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Activation of β-Adrenergic Receptors Inhibits Ca^{2+} Entry-Mediated Generation of Inositol Phosphates in the Guinea Pig Myometrium, a Cyclic AMP-Independent Event

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SUMMARY

In the guinea pig myometrium, carbachol, oxytocin, and fluoroaluminates stimulated the breakdown of phosphatidylinositol 4,5-bisphosphate, which was insensitive to pertussis toxin [*Biochem. J.* **255**:705–713 (1988)]. We now demonstrate that an increased accumulation of inositol phosphates, with an early production of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], could also be obtained with K^+ (30 mM) and the Ca^{2+} ionophore ionomycin. Removal of extracellular Ca^{2+} or addition of the Ca^{2+} channel antagonists nifedipine and verapamil almost totally abolished stimulations elicited by high K^+ and partially attenuated receptor- and fluoroaluminate-mediated increases in inositol phosphates. Isoproterenol similarly attenuated the accumulation of inositol phosphates elicited by carbachol, oxytocin, and fluoroaluminates (maximal inhibition, 35%; EC_{50}, 0.5 nM), with no change in the rate of Ins(1,4,5)P_3, inositol bisphosphate, and inositol monophosphate generation. The β-adrenergic receptor-induced inhibition was prevented by pertussis toxin and could not be reproduced by forskolin, indicating that cAMP was not involved. Experimental findings were, rather, consistent with a predominant role for Ca^{2+}. Thus, inhibition due to isoproterenol was lost in a Ca^{2+}-depleted medium and was not additive with that caused by nifedipine. Accumulation of inositol phosphates triggered by high K^+ was insensitive to the β-adrenergic receptor inhibition. The inhibitory effect of isoproterenol, similar to that of nifedipine, was counteracted by ionomycin and also by the Ca^{2+} channel agonist Bay K 8644. These data indicate that in the myometrium 1) phospholipase C can be activated through a voltage-gated Ca^{2+} entry-dependent process that contributes at least partially to the stimulations triggered by receptor- and/or guanine nucleotide-binding protein-mediated activation and 2) β-adrenergic receptor activation is linked via a cAMP-independent, pertussis toxin-sensitive pathway to an inhibition of voltage-gated Ca^{2+} channels, resulting in an attenuation of the Ca^{2+}-associated generation of inositol phosphates.

The initial response of many cells to Ca^{2+}-mobilizing receptor agonists is the activation of phospholipase C, catalyzing the hydrolysis of PtIns(4,5)P_2 to InsP(1,4,5)P_3 and diacylglycerol (1, 2). Both products have intracellular second messenger functions. Diacylglycerol acts by stimulating protein kinase C (3), whereas Ins(1,4,5)P_3 causes a release of Ca^{2+} from intracellular stores that is soon followed by an entry of Ca^{2+} across the plasma membrane (1, 2). A large body of evidence indicates that stimulation of phospholipase C induced by the binding of specific agonists to cell surface receptors occurs through the signal transduction action of a regulatory G protein, which has been shown, in some but not in all cases, to be insensitive to pertussis toxin (4–7).

Evidence is gradually accumulating that, in some tissues, activation of receptors that inhibit Ca^{2+} mobilization can be correlated with a decrease in the production of inositol phosphates (8–12). Two distinct mechanisms have been proposed for the negative regulation of phospholipase C mediated by receptor and G protein activation. The first implies that inhibition is not a primary consequence of receptor occupation but rather a late event reflecting an indirect regulation of phospholipase C. A role of receptor-mediated ion-gating mechanism has thus been proposed for the decrease in inositol phosphate production caused by dopamine in anterior pituitary (8) and lactotroph (9) cells and by excitatory amino acids in rat hippocampus slices (10). An alternate mechanism for phospholipase C inhibition implies the existence of an inhibitory coun-

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ABBREVIATIONS: PtdInsP(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns, phosphatidylinositol; InsP_3, inositol trisphosphate; InsP_2, inositol bisphosphate; InsP, inositol monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; TBAHS, tetrabutylammonium hydrogensulfate; G protein, guanine nucleotide-binding regulatory protein; G_s and G_i, stimulatory and inhibitory guanine nucleotide-binding regulatory proteins of the adenylate cyclase; G_o, guanine nucleotide-binding regulatory protein of unknown function; Bay K 8644, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate; Ins(1,3,4)P_3; inositol 1,3,4-trisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; HPLC, high performance liquid chromatography; PtdInsP(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate.

terpart (G_{pi}) of the stimulatory G protein (G_{ps}) that may be the direct link between the inhibitory receptors and phospholipase C. Such a purely second messenger- and Ca^{2+}-independent mechanism of inhibition of phospholipase C has been proposed for muscarinic receptor-mediated inhibition of inositol phosphate generation in rat FRTL5 thyroid cells (12) and for adenosine-mediated inhibition of inositol phosphate production in GH_3 pituitary cells (11).

