringently required to initiate contraction [1,2], whereas increases in cyclic AMP content have been demonstrated to contribute to uterine relaxation [3,4]. The increase in intracellular $Ca^{2+}$ evoked by stimulatory agonists is considered to originate, at least in part, from intracellular stores [5,6]. There is compelling evidence which indicates that $Ca^{2+}$-mobilizing receptors affect intracellular $Ca^{2+}$ levels via hydrolysis of PtdIns(4,5)$P_2$, with the generation of Ins(1,4,5)$P_3$ resulting in an initial release of internal $Ca^{2+}$ that is soon followed by an entry of $Ca^{2+}$ across the plasma membrane [7,8]. It has recently been demonstrated that the phosphoinositide-phospholipase C transducing mechanism can indeed be activated by contracting agonists, namely oxytocin and carbachol, in different myometrial preparations [9–11]. A number of reported findings further provide satisfactory correlations between the increased generation of inositol phosphates, the ability of Ins(1,4,5)$P_3$ to release $Ca^{2+}$ from intracellular sites [12,13] and the accompanying $Ca^{2+}$-induced uterine contractions [10,13,14].

The major pathway that contributes to the modulation of intracellular cyclic AMP, i.e. the adenylate cyclase cyclic AMP-generating system [15], has been under investigation in our laboratory for several years. It has been shown that both intracellular cyclic AMP levels and adenylate cyclase activity were enhanced by way of receptor activation ($\beta$-adrenergic, prostacylin) via the stimulatory regulatory protein $G_s$ [16–20]. More recently, it was demonstrated that the inhibitory arm of the adenylate cyclase, a pertussis-toxin-sensitive pathway regulated by the inhibitory protein $G_i$ [15], was expressed in the oestrogen-treated guinea pig [14,21] and pregnant-rat [19] myometrium, through activation of muscarinic (M2 subtype) and prostaglandin ($PGE_2$, $PGF_{2\alpha}$) receptors respectively. In this regard the characterization of $G_{i2}\alpha$ as the major $G_i\alpha$ species present in the rat myometrium is worth mentioning [22].

Beside the inhibitory pathway operating at the level of cyclic AMP synthesis, the concentration of cyclic AMP can also be negatively regulated by way of its degradation via a phosphodiesterase activity [23]. Multiple forms of cyclic nucleotide phosphodiesterases have been described, differing at least in substrate specificity and/or $Ca^{2+}$ sensitivity. Hormonal regulation of cyclic AMP degradation, mainly through activation of $Ca^{2+}$-mobilizing receptors, has been documented in a number of cells [23,24].

In the present paper, data are reported demonstrating that in the pregnant-rat myometrium (day 21 of gestation), the increases in cyclic AMP caused by the relaxing agonist isoprenaline

Abbreviations used: PG, prostaglandin; IBMX, 3-isobutyl-1-methylxanthine; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methobromide; AF-DX116, 11-({2-[(diethylamino)methyl]-1-piperidinyl}acetyl)-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one; $G_s$ and $G_i$, stimulatory and inhibitory guanine nucleotide regulatory proteins of the adenylate cyclase system; $G_s\alpha$, $G_i\alpha$, their $\alpha$ subunit; $EC_{50}$, concn. giving 50% of maximal response.

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(isoproterenol) may be negatively modulated by contracting agents, namely PGs and carbachol acting through two distinct mechanisms. PGs mediated attenuation of cyclic AMP via the inhibitory pathway of the cyclic AMP generating system. On the other hand, the carbachol-attenuated effect, which was $Ca^{2+}$-dependent, operated at the level of cyclic AMP degradation, consecutive to a sequence of events initiated by muscarinic-receptor stimulation of inositol phosphate generation, a pertussis-toxin-insensitive process.

## MATERIALS AND METHODS

### Materials

L-Isoprenaline bitartrate, LiCl, atropine, carbamoylcholine chloride (carbachol), pilocarpine hydrochloride and oxotremorine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). $PGE_2$ and $PGF_{2\alpha}$ were from the Upjohn Co. (Kalamazoo, MI, U.S.A.). L-Propranolol hydrochloride was a gift from I.C.I. Ltd (Macclesfield, Cheshire, U.K.). IBMX was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), ionomycin from Calbiochem (Los Angeles, CA, U.S.A.), and pertussis toxin from List Biological Laboratories (Campbell, CA, U.S.A.). *myo*-[2-$^3$H]Inositol (10–20 Ci/mmol) was from Amersham International (Amersham, Bucks., U.K.), and cyclic [$^3$H]AMP (30–50 Ci/mmol) from NEN Products Division (Dupont de Nemours, Paris, France). 4-DAMP was generously given by Dr. R. Barlow (Medical School, University of Bristol, Bristol, U.K.), and AF-DX116 was obtained from Dr. Thomae G.m.b.h. (Biberbach, Germany). Other chemicals were of the highest grade commercially available.

