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# Carbachol and oxytocin stimulate the generation of inositol phosphates in the guinea pig myometrium

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In the guinea pig myometrium prelabelled with *myo*-[2-$^3$H]inositol, carbachol and oxytocin enhanced a concentration-dependent and rapid release of $IP_3$ which preceded that of $IP_2$ and $IP_1$. The specific receptor-mediated phospholipase C activation degrading $PIP_2$ to $IP_3$ did not require the presence of extracellular $Ca^{2+}$. The ionophore A23187 as well as $K^+$ depolarization failed to increase inositol phosphate accumulation. It is proposed that $IP_3$ could have a role in the contraction of uterine smooth muscle elicited by the activation of muscarinic as well as of oxytocin receptors.

*(Myometrium) Muscarinic receptor Oxytocin receptor Inositol phosphate Phospholipase C Contraction*

## 1. INTRODUCTION

In many stimulated cells there is a striking association between elevation of cytosol $Ca^{2+}$ concentrations and the enhanced inositol lipid metabolism particularly the phosphodiesterasic cleavage of $PIP_2$ with the accumulation of $IP_3$ [1,2]. Evidence obtained so far is compatible with a second messenger role for $IP_3$ in the mobilization of $Ca^{2+}$ in different cell types [1–3]. Contraction of smooth muscle in response to appropriate agonists is considered to be mediated by an increase in the concentration of intracellular free $Ca^{2+}$, originating at least in part from internal stores [4]. It has been described that contraction induced by carbachol was associated with an increase in phosphoinositide metabolism in the trachea [5] and an enhanced formation of $IP_3$ in the rabbit iris [6]. $IP_3$ has also been demonstrated to release $Ca^{2+}$ from internal stores in permeabilized vascular smooth muscle cells [7] as well as to cause $Ca^{2+}$ release from a microsomal fraction derived from bovine uterine sarcoplasmic reticulum [8].

We recently reported that in the estrogen-dominated guinea pig myometrium two biochemical events viz. an increased synthesis of cGMP and a decreased synthesis of cAMP accompany contractions elicited by carbachol [9,10]. We further demonstrated the absence of a causal relationship between the carbachol-mediated responses at the cyclic nucleotide levels and the contractile response [10]. This work constitutes the first report on muscarinic receptor, as well as oxytocin receptor, mediated effects on phosphoinositide metabolism in the uterine smooth muscle. The characteristics of the accumulation of $IP_3$ in the guinea pig myometrium have been carefully examined in light of the possibility that

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*Abbreviations:* $PIP_2$, phosphatidylinositol 4,5-biphosphate; $IP_1$, inositol monophosphate; $IP_2$, inositol biphosphate; $IP_3$, inositol triphosphate; d($CH_2$)$_5$-D-Phe$^2$, Ile$^4$AVP, [1-$\beta$-(mercapto-$\beta$,$\beta$-cyclopentamethylenepropionic acid),2-D-phenylalanine,4-isoleucine]arginine-vasopressin

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this putative messenger could contribute to both carbachol- and oxytocin-elicited contractions.

## 2. MATERIALS AND METHODS

### 2.1. *Chemicals*

*myo*-[2-$^3$H]Inositol (14.3 Ci/mmol) was obtained from Amersham (England); lithium chloride, carbamoylcholine hydrochloride (carbachol), oxytocin and atropine were from Sigma (St Louis, MO); Dowex AG1-X8 ion-exchange resin (formate form 200–400 mesh) from Bio-Rad (Watford, England); d(CH$_2$)$_5$-D-Phe$^2$,Ile$^4$AVP was kindly provided by Dr M. Manning (Medical College of Ohio, Toledo, OH).

### 2.2. *Tissue preparation and incubation*

Uteri were obtained from immature estrogen-pretreated guinea pig and myometrium was prepared free of endometrium [9,10]. Myometrial strips (about 100 mg) were equilibrated in 5.0 ml of Krebs bicarbonate buffer and subsequently incubated with 6 $\mu$Ci of *myo*-[2-$^3$H]inositol (0.42 $\mu$M) in 1 ml of fresh Krebs buffer. The prelabelling incubation was continued for 3½ h by which time the incorporation of $^3$H into inositol lipids had reached a plateau (not shown). The tissues were then washed 3 times with 20 ml Krebs buffer, transferred to 1.5 ml of fresh buffer and allowed to equilibrate for 20 min before the addition of 10 mM LiCl or NaCl [11]. After 10 min, the agent to be tested was added at the indicated concentration and incubation was further continued for the time specifically indicated in the legends to the figures. Reactions were stopped by immersing the tissues in 1 ml of cold 7% trichloroacetic acid with an immediate homogenization and centrifugation (20 min, 3000 × *g*). The pellets were washed by resuspension in 0.5 ml trichloroacetic acid and centrifuged again. The resulting supernatants were combined with those obtained from the first centrifugation.