We have previously reported that, in the estrogen-treated guinea pig myometrium, the phosphoinositide-phospholipase C transducing mechanism can be activated by contracting agonists, namely oxytocin and carbachol (6, 13). Stimulations at the level of both inositol phosphates and tension were mimicked by the fluoroaluminate complex (AlF_4^-), suggesting the involvement of a G protein. For both carbachol and oxytocin, the receptor-phospholipase C coupling is insensitive to pertussis toxin (6). Isoproterenol, a β-adrenergic agonist, causes uterine relaxation and counteracts contractions elicited by carbachol and oxytocin. We previously documented that the β-adrenergic relaxing effect could not be entirely explained by the accompanying rises in intracellular cAMP, suggesting an additional target for β-adrenergic receptors in the myometrium (14–16).

In the present work, we have analyzed the effect of isoproterenol on the accumulation of inositol phosphates in the guinea pig myometrium, with the aim of demonstrating possible cross-talk between contractants and relaxants at the level of phospholipase C activation. In the course of this study, we found that entry of Ca^{2+} through voltage-operated Ca^{2+} channels accounted partially for the accumulation of inositol phosphates induced by carbachol, oxytocin, and fluoroaluminates. The reported data further demonstrate an inhibitory effect of isoproterenol on receptor-mediated as well as on direct G protein-mediated phospholipase C activation. The findings suggest that the ability of the β-adrenergic agonist to attenuate the degradation of PtdIns(4,5)P_2 resulted from an inhibition of Ca^{2+} influx through nifedipine-sensitive Ca^{2+} channels, a cAMP-independent, pertussis toxin-sensitive process.

Materials and Methods

Chemicals. *myo*-[2-^{3}H]Inositol (10–20 Ci/mmol), [^{3}H]Ins(1,4,5)P_3, and [^{3}H]Ins(1,3,4)P_3 were from Amersham International (Amersham, Bucks., UK), carbamoycholine chloride (carbachol), β-estradiol 3-benzoate, oxytocin, isoproterenol, alprenolol, verapamil, and nifedipine from Sigma Chemical Co. (St. Louis, MO), forskolin and ionomycin from Calbiochem (Los Angeles, CA), and pertussis toxin from List Biological Laboratories (Campbell, CA). Silica gel plates and TBAHS were from Merck. Bay K 8644 was a gift from Bayer Co. (Leverkusen, Germany). Other chemicals were of the highest grade commercially available. Nifedipine and ionomycin were dissolved in ethanol or dimethylsulfoxide. At the concentration used in the incubation medium, both solvents were without effect on inositol phosphate accumulation.

Animals and tissue processing. Immature female Hartley guinea pigs (Charles River, 300–350 g) were treated with 160 μg of estradiol for 2 days and used on the following day. Animals were killed by decapitation, their uteri were removed immediately, and the myometrium was prepared free of endometrium, as described previously (6, 13).

Measurement of [^{3}H]inositol phosphates. Incubations were carried out as described previously (6, 13). Briefly, tissues were incubated at 37° in Krebs bicarbonate buffer (pH 7.4), containing 117 mM NaCl, 4.7 mM KCl, 1.1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.4 mM $NaHCO_3$, 2.4 mM $CaCl_2$, and 1 mM glucose (gas phase O_2/CO_2, 19:1), under constant agitation. Myometrial strips (about 40–50 mg, wet weight) were equilibrated in 5 ml of buffer for 20 min and subsequently incubated with 5 μCi of *myo*-[2-^{3}H]inositol (0.4 μM), in 1 ml of fresh buffer, for 4 hr, by which time the incorporation of ^{3}H into inositol lipids had reached a plateau. Tissues were washed with 3 × 20 ml of Krebs buffer and then transferred into 1 ml of fresh Krebs buffer whose Ca^{2+} concentration was decreased to 0.8 mM to avoid the formation of insoluble Ca_2F_2. This decrease in Ca^{2+} did not impair the effects of agonists on the stimulation of inositol phosphate accumulation (6). Tissues were allowed to equilibrate for 20 min before the addition of 10 mM LiCl. Ten minutes later, the various agents to be tested were added and incubations were carried out for an additional 20 min. Isoproterenol and nifedipine, when present, were added 1 min before the stimulatory agents. In the pertussis toxin experiments, the toxin (400 ng/ml) was added 2 hr before the addition of *myo*-[2-^{3}H]inositol and the incubations were continued as described above. In all cases, the reactions were stopped by immersing the tissue strips in 2 ml of cold 7% (w/v) trichloroacetic acid, followed by homogenization and centrifugation at 3000 × *g* for 15 min at 4°.