### Animals and tissue processing

Pregnant rats (Wistar) were used at day 21 of gestation, near term. The period of gestation was calculated from the single day that the male was caged with the female rat. Rats were killed by decapitation. Their uteri were removed immediately and immersed in Krebs–Ringer bicarbonate buffer (pH 7.4) at 4 °C. The uterine horns were quickly cleared of adhering fat and excised out longitudinally. After removal of fetuses, their sites of attachment and placenta tissues, myometrium was prepared free of endometrium essentially as described previously [19].

### Incubation experiments for assay of cyclic AMP levels

Myometrial strips were equilibrated at 37 °C during 25 min in 5 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) containing 117 mM-NaCl, 4.7 mM-KCl, 1.1 mM-$MgSO_4$, 1.1 mM-$KH_2PO_4$, 24.7 mM-$NaHCO_3$, 2.4 mM-$CaCl_2$ and 1 mM-glucose (gas phase, $O_2/CO_2$ 19:1) under constant agitation. The strips were then transferred into 1 ml of fresh buffer and further allowed to equilibrate for 25 min at 37 °C. After the addition of IBMX (final concn. 240 $\mu$M), incubations were continued in the presence of the different agents for the times indicated under the specific experiments. The reactions were stopped by immersing the tissues in 1 ml of cold 7% (w/v) trichloroacetic acid, followed by homogenization and centrifugation at 10000 *g* for 15 min. Cyclic AMP was measured in the trichloroacetic acid-soluble extract, in accordance with Gilman [25] as described previously [16]. The centrifuged pellets were dissolved in 1 M-NaOH for protein determination [26]. Cyclic AMP levels were expressed as pmol/mg of protein.

### Measurement of [$^3$H]inositol phosphates

Incubations were carried out at 37 °C in the same Krebs–Ringer buffer as was used for cyclic AMP experiments. Myometrial strips (about 50 mg) were allowed to equilibrate in 5 ml of buffer for 25 min and subsequently incubated for $3\frac{1}{2}$ h with 5 $\mu$Ci of *myo*-[2-$^3$H]inositol (0.4 $\mu$M) in 1 ml of fresh buffer, by which time the incorporation of $^3$H into inositol lipids had reached a plateau [9]. Tissues were washed with 3 × 10 ml of non-radioactive Krebs–Ringer buffer and then transferred into 1 ml of fresh buffer during 20 min before the addition of 10 mM-LiCl. After 10 min, the muscarinic agonist to be tested was added at the indicated concentrations, and incubation was further continued for the time indicated in the specific experiments. Reactions were stopped by immersing the myometrial strips in 1.5 ml of cold 7% (w/v) trichloroacetic acid, followed by homogenization and centrifugation at 10000 *g* for 15 min at 4 °C. The trichloroacetic acid supernatants were extracted with 4 × 6 ml of diethyl ether, neutralized with Tris base and applied to a column of anion-exchange resin (AG1-X8: formate form, 200–400 mesh) for separation of the different inositol phosphates as described previously [27]. Free inositol, glycerophosphoinositol, Ins*P*, Ins$P_2$ and Ins$P_3$ were successively eluted with 10 ml of water, 10 ml of 60 mM-ammonium formate/5 mM-sodium tetraborate, 10 ml of 0.2 M-ammonium formate/0.1 M-formic acid, 12 ml of 0.5 M-ammonium formate/0.1 M-formic acid and 6 ml of 1 M-ammonium formate/0.1 M-formic acid. Alternatively, total inositol phosphates (Ins$P_3$ + Ins$P_2$ + Ins*P*) were eluted together in a single step with 12 ml of 1 M-ammonium formate/0.1 M-formic acid. The $^3$H content of the fractions was determined by scintillation counting of 1.4 ml samples in Instagel (Packard). Results were expressed as c.p.m./100 mg of tissue, or alternatively as the percentage stimulation over the basal value obtained before addition of the stimulatory agonist. We verified (results not shown) that 240 $\mu$M-IBMX, which was present only in the cyclic AMP incubations, did not modify the inositol phosphate response induced by carbachol. We similarly checked that the presence of 10 mM-LiCl, which was added routinely in the inositol phosphate incubations, had no effect on the muscarinic cyclic AMP responses.