### 2.3. *Separation of [$^3$H]inositol phosphates*

The trichloroacetic acid supernatants (1.5 ml) which contained the water-soluble inositol metabolites were extracted 4 times with 4 ml of diethyl ether. The neutralized extract was applied to a column (0.7 × 3 cm) of Dowex AG1-X8 formate form as in [11]. Free inositol was totally eluted by 10 ml of water and glycerophosphoinositol by 10 ml of 5 mM sodium tetraborate and 60 mM ammonium formate (the level of glycerophosphoinositol did not change significantly under the conditions used in this study). $IP_1$, $IP_2$ and $IP_3$ were then eluted successively by 10 ml of 0.1 N formic acid and 0.2 M ammonium formate; 16 ml of 0.1 N formic acid and 0.5 M ammonium formate; and 10 ml of 0.1 N formic acid and 1 M ammonium formate. Initially 2 ml fractions were collected in order to characterize the separation system but routinely each peak was collected as a single fraction. 1.4 ml of each fraction was taken for determination of radioactivity in Instagel scintillation fluid. Results were expressed as cpm/100 mg tissue or as a percentage of stimulation compared to control.

### 2.4. *Methods for recording uterine contractions*

The contractile activity of isolated myometrial strips was measured using an isometric transducing device [10].

## 3. RESULTS AND DISCUSSION

The data in fig.1 illustrate the radioactivity associated with each specific water-soluble $^3$H-labelled inositol phosphate ($IP_1$, $IP_2$, $IP_3$) separated on Dowex 1-X8 columns. In the unstimulated guinea pig myometrium the major $^3$H-labelled component was $IP_1$ while $IP_2$ and particularly $IP_3$ were less important. After 5 min of incubation in the presence of 50 $\mu$M carbachol or 0.2 $\mu$M oxytocin, the levels of $IP_1$, $IP_2$ and $IP_3$ all increased. $Li^+$ which inhibits the phosphomonoesterase that degrades $IP_1$ to inositol [1,12] caused by itself a modest but significant increase not only in $IP_1$ but similarly in $IP_2$ and $IP_3$ (1.5-fold). In the simultaneous presence of 10 mM LiCl plus carbachol or LiCl plus oxytocin, the accumulation of each inositol phosphate was higher than that reached with each agent separately. The liberation of all inositol phosphates mediated by carbachol was blocked by the muscarinic antagonist atropine (100% inhibition at 1 $\mu$M). Similarly the oxytocin response was markedly affected by the oxytocin receptor antagonist d(CH$_2$)$_5$-D-Phe$^2$,Ile$^4$AVP [13] (inhibition averaged 40 and 100% at an antagonist concentration of 50

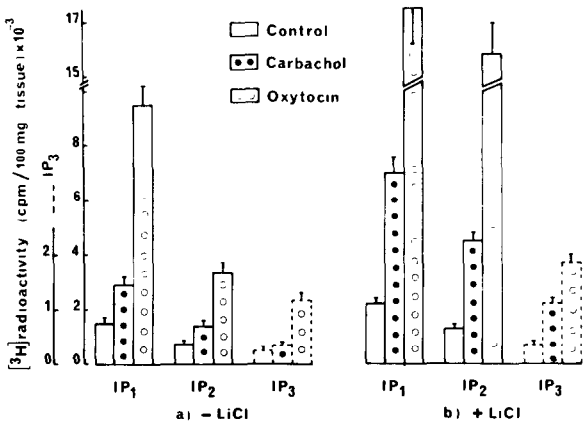


Fig.1. Effect of carbachol and oxytocin on the accumulation of inositol phosphates in the guinea pig myometrium. Role of lithium. [$^3$H]Inositol-labelled myometrial strips were transferred to a fresh medium with (b) or without (a) the addition of 10 mM LiCl. After 10 min, tissues were stimulated by 50 $\mu$M carbachol or 0.2 $\mu$M oxytocin for 5 min followed by trichloroacetic acid extraction and centrifugation. The $^3$H-labelled water-soluble components were applied to a Dowex AG1-X8 column and eluted as described in section 2. Aliquots (1.4 ml) of the relevant $IP_1$, $IP_2$ and $IP_3$ fractions were used for radioactivity measurement. Results are expressed as cpm/100 mg tissue. Values are means ± SE of 4 experiments.