The trichloroacetic acid-soluble supernatants were extracted with 4 × 6 ml of diethyl ether, neutralized with Tris base, and applied to a column (0.7 × 2 cm) of the anion exchange resin (AG1-X8, formate form, 200–400 mesh) for the separation of individual inositol phosphates (13, 17). Free inositol, glycerophosphoinositol, InsP, $InsP_2$, and $InsP_3$ were successively eluted with 1) 10 ml of water, 2) 10 ml of 60 mM ammonium formate/5 mM sodium tetraborate, 3) 10 ml of 0.2 M ammonium formate/0.1 M formic acid, 4) 12 ml of 0.5 M ammonium formate/0.1 M formic acid, and 5) 6 ml of 1 M ammonium formate/0.1 M formic acid. Alternatively, total inositol phosphates 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 cpm/100 mg of tissue.

Separation of Ins(1,4,5)P_3 and Ins(1,3,4)P_3 by HPLC. In some cases, the $InsP_3$ fraction was separated from the other metabolites of inositol phospholipids by HPLC, according to a method devised by Sulpice *et al.* (18). The technique, which is based on ion pair chromatography, included an octadecyl reverse phase 25- × 0.46-cm Ultrasphere IP (5 μm) column (Beckman); the eluant used was 2% acetonitrile in 100 mM KH_2PO_4 and 25 mM TBAHS, pH 5.5. The eluant flow was 1 ml/min. Tissues were processed as described above in Measurement of [^{3}H]inositol phosphates. The trichloroacetic acid supernatants were extracted with 4 × 6 ml of diethyl ether and lyophilized. The residues were dissolved in 1 ml of the HPLC eluant and filtered on Millex-HV 13 filters. The resulting filtrates were injected onto the column, and the eluant was run for 30 min. Fractions were collected every 30 sec and were used to determine radioactivity by liquid scintillation counting. Ins(1,4,5)P_3 and Ins(1,3,4)P_3 were identified by coelution with authentic ^{3}H-labeled standards in separate runs and/or by running of the samples with the corresponding ^{3}H-labeled standards. To overcome problems associated with variations in retention times due to column aging, the column was standardized before injection by running 5′-AMP, whose absorbance was monitored at 254 nm.

Measurement of [^{3}H]phosphoinositides. The pellets obtained after centrifugation of the trichloroacetic homogenates were washed with 0.5 ml of trichloroacetic acid to remove any residual [^{3}H]inositol. Chloroform/methanol/12 M HCl (40:80:1) (2.8 ml) was then added, and the phospholipids were extracted for 30 min at room temperature (19). Chloroform (930 μl) and 0.1 N HCl (1700 μl) were then added, and two phases were obtained by centrifugation. The upper phase was discarded; the lower phase was dried under N_2. The dried lipid residues were dissolved in 100 μl of chloroform/methanol (95:5), and an aliquot of the extracts was spotted on silica gel thin layer plates presoaked in 2 mM EDTA. PtdIns, PtdIns4P, and PtdIns(4,5)P_2 were separated by developing the plates in chloroform/methanol/4 M NH_4OH (90:70:20) (20). The phospholipids were located according to their migration compared with authentic standards, detected with iodine vapor. The corresponding areas were cut into vials, to determine the radioactivity by liquid scintillation counting.

Assay of cAMP levels. cAMP was estimated in the trichloroacetic acid-soluble extracts according to the method of Gilman (21), as reported previously (14, 15). cAMP levels were expressed as pmol/mg of protein.