## RESULTS AND DISCUSSION

### Stimulation and inhibition of intracellular cyclic AMP in the pregnant-rat myometrium

Fig. 1 shows the kinetics for cyclic AMP accumulation in myometrial strips incubated with a maximal effective concentration (0.3 $\mu$M) of isoprenaline in the presence of 240 $\mu$M-IBMX, a phosphodiesterase inhibitor. A peak response was obtained within 5 min, with a plateau that remained sustained for at least 15 min. IBMX at 240 $\mu$M alone also enhanced cyclic AMP accumulation and markedly potentiated the increase in cyclic AMP caused by isoprenaline: cyclic AMP values under basal conditions and after 7 min stimulation with isoprenaline were $5 \pm 1$ and $45 \pm 5$ pmol/mg of protein respectively in the absence of IBMX and $10 \pm 2$ and $180 \pm 15$ pmol/mg of protein respectively in the presence of IBMX. Data in Fig. 1 further show that the simultaneous addition of isoprenaline and 50 nM-$PGE_2$ in the presence of IBMX resulted in a rapid and substantial attenuation of the $\beta$-adrenergic cyclic AMP response. A similar inhibitory pattern could also be obtained when isoprenaline was added in combination with carbachol (50 $\mu$M). Attenuation of cyclic AMP accumulation caused by both agonists was not restricted to the $\beta$-adrenergic stimulatory response, but was also observed when forskolin was the stimulatory agent. Cyclic AMP values were $575 \pm 47$ pmol/mg of protein with 10 $\mu$M-forskolin alone, compared with $380 \pm 20$ and $340 \pm 17$ pmol/mg of protein when forskolin was added simultaneously with 50 nM-$PGE_2$ and 50 $\mu$M-carbachol respectively.

Data in Fig. 2 show that $PGE_2$ and $PGF_{2\alpha}$ displayed similar

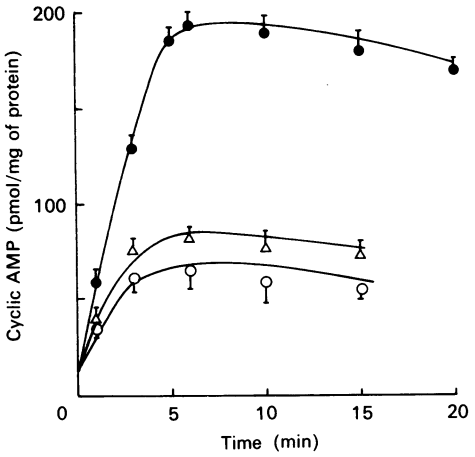


**Fig. 1. Time course of isoprenaline-induced cyclic AMP accumulation in the pregnant-rat myometrium: inhibitory effects of $PGE_2$ and carbachol**

Myometrial strips were incubated for 25 min in a Krebs bicarbonate buffer at 37 °C. After 5 min treatment with 240 $\mu$M-IBMX, incubations were continued for the indicated times with 0.3 $\mu$M-isoprenaline alone (●) or combined with 50 nM-$PGE_2$ (○) or 50 $\mu$M-carbachol (△). Cyclic AMP was measured as described in the Materials and methods section. Values represent means ± S.E.M. for four independent experiments.

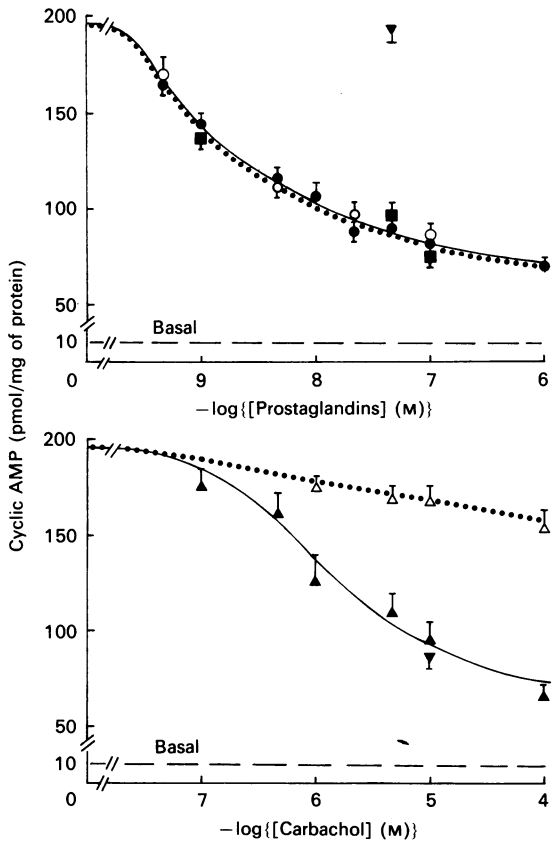


**Fig. 2. Concentration-dependent inhibition induced by prostaglandins ($PGE_2$, $PGF_{2\alpha}$) and carbachol on cyclic AMP accumulation caused by isoprenaline: differential effects of $Ca^{2+}$ and pertussis toxin**

After the 25 min incubation in Krebs–Ringer bicarbonate buffer, myometrial strips were further incubated for 15 min in a normal (2.4 mM-$Ca^{2+}$) Krebs–Ringer bicarbonate buffer (black symbols) or in a $Ca^{2+}$-deprived medium containing 1 mM-EGTA (white symbols). After 5 min treatment with 240 $\mu$M-IBMX, incubations were continued for 7 min with 0.3 $\mu$M-isoprenaline added alone or combined with the indicated concentration of carbachol (△, ▲), $PGE_2$ (○, ●) or $PGF_{2\alpha}$ (■). When indicated (▼), myometrial strips were incubated for 6 h in the presence of pertussis toxin (200 ng/ml). Tissues were then washed and re-challenge incubations were performed in the presence of 0.3 $\mu$M-isoprenaline added simultaneously with 10 $\mu$M-carbachol or 50 nM-$PGE_2$. Cyclic AMP was measured as described in the Materials and methods section. Values are means ± S.E.M. for five independent experiments.