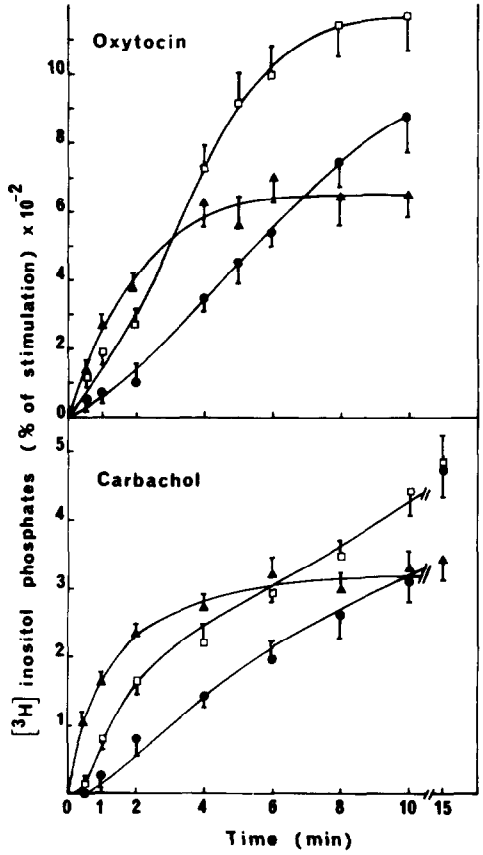


Fig.2. Time courses of carbachol- and oxytocin-induced accumulation of inositol phosphates. [$^3$H]Inositol-labelled myometrial strips were incubated with 10 mM LiCl for 10 min before the addition of 50 $\mu$M carbachol or 0.2 $\mu$M oxytocin. Incubations were stopped at the indicated times. The changes in the accumulation of $IP_1$ (●), $IP_2$ (□) and $IP_3$ (▲) have been expressed as a percentage of the control value obtained before the addition of the agonist. Values are means ± SE of 3–4 experiments.

and 500 nM, respectively). Therefore, the increased formation of water-soluble inositol phosphates due to both carbachol and oxytocin was consistent with a specific receptor-mediated activation of phospholipase C acting on phosphoinositides.

Fig.2 shows the time courses of the effects of 50 $\mu$M carbachol and of 0.2 $\mu$M oxytocin in the presence of 10 mM of $Li^+$ on [$^3$H]$IP_3$, [$^3$H]$IP_2$ and [$^3$H]$IP_1$ levels. The addition of either carbachol or oxytocin caused a rapid increase in $IP_3$ (100% stimulation at 30 s) which reached a plateau (300 and 500% stimulation with carbachol and oxytocin, respectively) at 3 min. The accumulation of $IP_2$ and $IP_1$ was delayed (100% stimulation for $IP_2$ at 1 min and 20% for $IP_1$). $IP_2$ was maximally enhanced at 8–10 min (500–1000% stimulation), whereas $IP_1$ accumulated further and was not stabilized at 15 min. The time sequential generation of inositol phosphates in the order of $IP_3$, $IP_2$ and $IP_1$ indicated that most probably, the primary substrate of both carbachol- and oxytocin-mediated activation of phospholipase C was $PIP_2$ and that $IP_3$ was subsequently degraded to $IP_2$ and further to $IP_1$ through the action of the respective phosphatases [1].

Fig.3 illustrates the concentration-dependent effects of carbachol and oxytocin on the accumulation of inositol phosphates during 5 min incubation of the guinea pig myometrium. The sensitivity to carbachol concentrations for the generation of $IP_3$, $IP_2$ and $IP_1$ was the same with a half-maximal effect at 15 $\mu$M and a maximal response achieved at 100 $\mu$M. Similarly the dose-dependent patterns