Data analysis. The results are expressed as means ± standard errors and were analyzed statistically using Student's *t* test. *p* values of < 0.05 were considered as significant.

Results

Inhibition by isoproterenol of carbachol, oxytocin, and fluoroaluminate stimulatory effects on the accumulation of inositol phosphates in the guinea pig myometrium, a cAMP-independent event. Data in Fig. 1 illustrate the increased generation of inositol phosphates in the guinea pig myometrium caused by carbachol and oxytocin, both used at their half-maximal effective concentration (6, 13). Prior exposure of the tissue to 20 nM isoproterenol for 1 min significantly reduced the stimulatory effects exerted by both agonists. The decrease in the generation of inositol phosphates for carbachol and oxytocin averaged 38% and 32%, respectively. Isoproterenol was similarly able to attenuate (25%) the inositol phosphate response elicited by fluoroaluminates, indicating that the inhibitory effect could be exerted on receptor-mediated as well as on G protein-mediated inositol phosphate accumulation. No significant effect of isoproterenol on basal inositol phosphates was detected. Alprenolol (Fig. 1) completely reversed the inhibition evoked by isoproterenol, indicating a β-adrenergic receptor-mediated inhibitory effect. Isoproterenol inhibited, in a dose-dependent manner, the production of inositol phosphates due to carbachol and oxytocin (Fig. 2). With both stimulatory agonists, maximal inhibition was achieved at 20 nM isoproterenol, with an EC_{50} of 0.5 nM.

Data in Table 1 further illustrate that isoproterenol at a concentration (20 nM) that caused a maximal inhibition of the inositol phosphate response was unable to evoke any detectable increase in intracellular cyclic AMP. Furthermore, forskolin (Table 1) and cholera toxin (6), under conditions that markedly increased cAMP accumulation, failed to alter the inositol phosphate response due to oxytocin.

The findings support the contention that cAMP was not involved in the inhibitory effect exerted by isoproterenol on the accumulation of inositol phosphates.

Phospholipase C degradation of PtdIns(4,5)P_2 as the target of the inhibitory effect of isoproterenol. Data in Fig. 3 illustrate the kinetics of the carbachol-mediated accumulation of each of the three inositol phosphates in a 10 mM LiCl-containing medium in the absence and presence of isoproterenol. In agreement with our previous report (13), upon addition of carbachol there was a rapid increase in $InsP_3$ (100%