dose-dependent inhibitory curves for the accumulation of cyclic AMP owing to isoprenaline ($EC_{50}$ = 2 nM), with a maximal inhibition at 50 nM. The inhibitory effect exerted by carbachol on the increased accumulation of cyclic AMP caused by isoprenaline was dose-dependent, with a maximal inhibition at 50 $\mu$M ($EC_{50}$ = 2 $\mu$M). The carbachol-induced decrease in cyclic AMP was blocked by atropine, indicating a muscarinic-receptor-mediated event (results not shown). When myometrial strips were incubated with 200 ng of pertussis toxin/ml for 6 h to cause exhaustive ADP-ribosylation of pertussis-toxin-sensitive G protein in the cells [19], inhibition caused by $PGE_2$ on the accumulation of cyclic AMP was completely reversed, supporting our previous interpretation for the involvement of $G_i$ [19]. In marked contrast, such a pertussis-toxin treatment failed to alter carbachol-induced cyclic AMP attenuation, suggesting that the inhibitory pathway of the adenylate cyclase was not involved (Fig. 2). It was worth noting that the efficiency of carbachol to decrease cyclic AMP in the pregnant-rat myometrium was 500–1000-fold lower than the efficiency of the muscarinic agonist to attenuate cyclic AMP accumulation, via the inhibitory pathway of the adenylate cyclase [14,21], in the oestrogen-treated guinea-pig myometrium. However, an $EC_{50}$ of 2 $\mu$M (low-affinity interaction) did not differ significantly from the $EC_{50}$ (10 $\mu$M) displayed by carbachol in mediating phospholipase C activation in the latter tissue [9,14].

### Differential effects of $Ca^{2+}$ on carbachol- and $PGE_2$-mediated attenuation of cyclic AMP accumulation

Previous studies [1,6], including our own (results not shown), demonstrated that carbachol was able to trigger a transient contractile activity when applied to myometrial strips incubated in a $Ca^{2+}$-free medium containing 1 mM-EGTA. This was followed by a progressive decline in the ability of the muscarinic agonist to induce uterine contractions, with no more than 10% of the stimulatory effect being expressed after 10 min exposure of the tissue to a $Ca^{2+}$-free medium, indicating that removal of extracellular $Ca^{2+}$ could ultimately limit the ability of the sarcoplasmic reticulum to maintain its $Ca^{2+}$ content [2,28]. We further documented that incubation of rat myometrial strips for 15 min at 37 °C in a $Ca^{2+}$-free medium supplemented with 1 mM-EGTA was similarly associated with an almost total disappearance of the phosphorylated form of myosin light chain, reflecting the inability of calmodulin to mediate myosin light-chain kinase activation [29]. Data in Fig. 2 show that such conditions of $Ca^{2+}$ deprivation had no effect on cyclic AMP level both under basal conditions and under $\beta$-adrenergic stimulations. Likewise, the inhibitory effect of $PGE_2$ on cyclic AMP accumulation caused by isoprenaline was not affected, indicating that both the stimulatory and inhibitory pathways of the adenylate cyclase system appeared to be independent of $Ca^{2+}$. In contrast, the ability of carbachol to attenuate cyclic AMP accumulation in the pregnant-rat myometrium was markedly prevented in $Ca^{2+}$-depleted preparations (Fig. 2). $Ca^{2+}$ was also required for the inhibitory effect of carbachol on forskolin-