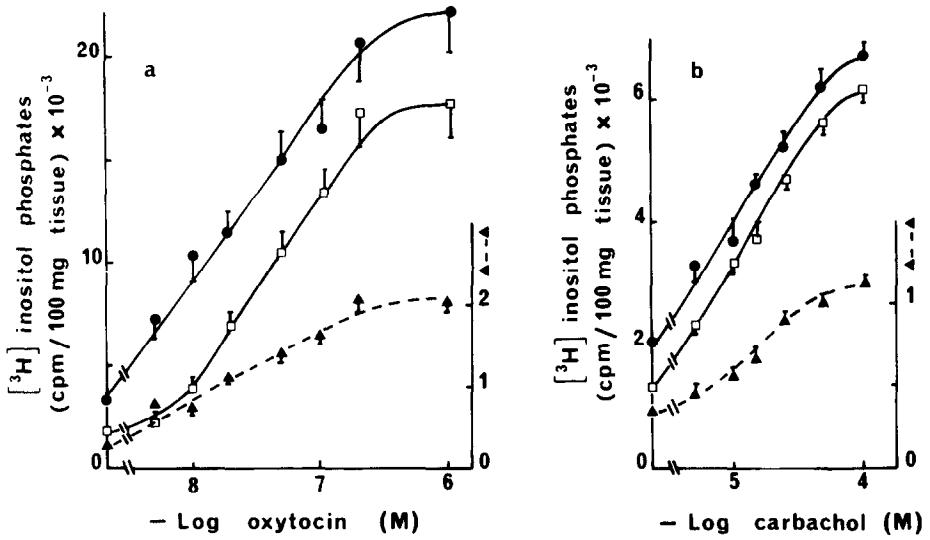


Fig.3. Dose-dependent effects of oxytocin (a) and carbachol (b) on the accumulation of inositol phosphates in the guinea pig myometrium. [$^3$H]Inositol-labelled myometrial strips were incubated for 10 min with 10 mM LiCl before exposure for 5 min to the indicated concentrations of oxytocin (a) or of carbachol (b). (●) $IP_1$, (□) $IP_2$, (▲) $IP_3$. Values represent means ± SE of 3–4 experiments.

for oxytocin-mediated $IP_3$, $IP_2$ and $IP_1$ accumulation were comparable; half-maximal and maximal levels of all inositol phosphates were achieved at 30 and 200 nM, respectively. The maximal amount of $IP_3$, $IP_2$ and $IP_1$ released by oxytocin was 2–3-fold higher than that reached by a maximal effective concentration of the muscarinic agonist.

As shown in fig.4, treatment of *myo*-[$^3$H]-inositol prelabelled guinea pig myometrium with the $Ca^{2+}$ ionophore A23187 (10 $\mu$M) in the presence of 2.4 mM $Ca^{2+}$ for 5 min did not result in any modification in basal inositol phosphate levels. Under these conditions, A23187 has been demonstrated to activate in the myometrium a $Ca^{2+}$-dependent phospholipase A2 activity and enhance the release of arachidonic acid from phospholipids [14]. Similarly, high $K^+$ concentrations which depolarize smooth muscles and cause influx of intracellular $Ca^{2+}$ leading to contraction [6,15] failed to induce the accumulation of any of the inositol phosphates over the 5 min period examined (fig.4). Furthermore incubation of myometrial strips in a Krebs buffer where $Ca^{2+}$ was omitted had no significant effect on the inositol phosphate responses to either carbachol or oxytocin. Nevertheless substantial depletion of intracellular $Ca^{2+}$ by a 5 min treatment of the myometrium with 4 mM EGTA in the absence of $Ca^{2+}$ did lower basal inositol phosphate levels in the presence of $Li^+$ and particularly altered the stimulation of inositol phosphate accumulation due to carbachol and oxytocin (2 and 15% of the respective optimal responses could only be expressed). Thus it appears that with both oxytocin and carbachol, the receptor-mediated stimulation of phosphoinositide breakdown, reflected by the enhanced accumulation of inositol phosphates, is not regulated by increases of cytosolic free $Ca^{2+}$ as in [11,16] but that it is apparently dependent on a certain minimum content of this ion [11,17].

Fig.5 illustrates the efficacy of both carbachol and oxytocin to elicit contractions in the guinea pig myometrium (half-maximal effect at 3 $\mu$M and 3 nM for carbachol and oxytocin, respectively). This order of potency for the two agonists coincided with their order of potency to cause inositol phosphate accumulation (fig.3). However with both carbachol and oxytocin, maximal activation of contraction required a 7–10-fold less concentration of each agonist than did maximal increase in $IP_3$ ($EC_{50}$ 15 $\mu$M and 30 nM for carbachol and oxytocin, respectively). Such disparities between the