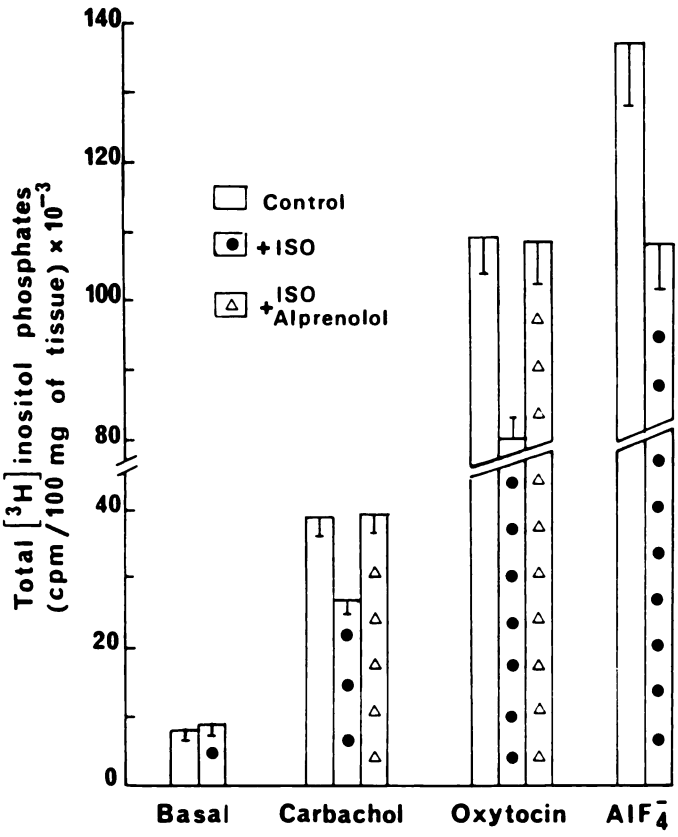

Fig. 1. Inhibitory effect of isoproterenol on inositol phosphate generation elicited by carbachol, oxytocin, and fluoroaluminate in the guinea pig myometrium. [^{3}H]Inositol-prelabeled myometrial strips were incubated with 10 mM LiCl for 10 min, followed by a 1-min treatment with or without 20 nM isoproterenol (*ISO*) before the addition of 15 μM carbachol, 20 nM oxytocin, or 20 mM NaF plus 10 μM $AlCl_3$. Incubations were stopped 20 min later. When used, alprenolol at 0.1 μM was added 5 min before isoproterenol. At the end of the incubation, tissues were extracted with 7% trichloroacetic acid and total [^{3}H]inositol phosphates were eluted from AG1-X8 columns in a single step, with 12 ml of 1 M ammonium formate/0.1 M formic acid, as described in Materials and Methods. Values are means ± standard errors of four to six independent experiments, each done in duplicate.

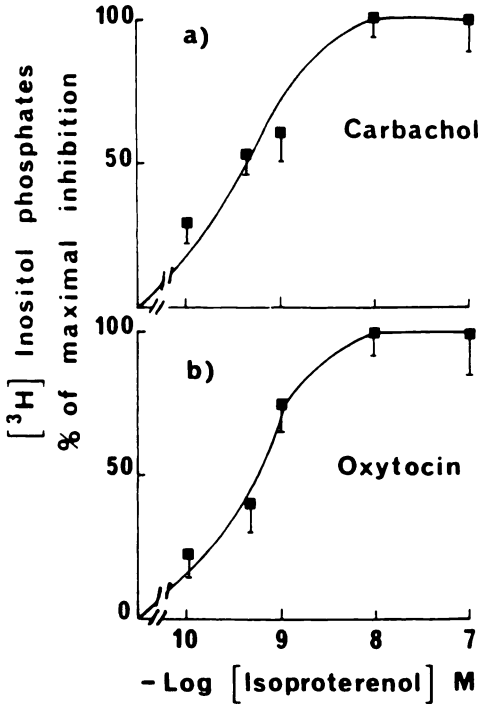

Fig. 2. Dose-dependent inhibitory effect of isoproterenol on carbachol- and ocytocin-induced inositol phosphate accumulation. [^{3}H]Inositol-labeled myometrial strips were treated for 10 min with 10 mM LiCl and incubated further for 1 min in the absence and presence of the indicated concentration of isoproterenol before the 20-min exposure to 20 nM oxytocin and 15 μM carbachol. Total [^{3}H]inositol phosphates were determined as described in the legend to Fig. 1. Results are expressed as the percentage of the maximal inhibition induced by isoproterenol on inositol phosphate accumulation due to carbachol and oxytocin (35% and 32%, respectively). Data are means ± standard errors of three independent experiments, each done in duplicate.

TABLE 1

Isoproterenol- but not forskolin-inhibited oxytocin-induced inositol phosphate accumulation in the myometrium

For determination of cAMP, myometrial strips were incubated for 7 min with isoproterenol or forskolin at the indicated concentration, each added alone or combined with oxytocin. Values for cAMP represent means ± standard errors for four experiments, each done in duplicate. For the determination of total inositol phosphates, ^{3}H-prelabeled strips were treated with 10 mM LiCl and incubated further for 1 min in the absence and presence of isoproterenol, at the indicated concentration, before the 20-min exposure to 20 nM oxytocin. When present, forskolin was added during the last 5 min of LiCl treatment. Total inositol phosphates were estimated as described in Materials and Methods. Values are means ± standard errors for three independent experiments, each done in duplicate.

Treatment	cAMP	[^{3}H]Inositol phosphates
	pmol/mg of protein	*cpm/100 mg of tissue*
None	16.0 ± 1.5	8,211 ± 975
Oxytocin (20 nM)	17.2 ± 1.5	115,527 ± 9,246
Isoproterenol (20 nM)	15.4 ± 2.1	8,742 ± 811
Isoproterenol (0.1 μM)	20.4 ± 1.8	8,109 ± 750
Isoproterenol (20 nM) + oxytocin (20 nM)	16.4 ± 1.8	80,038 ± 7,459
Isoproterenol (0.1 μM) + oxytocin (20 nM)	20.4 ± 1.8	81,215 ± 7,900
Forskolin (5 μM)	216 ± 31	8,166 ± 855
Forskolin (5 μM) + oxytocin (20 nM)	213 ± 25	118,992 ± 1,067