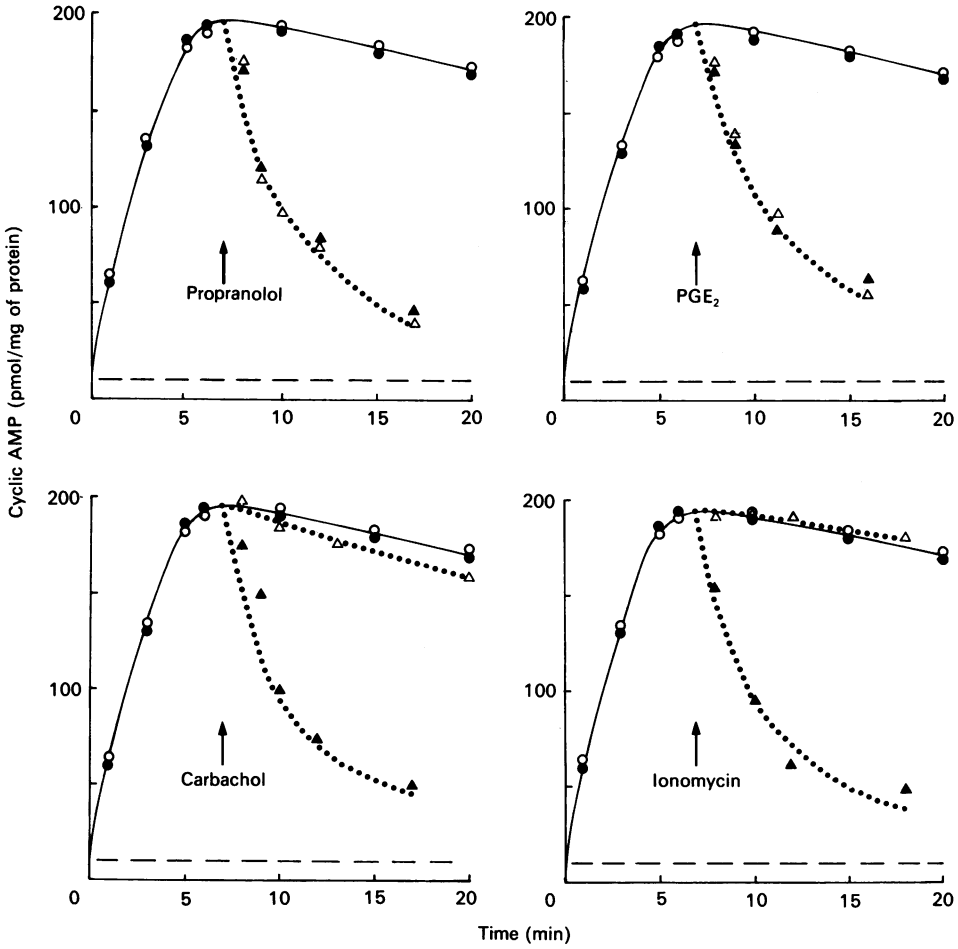


**Fig. 3. Reversibility by propranolol, $PGE_2$, carbachol and ionomycin of isoprenaline-induced cyclic AMP accumulation in the pregnant-rat myometrium: differential effects of $Ca^{2+}$**

Myometrial strips were incubated for 15 min in a normal (2.4 mM-$Ca^{2+}$) Krebs–Ringer bicarbonate buffer (black symbols) or in a $Ca^{2+}$-deprived medium supplemented with 1 mM-EGTA (white symbols). This was followed by a 5 min incubation in the presence of 240 $\mu$M-IBMX before addition of 0.3 $\mu$M-isoprenaline. Incubations were continued without (●, ○) or with the addition (△, ▲), as indicated by the arrow, of 0.1 $\mu$M-propranolol, 50 nM-$PGE_2$, 50 $\mu$M-carbachol and 0.1 $\mu$M-ionomycin. Incubations were stopped at the times indicated and cyclic AMP was assayed as described in the Materials and methods section. Values are means for three independent experiments, each done in duplicate, and the S.E.M. did not exceed 10% of the mean.

induced cyclic AMP accumulation (results not shown). Furthermore, the $Ca^{2+}$ ionophore ionomycin was able to decrease, like carbachol, the isoprenaline-induced cyclic AMP accumulation: cyclic AMP values with isoprenaline were $185 \pm 12$ and $75 \pm 5$ pmol/mg of protein in the absence and the presence of 1 $\mu$M-ionomycin respectively (see also Fig. 3). These findings substantiated the interpretation that the muscarinic-receptor-mediated decrease in cyclic AMP did not involve the inhibitory pathway of the adenylate cyclase. At first instance, the data could be interpreted to imply that elevated $Ca^{2+}$ levels might exert an inhibitory effect on the adenylate cyclase activity through a mechanism distinct from the $G_i$ inhibitory process. This possibility seems somewhat unlikely, in view of the numerous observations describing an activation, rather than an inhibition, mediated by the $Ca^{2+}$–calmodulin complex, of the catalytic activity of the cyclase in membrane preparations of numerous, although not all, cells [15]. A more plausible interpretation for the decrease in cyclic AMP observed with carbachol and ionomycin would rather be to invoke the known ability of the $Ca^{2+}$–calmodulin complex to activate a phosphodiesterase activity [23]. This would be consistent with similar observations demonstrating the contribution of a $Ca^{2+}$-dependent activation of phosphodiesterase to the muscarinic-receptor-mediated attenuation of cyclic AMP in different cell types [24,30].

### Reversibility of the $\beta$-adrenergic stimulatory cyclic AMP response by propranolol, $PGE_2$, carbachol and ionomycin: differential effects of $Ca^{2+}$

In contradistinction with the above-mentioned reports [24,30], cyclic AMP attenuation elicited by carbachol in the pregnant-rat myometrium could still be evoked in the presence of 240 $\mu$M-IBMX. The extent of cyclic AMP attenuation was nevertheless slightly improved by omitting the phosphodiesterase inhibitor (52% inhibition of the $\beta$-adrenergic response, versus 45% in the presence of 240 $\mu$M-IBMX). Consequently a series of experiments was undertaken in order to assess the contribution of cyclic AMP degradation to the overall cyclic AMP levels attained in intact myometrial preparations under different incubation conditions. When propranolol, a $\beta$-adrenergic antagonist, was added after a 7 min stimulation with isoprenaline, there was a progressive return to basal cyclic AMP values with almost total reversibility of stimulation within 10 min (Fig. 3). The decline was not due to