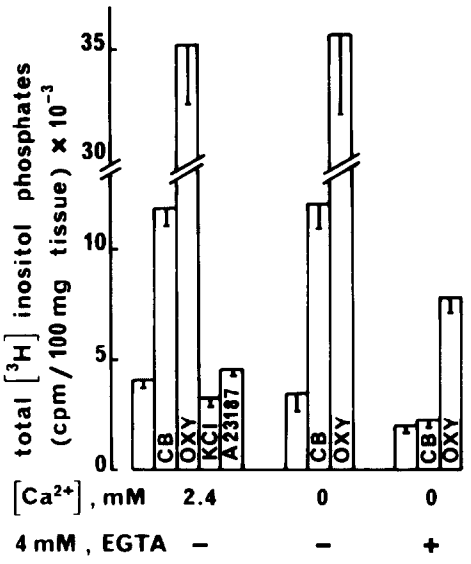


Fig.4. Effects of ionophore A23187 and KCl on inositol phosphate levels in the guinea pig myometrium. Role of $Ca^{2+}$ in the oxytocin- and carbachol-mediated stimulations. Following the [$^3$H]inositol-labelling step, myometrial strips were transferred to a fresh normal medium (2.4 mM $Ca^{2+}$), a medium without $Ca^{2+}$, or a medium without $Ca^{2+}$ and with 4 mM EGTA and allowed to equilibrate for 5 min before the addition of 10 mM LiCl. After 10 min further incubation, tissues were treated with or without 50 $\mu$M carbachol, 1 $\mu$M oxytocin, 100 mM KCl or 10 $\mu$M A23187 for 5 min. For incubations with 100 mM KCl, the increase in $K^+$ concentration from 6–100 mM was compensated by an equivalent decrease in $Na^+$ concentration. Values correspond to the combined inositol phosphate peaks ($IP_1$ + $IP_2$ + $IP_3$), expressed as cpm/100 mg tissue (mean ± SE of 3 different experiments).

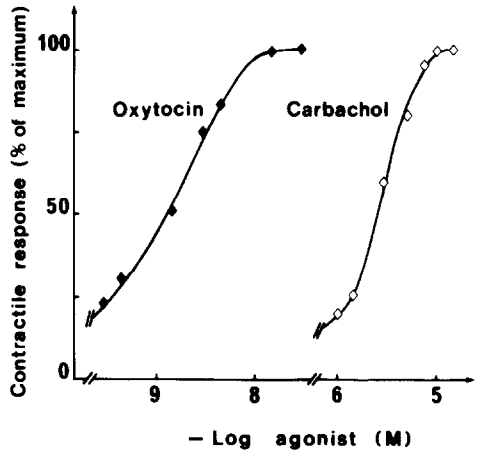


Fig.5. Comparison of the contractile response of the isolated guinea pig myometrium to increasing concentrations of oxytocin and carbachol. Isometric contractions were recorded during 2 min exposure of the loaded myometrial segments to the indicated concentrations of oxytocin or carbachol. The degree of the contractile response to the specific agonist was expressed as the percentage of the response to a maximal effective concentration of either oxytocin or carbachol (maximal contractile responses were identical for both agonists). Values are means of 3 experiments and they agreed within 10%.

dose-response curves for agonist-stimulated $IP_3$ release and the $Ca^{2+}$-dependent physiological events have previously been observed [16,18]. Our present findings could thus possibly indicate that a very small and submaximal elevation of $IP_3$ would be sufficient to maximally activate contraction. It is worth noting that oxytocin and carbachol produced the same maximal contractile response although the maximum capacity for oxytocin to generate $IP_3$ exceeded by 2–3-fold that of the muscarinic effector.

In summary, the foregoing data clearly establish that muscarinic, as well as oxytocin, receptor activation in the guinea pig myometrium is coupled to an enhanced phosphoinositide metabolism. Evidence is provided that both carbachol and oxytocin which caused uterine contractions, concomitantly stimulated a rapid phosphodiesterasic cleavage of $PIP_2$ to $IP_3$ and that the accumulation of $IP_3$ preceded that of $IP_2$ and $IP_1$. The relative contribution of the two isomers viz. the active 1,4,5-$IP_3$ and the inactive 1,3,4-$IP_3$ to the total $IP_3$ pool [19] has not been examined. The demonstration that the stimulation of $IP_3$ release due to carbachol and oxytocin was not affected by omission of $Ca^{2+}$ from the extracellular medium together with the failure of $IP_3$ to accumulate during $K^+$-induced contractions as well as in the presence of A23187 strongly imply that the breakdown of phosphoinositides (i) is not controlled by the potential-dependent $Ca^{2+}$ channel, (ii) is not the consequence of an increase in intracellular $Ca^{2+}$ concentration but (iii) is rather a specific receptor-mediated event that precedes $Ca^{2+}$ mobilization during uterine muscle activation. With both carbachol and oxytocin, very small increases in $IP_3$

correlated with maximal tension. This, as proposed for the hepatocytes [16,18] may reflect a biological amplification between the step of lipid breakdown and activation of contraction by elevated cytosolic $Ca^{2+}$. In light of the present findings and of recent reports [5–8] it would be tentative to consider $IP_3$ as the potential physiological link between the two activated receptors (muscarinic and oxytocin) and the resulting $Ca^{2+}$-dependent contractile event in the uterine smooth muscle.

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