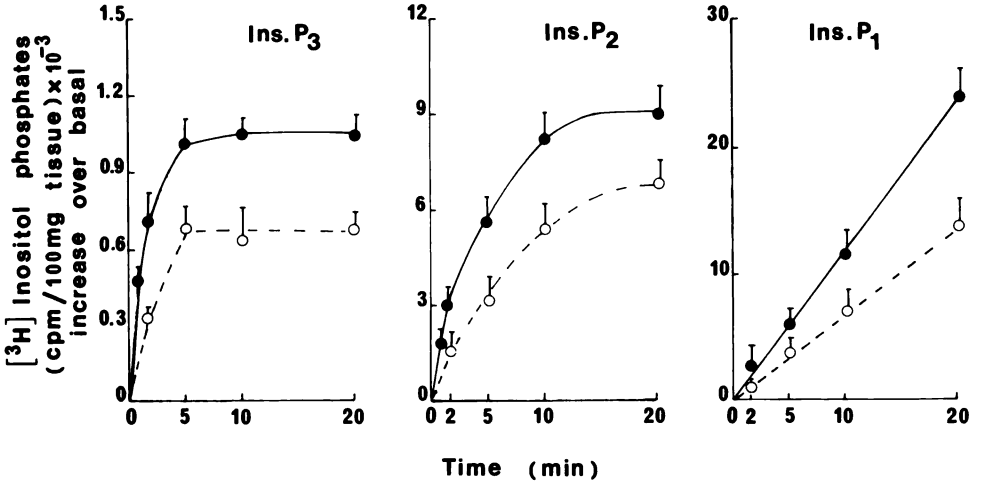

Fig. 3. Time course of carbachol-stimulated inositol phosphate generation in the absence and presence of isoproterenol. After alo-min incubation in the presence of 10 mM LiCl, [^{3}H]inositol-prelabeled strips were treated for 1 min with (○) or without (●) 20 nM isoproterenol before the addition of 15 μM carbachol. Incubations were stopped at the times indicated and individual [^{3}H]inositol phosphates were separated on AG1-X8 columns, as described in Materials and Methods. $InsP_3$, $InsP_2$, and InsP accumulation was expressed as cpm/100 mg of tissue. Values are means ± standard errors of six independent experiments, each done in duplicate.

stimulation at 30 sec), which reached a plateau at 3 min. The accumulation of $InsP_2$ and InsP was delayed (100% and 20% stimulation for $InsP_2$ and InsP, respectively). The simultaneous addition of isoproterenol resulted in a similar attenuation (35%) in the accumulation of each of the three inositol phosphates, without modifying the kinetic patterns. The sequential precursor-product relationship in the order of $InsP_3$, $InsP_2$, and InsP was still observed, reflecting that any decrease in the formation of $InsP_3$ was followed by a decrease in the accumulation of $InsP_2$ and then in the accumulation of InsP.

Data in Fig. 4 illustrate the HPLC analysis of the isomeric pattern of the $InsP_3$ fraction. It can be seen that, in the myometrium, the basal level of the $InsP_3$ pool consisted essentially of the Ins(1,4,5)P_3 isomer, with no significantly detectable Ins(1,3,4)P_3. After 1-min exposure to carbachol, there was an increased accumulation of both InsP(1,4,5)P_3 and Ins(1,3,4)P_3, but Ins(1,4,5)P_3 still remained the predominant species, accounting for 65% of the total $InsP_3$ pool. As incubation with carbachol proceeded, the $InsP_3$ fraction increased but the ratio of the two isomers differed substantially, with Ins(1,3,4)P_3 predominating gradually to reach 75% of the total $InsP_3$ pool at 20 min. These results indicate that, over a 20-min incubation with carbachol, there was a continued production of Ins(1,4,5)P_3 acting as a precursor for the generation of Ins(1,3,4)P_3, most probably through the intermediate Ins(1,3,4,5)P_4 (2). Data in Fig. 4 further show that, in the simultaneous presence of isoproterenol and carbachol, at all time points tested, the decrease in the total $InsP_3$ fraction was reflected at the level of both Ins(1,4,5)P_3 and Ins(1,3,4)P_3. Moreover, the relative proportions of both isomers were maintained, indicating that any alteration of Ins(1,4,5)P_3 formation was paralleled quantitatively by that of Ins(1,3,4)P_3. These data support the interpretation that phospholipase C degrading PtdIns(4,5)P_2 was the target for the stimulatory and inhibitory effects of carbachol and isoproterenol, respectively.