cyclic AMP extrusion in the medium (results not shown), but could rather be attributed to its degradation by a phosphodiesterase activity. These data suggested that at 240 $\mu$M-IBMX a phosphodiesterase activity could still be expressed when receptor-mediated activation of adenylate cyclase was prevented. This also indicated that the plateau of cyclic AMP accumulation obtained with the $\beta$-adrenergic agonist in the presence of IBMX should be interpreted to reflect, at least at the earliest time, an equilibrium between synthesis (i.e. adenylate cyclase activity) and degradation (i.e. phosphodiesterase activity) of cyclic AMP. Data from Fig. 3 revealed that the latent phosphodiesterase activity was insensitive to $Ca^{2+}$ deprivation, inasmuch as the reversibility by propranolol of isoprenaline-mediated cyclic AMP accumulation could equally be demonstrated in both the presence and the absence of $Ca^{2+}$. The data further show that the inhibitory effect of $PGE_2$ could also be evidenced when $PGE_2$ was added at the plateau of isoprenaline-induced cyclic AMP accumulation in the presence of IBMX. In this case, the stimulatory pathway of the adenylate cyclase was blunted, owing to $PGE_2$-mediated activation of $G_i$, thus allowing the latent phosphodiesterase activity to be expressed. The reversibility pattern obtained with $PGE_2$ was, as for propranolol, insensitive to $Ca^{2+}$ deprivation.

This markedly contrasted with the findings obtained with carbachol (Fig. 3). The muscarinic agonist was also able to reverse the stimulatory effect of isoprenaline, but this reversibility was dependent on $Ca^{2+}$. Similar reversibility of $\beta$-adrenergic stimulation occurred with the addition of 1 $\mu$M-ionomycin and, like carbachol, the reversibility by ionomycin could not be observed in the absence of $Ca^{2+}$. Assuming, as commented above, that for the $Ca^{2+}$-mediated effects of both carbachol and ionomycin the contribution of the adenylate cyclase activity [15] to the decline in cyclic AMP accumulation was not retained, then the decrease in cyclic AMP could most probably be attributed to its increased degradation, i.e. an activation of a phosphodiesterase which appeared to require $Ca^{2+}$. These data would then provide indirect evidence for the presence in the pregnant-rat myometrium of more than one type of phosphodiesterase, with one type exhibiting no sensitivity to $Ca^{2+}$ omission and one type that is $Ca^{2+}$-sensitive and that is activated by an increase in intracellular $Ca^{2+}$, whether induced by carbachol or ionomycin. The latter form does not appear to contribute to a great extent to the degradation of cyclic AMP in the absence of muscarinic stimulation. The different forms of phosphodiesterases have not yet been characterized in the pregnant-rat myometrium. Nevertheless, in experiments not reported here, phosphodiesterase activity estimated in a crude myometrial extract appeared to be inhibited by no more than 30% when the assay medium was severely deprived of $Ca^{2+}$. Additionally, a recent report describing the characterization in the pregnant human myometrium of both $Ca^{2+}$–calmodulin-sensitive and -insensitive forms of phosphodiesterases is worth mentioning [31].

### Stimulation by carbachol of the accumulation of inositol phosphates in the pregnant-rat myometrium: a close relationship with muscarinic-mediated attenuation of cyclic AMP responses

Data in Fig. 4 illustrate that incubation of [$^3$H]inositol-prelabelled myometrial strips with 50 $\mu$M-carbachol, in the presence of 10 mM-LiCl, caused a marked increase in the generation of the three inositol phosphates (Ins$P$, Ins$P_2$ and Ins$P_3$). The time course of the effects of carbachol revealed a rapid increase in Ins$P_3$ which reached a plateau (280% stimulation) at 3 min. The accumulation of Ins$P_2$ and Ins$P$ was delayed. Ins$P_2$ was maximally enhanced at 10 min (350% stimulation), whereas Ins$P$ accumulated further and was not stabilized up to 15 min. The time-sequential generation of inositol phosphates in the order Ins$P_3$, Ins$P_2$ and Ins$P$ indicated that the primary substrate of carbachol-mediated phospholipase C was PtdIns(4,5)$P_2$ [7,8].

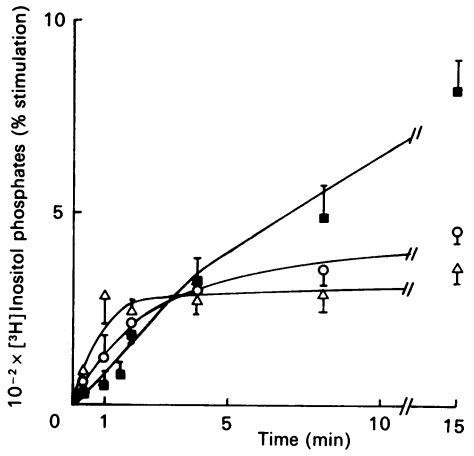


**Fig. 4. Time course of carbachol-induced accumulation of inositol phosphates in the pregnant-rat myometrium**

[$^3$H]Inositol-labelled myometrial strips were incubated with 10 mM-LiCl for 10 min before the addition of 50 $\mu$M-carbachol. Incubations were stopped at the times indicated, and individual [$^3$H]inositol phosphates were separated on AG1-X8 columns as described in the Materials and methods section. The changes in accumulation of Ins$P_3$ (△), Ins$P_2$ (○) and Ins$P$ (■) were expressed as percentage stimulations over the control value (Ins$P_3$ 780 ± 60, Ins$P_2$ 1755 ± 118, Ins$P$ 4030 ± 320 c.p.m./100 mg of tissue) obtained before the addition of carbachol. Values are means ± S.E.M. for three different experiments each done in duplicate.

