

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*-[2-³H]inositol, carbachol and oxytocin enhanced a concentration-dependent and rapid release of IP₃ which preceded that of IP₂ and IP₁. The specific receptor-mediated phospholipase C activation degrading PIP₂ to IP₃ did not require the presence of extracellular Ca²⁺. The ionophore A23187 as well as K⁺ depolarization failed to increase inositol phosphate accumulation. It is proposed that IP₃ 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 Ca²⁺ concentrations and the enhanced inositol lipid metabolism particularly the phosphodiesterase cleavage of PIP₂ with the accumulation of IP₃ [1,2]. Evidence obtained so far is compatible with a second messenger role for IP₃ in the mobilization of Ca²⁺ 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 Ca²⁺, 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 IP₃ in the rabbit iris [6]. IP₃ has also been demonstrated to release Ca²⁺ from internal stores in permeabilized vascular smooth muscle cells [7] as well as to cause Ca²⁺ 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 IP₃ in the guinea pig myometrium have been carefully examined in light of the possibility that

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Abbreviations: PIP₂, phosphatidylinositol 4,5-bisphosphate; IP₁, inositol monophosphate; IP₂, inositol bisphosphate; IP₃, inositol triphosphate; d(CH₂)₅-D-Phe², Ile⁴AVP, [1-β-(mercapto-β,β-cyclopentamethylenepropionic acid),2-D-phenylalanine,4-isoleucine]arginine-vasopressin

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

2. MATERIALS AND METHODS

2.1. Chemicals

myo-[2-³H]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(CH₂)₅-D-Phe²,Ile⁴AVP 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 μ Ci of *myo*-[2-³H]inositol (0.42 μ M) in 1 ml of fresh Krebs buffer. The prelabelling incubation was continued for 3½ h by which time the incorporation of ³H 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 \times 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 [³H]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 \times 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). IP₁, IP₂ and IP₃ 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 ³H-labelled inositol phosphate (IP₁, IP₂, IP₃) separated on Dowex 1-X8 columns. In the unstimulated guinea pig myometrium the major ³H-labelled component was IP₁ while IP₂ and particularly IP₃ were less important. After 5 min of incubation in the presence of 50 μ M carbachol or 0.2 μ M oxytocin, the levels of IP₁, IP₂ and IP₃ all increased. Li⁺ which inhibits the phosphomonoesterase that degrades IP₁ to inositol [1,12] caused by itself a modest but significant increase not only in IP₁ but similarly in IP₂ and IP₃ (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 μ M). Similarly the oxytocin response was markedly affected by the oxytocin receptor antagonist d(CH₂)₅-D-Phe²,Ile⁴AVP [13] (inhibition averaged 40 and 100% at an antagonist concentration of 50