In order to define more clearly the nature of the inositol phospholipid species whose degradation would be preferentially affected by carbachol and isoproterenol, further experiments were conducted to assess the distribution of [^{3}H]inositol in PtdIns, PtdIns4P, and PtdIns(4,5)P_2. The results in Table 2 indicate that, in control tissue, most of the [^{3}H]inositol was incorporated into PtdIns (86% of the total inositol phospholipid pool) whereas PtdIns4P and PtdIns(4,5)P_2 accounted for 6.5% and 7.5%, respectively. After the addition of carbachol to myometrial strips, PtdIns(4,5)P_2 level decreased rapidly below the initial level, to reach a value of 67% of control within 1 min. The decrease of PtdIns(4,5)P_2 persisted for at least 3 min. The amount of PtdIns4P was slightly decreased, whereas that of PtdIns was not altered significantly; they were 88% and 100% of the control value, respectively. The levels of PtdIns4P and PtdIns remained constant for incubation times as long as 20 min in the presence of the muscarinic agonist. At that time, the level of PtdIns(4,5)P_2 had almost totally recovered, indicating that it was continuously replenished from the labeled

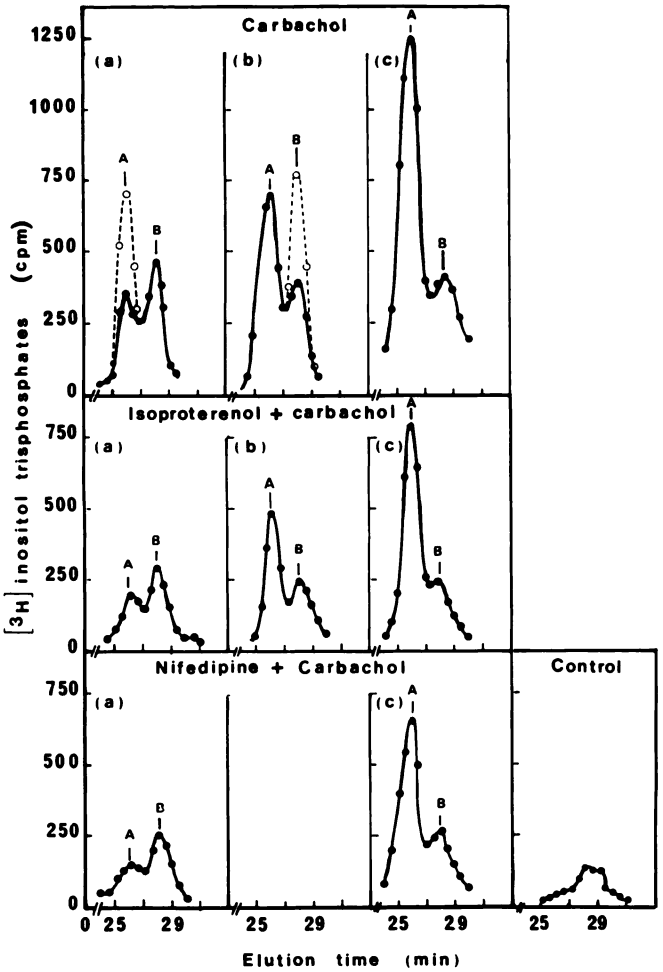

Fig. 4. Separation by HPLC of [^{3}H]Ins(1,4,5OP$_3$ and [^{3}H]Ins(1,3,4)P$_3$ in carbachol-treated myometrium. Effect of isoproterenol and nifedipine. After a 10-min exposure to 10 mM LiCl, myometrial strips prelabeled with [^{3}H]inositol (17 μCi/ml) were treated without (control) and with 20 nM isoproterenol or 250 nM nifedipine. Carbachol (15 μM) was then added and the incubations were terminated after 1 (a), 3 (b), or 20 min (c), by the addition of 7% trichloracetic acid. After four successive washings with diethyl ether, the extracts were lyophylized and the residues were dissolved in the HPLC eluant before application to the column and elution, as described in Materials and Methods. ——, Elution profile of the InsP$_3$ fraction for the different samples. Where indicated, two successive runs were conducted with aliquots of the same sample, without (——) and with (– – –) the addition of standard [^{3}H]Ins(1,3,4)P$_3$ or [^{3}H]Ins(1,4,5)P$_3$. *Peaks A* and *B*, Ins(1,3,4)P$_3$ and Ins(1,4,5)P$_3$, respectively. Results are expressed as cpm/100 mg of tissue versus elution time. Data are from one of three experiments, giving similar results.