The dose-dependency of carbachol-induced increases in inositol phosphates was strikingly similar to the dose-dependency of carbachol-mediated attenuation of cyclic AMP accumulation, with virtually the same $EC_{50}$: 2 and 3 $\mu$M respectively. Worth noting was also the failure of pertussis toxin to alter the inositol phosphate responses caused by carbachol, similar to the inability of pertussis toxin to affect carbachol-induced attenuation of cyclic AMP (Fig. 5), under conditions where $PGE_2$-mediated inhibition of cyclic AMP generation was totally abolished. These data provide further evidence that a pertussis-toxin-insensitive G protein is involved in the coupling of muscarinic receptors to phospholipase C activation in the pregnant-rat myometrium. An additional correlation between the increased generation of inositol phosphates and the attenuated cyclic AMP responses in the pregnant-rat myometrium was further provided by the data obtained with partial muscarinic agonists [14,32]. Results in Table 1 illustrate the weak ability of oxotremorine to cause an increased accumulation of inositol phosphates in the pregnant-rat myometrium. Compared with carbachol, oxotremorine displayed also a weaker activity in attenuating cyclic AMP accumulation; maximal inhibition reached a plateau at 1 $\mu$M-oxotremorine and averaged 35% of the maximal inhibitory cyclic AMP response to carbachol. Nevertheless, for an equivalent accumulation of [$^3$H]inositol phosphates there coincided for both carbachol and oxotremorine an identical amplitude of the inhibitory cyclic AMP response. Pilocarpine at concentrations as high as 100 $\mu$M was unable to elicit any increase in the generation of inositol phosphates. Likewise, pilocarpine failed to cause any attenuation in the accumulation of cyclic AMP. The cumulative similarities of the results obtained when measuring increased Ins$P_3$ and cyclic AMP attenuation support the contention that the muscarinic-mediated inhibitory effect on cyclic AMP accumulation is closely related to the muscarinic-induced activation of the phospholipase C pathway. This interpretation was

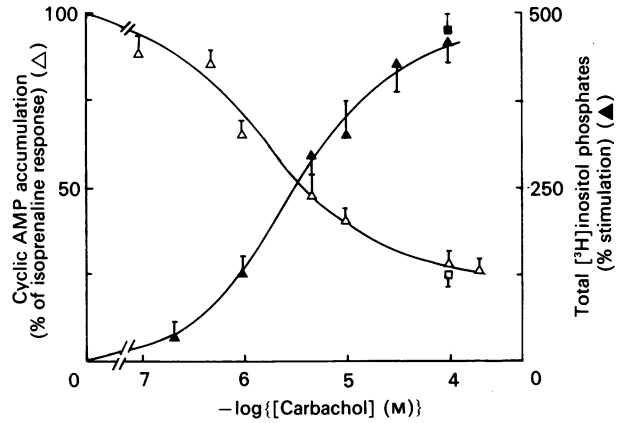


**Fig. 5. Correlation between the dose-dependent effects of carbachol on the increased generation of inositol phosphates and on the attenuation of cyclic AMP accumulation in the pregnant-rat myometrium: absence of pertussis-toxin effect**

(*a*) For the determination of cyclic AMP (△, □), myometrial strips were treated with 240 μM-IBMX for 5 min, followed by 7 min incubation with isoprenaline (0.3 μM) added individually or simultaneously with the indicated concentration of carbachol. Treatment with pertussis toxin (□) was carried out as described in the legend to Fig. 2, and re-challenge incubations were performed in the simultaneous presence of 0.3 μM-isoprenaline and 50 μM-carbachol. Results were expressed as percentages of the cyclic AMP response elicited by isoprenaline alone (185±15 pmol/mg of protein), and are means±S.E.M. for five independent experiments. (*b*) For the determination of total inositol phosphates (▲, ■), [$^3$H]inositol-prelabelled strips were incubated in the presence of 10 mM-LiCl and stimulated for 10 min with the indicated concentration of carbachol. When indicated (■), tissues were incubated for 6 h with pertussis toxin at 200 ng/ml; [$^3$H]inositol was present during the last 3½ h of the treatment. Tissues were then washed and processed for the re-challenge experiment in the presence of 10 mM-LiCl and 50 μM-carbachol. Values correspond to the combined inositol phosphate fraction ($InsP+InsP_2+InsP_3$) eluted as described in the Materials and methods section, and were expressed as the percentage stimulation over the control (7800±680 c.p.m./100 mg of tissue) obtained without carbachol. Values are means±S.E.M. for three independent experiments each done in duplicate.