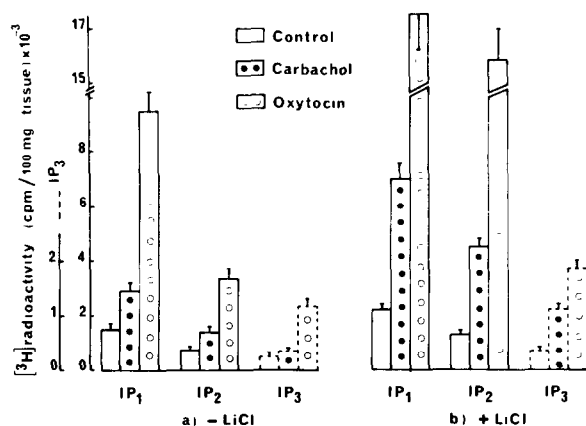


Fig.1. Effect of carbachol and oxytocin on the accumulation of inositol phosphates in the guinea pig myometrium. Role of lithium. [^3H]Inositol-labelled myometrial strips were transferred to a fresh medium with (b) or without (a) the addition of 10 mM LiCl. After 10 min, tissues were stimulated by 50 μM carbachol or 0.2 μM oxytocin for 5 min followed by trichloroacetic acid extraction and centrifugation. The ^3H -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 IP₁, IP₂ and IP₃ fractions were used for radioactivity measurement. Results are expressed as cpm/100 mg tissue. Values are means \pm SE of 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 μM carbachol and of 0.2 μM oxytocin in the presence of 10 mM of Li⁺ on [^3H]IP₃, [^3H]IP₂ and [^3H]IP₁ levels. The addition of either carbachol or oxytocin caused a rapid increase in IP₃ (100% stimulation at 30 s) which reached a plateau (300 and 500% stimulation with carbachol and oxytocin, respectively) at 3 min. The accumulation of IP₂ and IP₁ was delayed (100% stimulation for IP₂ at 1 min and 20% for IP₁). IP₂ was maximally enhanced at 8–10 min (500–1000% stimulation), whereas IP₁ accumulated further and was not stabilized at 15 min. The time sequential generation of inositol phosphates in the order of IP₃, IP₂ and IP₁ indicated that most probably, the primary substrate of both carbachol- and oxytocin-mediated activation of phospholipase C was PIP₂

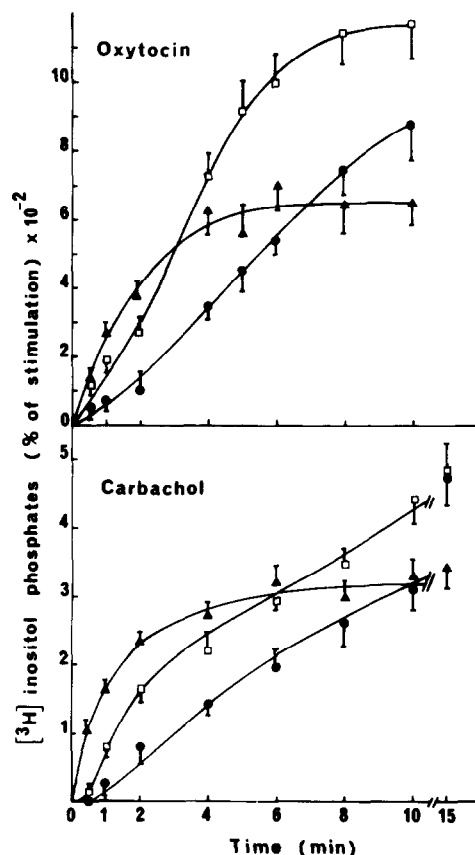


Fig.2. Time courses of carbachol- and oxytocin-induced accumulation of inositol phosphates. [^3H]Inositol-labelled myometrial strips were incubated with 10 mM LiCl for 10 min before the addition of 50 μM carbachol or 0.2 μM oxytocin. Incubations were stopped at the indicated times. The changes in the accumulation of IP₁ (●), IP₂ (□) and IP₃ (▲) have been expressed as a percentage of the control value obtained before the addition of the agonist. Values are means \pm SE of 3–4 experiments.

and that IP₃ was subsequently degraded to IP₂ and further to IP₁ 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 IP₃, IP₂ and IP₁ was the same with a half-maximal effect at 15 μM and a maximal response achieved at 100 μM . Similarly the dose-dependent patterns