**Table 1. Comparison of effects of muscarinic agonists and antagonists on the cyclic AMP and inositol phosphate responses in the pregnant rat myometrium**

For determination of cyclic AMP, myometrial strips were treated with 240 μM-IBMX for 5 min, followed by 7 min incubation with 0.3 μM-isoprenaline added alone (control) or simultaneously with the indicated concentration of carbachol, oxotremorine or pilocarpine. Values for cyclic AMP, expressed as pmol/mg of protein, represent means±S.E.M. for three to four experiments. For the accumulation of total inositol phosphates, [$^3$H]inositol-prelabelled myometrial strips were incubated in the presence of 10 mM-LiCl (control) and stimulated for 10 min with the indicated concentration of carbachol, oxotremorine or pilocarpine. Total [$^3$H]inositol phosphates, expressed as c.p.m./100 mg of tissue, represent means±S.E.M. for four independent experiments. When used, muscarinic antagonists [4-DAMP or AF-DX116 (*b*)] were added at the indicated concentration 10 min before carbachol.

| Treatment | Cyclic AMP (pmol/mg of of protein) | Total [$^3$H]inositol phosphates (c.p.m./100 mg of tissue) |
|---|---|---|
| Control | 190±8 | 7810±680 |
| (*a*) Carbachol (1 μM) | 122±6 | 17965±818 |
| (100 μM) | 68±6 | 46800±3980 |
| Oxotremorine (1 μM) | 118±4 | 17042±767 |
| (100 μM) | 107±2 | 18046±1517 |
| Pilocarpine (100 μM) | 170±12 | 7767±840 |
| (*b*) Carbachol (15 μM) | 94±7 | 39033±2422 |
| Carbachol+4-DAMP (10 nM) | 179±10 | 9883±1074 |
| Carbachol+AF-DX116 (1 μM) | 95±4 | 41071±3120 |

reinforced by further experiments using two selective muscarinic antagonists, i.e. AF-DX116 and 4-DAMP [33]. AF-DX116, a selective antagonist for the M2-receptor, which blocked carbachol-mediated adenylate cyclase inhibition via $G_i$ in the guinea-pig myometrium [14], remained without effect on carbachol-mediated cyclic AMP attenuation in the pregnant-rat myometrium, and similarly was unable to affect the muscarinic-induced inositol phosphate response. On the other hand 4-DAMP, which abolished the carbachol-mediated activation of phospholipase C in guinea pig [14] and rat myometrium [34], as well as the $Ca^{2+}$-dependent contractile event [14], markedly prevented the stimulatory effect of carbachol on the generation of inositol phosphates, and similarly antagonized its inhibitory cyclic AMP response in the pregnant-rat myometrium.

In conclusion, the data reported in the present study demonstrate that in the pregnant-rat myometrium cyclic AMP can be negatively regulated by way of two distinct mechanisms. Attenuation of cyclic AMP evoked by prostaglandins ($PGE_2$, $PGF_{2\alpha}$) is mediated via the inhibitory pathway of the adenylate cyclase involving $G_i$, a pertussis-toxin-sensitive G-protein. The adenylate cyclase pathway does not contribute to the attenuated cyclic AMP response evoked by carbachol. A number of findings are rather in favour of the involvement of the phosphoinositide-phospholipase C signal-transducing pathway, whose stimulation is usually associated with a rise in intracellular $Ca^{2+}$ [7,8], and which has been shown to contribute to uterine contractions [10,14]. Indeed, the carbachol-induced decrease in cyclic AMP was $Ca^{2+}$-dependent and was mimicked by ionomycin. The relative potencies of carbachol in stimulating the generation of inositol phosphates, with an earlier accumulation of $InsP_3$, parallel its activity in attenuating cyclic AMP. Among the muscarinic agonists, only those demonstrated to increase the generation of inositol phosphates inhibited cyclic AMP accumulation. To these observations should be added the failure of pertussis toxin to affect both the attenuated cyclic AMP response and the increased generation of inositol phosphates owing to carbachol. It is conceivable to propose that muscarinic-receptor (distinct from the M2 subtype) activation, associated with the $InsP_3$ pathway and the accompanying rise in intracellular $Ca^{2+}$, attenuated the cyclic AMP level by stimulating its degradation through a $Ca^{2+}$-activated phosphodiesterase [24,30]. Of much interest was the finding that oxytocin, a contractile agonist, which has been reported to stimulate the generation of inositol phosphates [10,11], was similarly able to evoke an attenuated cyclic AMP response in the pregnant-rat myometrium (results not shown). In light of the generally accepted second-messenger role for cyclic AMP in uterine relaxation [4], such a cross-talk between the second-messenger generating systems may be of functional significance. The combined increases in intracellular $Ca^{2+}$ and decrease in cyclic AMP would tend to enhance the contractile activity of the uterus, which is of crucial importance at the onset of parturition.

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