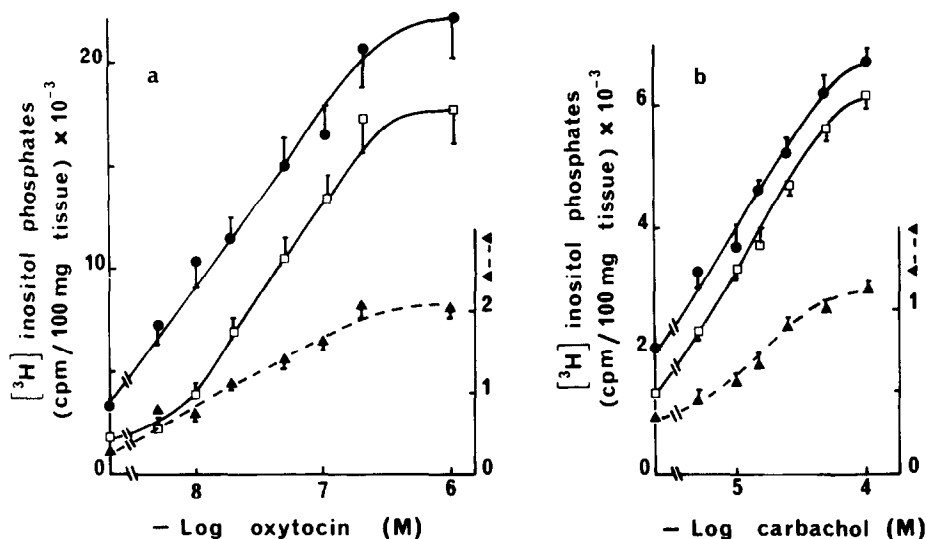


Fig.3. Dose-dependent effects of oxytocin (a) and carbachol (b) on the accumulation of inositol phosphates in the guinea pig myometrium. [^3H]Inositol-labelled myometrial strips were incubated for 10 min with 10 mM LiCl before exposure for 5 min to the indicated concentrations of oxytocin (a) or of carbachol (b). (●) IP_1 , (□) IP_2 , (▲) IP_3 . Values represent means \pm 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*-[^3H]-inositol prelabelled guinea pig myometrium with the Ca^{2+} ionophore A23187 (10 μM) 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 in-

tracellular 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 μM 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 μM and 30 nM for carbachol and oxytocin, respectively). Such disparities between the

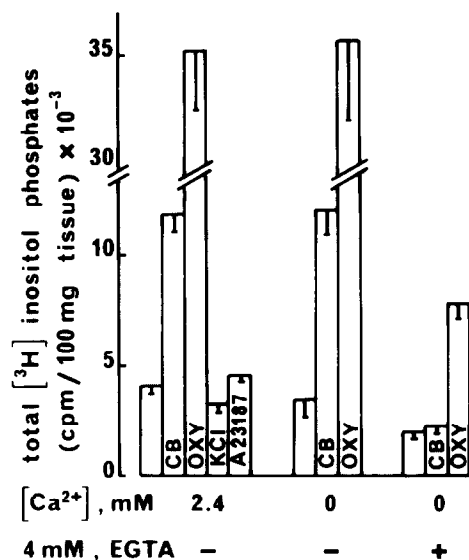


Fig.4. Effects of ionophore A23187 and KCl on inositol phosphate levels in the guinea pig myometrium. Role of Ca^{2+} in the oxytocin- and carbachol-mediated stimulations. Following the [^3H]inositol-labelling step, myometrial strips were transferred to a fresh normal medium (2.4 mM Ca^{2+}), a medium 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 μM carbachol, 1 μM oxytocin, 100 mM KCl or 10 μM 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 ($\text{IP}_1 + \text{IP}_2 + \text{IP}_3$), expressed as cpm/100 mg tissue (mean \pm SE of 3 different experiments).

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

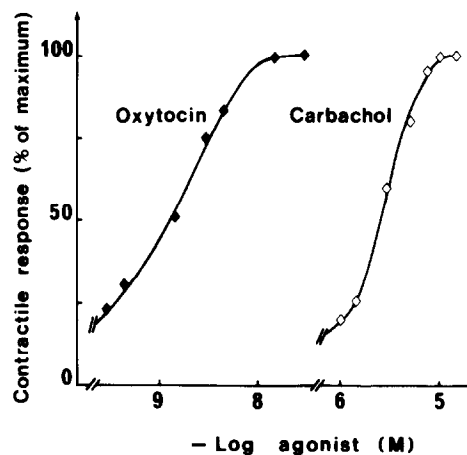


Fig.5. Comparison of the contractile response of the isolated guinea pig myometrium to increasing concentrations of oxytocin and carbachol. Isometric contractions were recorded during 2 min exposure of the loaded myometrial segments to the indicated concentrations of oxytocin or carbachol. The degree 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%.

to an enhanced phosphoinositide metabolism. Evidence is provided that both carbachol and oxytocin which caused uterine contractions, concomitantly stimulated a rapid phosphodiesterase 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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