PtdIns4P and PtdIns pools. In the simultaneous presence of isoproterenol and carbachol, the decline in the levels of PtdIns(4,5)P$_2$ could still be observed, but to a lesser extent than that obtained with the muscarinic agonist alone [32% and 20% decrease in PtdIns(4,5)P$_2$ for tissues incubated with carbachol alone and carbachol combined with isoproterenol, respectively]. Similar to the situation with carbachol, the levels of PtdIns(4,5)P$_2$ progressively increased and reached the control values after a 20-min exposure to both carbachol and isoproterenol. Thus, the presence of isoproterenol up to 20 min did not affect the levels of PtdIns4P and PtdIns. These data provide additional evidence that, in the myometrium, PtdIns(4,5)P$_2$ is the first target for phospholipase C activation due to the muscarinic agonist and that isoproterenol exerts its inhibitory effect on the accumulation of inositol phosphates by decreasing the hydrolytic activity of phospholipase C acting on PtdIns(4,5)P$_2$. It is also evident that the β-adrenergic-induced inhibition is not due to an impaired availability of the inositol phospholipid substrate(s). It is also worth noting that, despite the presence of LiCl, which, by inhibiting inositol phosphate phosphatases, would have limited the availability of free inositol for phosphatidylinositol recycling, the levels of the different phosphoinositide pools remained unaffected after a prolonged muscarinic receptor-induced stimulation of the generation of inositol phosphates. The constant recycling of the inositol phospholipids observed under these conditions is certainly due to the substantial retention of free [^{3}H]inositol in the myometrial strips,[1] despite extensive washing of the tissue after the [^{3}H]inositol prelabeling step.

Involvement of a dihydropyridine-sensitive Ca^{2+} component in the accumulation of inositol phosphates elicited by carbachol, oxytocin, and fluoroaluminates. Fig. 5 shows that high K^+ (30 mM), which was reported to cause membrane depolarization and uterine contractions (22), resulted in an increased accumulation of total inositol phosphates. A similar enhancement of inositol phosphate accumulation was also obtained with the calcium ionophore ionomycin. Both ionomycin and K^+ stimulatory effects were abolished when Ca^{2+} was omitted from the incubation medium, indicating that the accumulation of inositol phosphates induced by both compounds was related to an influx of extracellular Ca^{2+}. Table 3 shows that ionomycin stimulated the formation of InsP$_3$, InsP$_2$, and InsP. At 1 min, a 150% stimulation of InsP$_3$ accumulation could already be detected, with no significant effect on InsP$_2$ and InsP accumulation. An increase of these two latter compounds could be evidenced only after 3 min of ionomycin treatment. As also shown in Table 3, the isomeric composition of the InsP$_3$ fraction was made of both Ins(1,4,5)P$_3$ and Ins(1,3,4)P$_3$, and the relative levels of the two isomers changed with time; Ins(1,4,5)P$_3$ predominated at the earliest time (0.5–1 min), whereas Ins(1,3,4)P$_3$ gradually increased and predominated at the latest time (3–20 min). Similar to the data obtained with ionomycin, stimulation by high K^+ resulted also in the production of both Ins(1,4,5)P$_3$ and Ins(1,3,4)P$_3$ (data not shown). It has been verified (data not shown) that the stimulatory effects of K^+ and ionomycin on inositol phosphate accumulation persisted in the presence of inhibitors of the cyclooxygenase and lipoxygenases, i.e., indomethacin and eicosatetraynoic acid, respectively, ruling out the possibility that the Ca^{2+}-induced stimulations resulted from the generation of arachidonic acid metabolites (23). Our findings support, rather, the contention that, in guinea pig myometrium, rises in intracellular Ca^{2+} due to an increased influx of the cation may trigger activation of phospholipase C, degrading PtdIns(4,5)P$_2$.

It seemed, therefore, of interest to examine the role of extracellular Ca^{2+} in controlling the generation of inositol phosphates induced by agonists. The results presented in Fig. 5 show that omission of Ca^{2+} from the incubation medium resulted in a slight but consistent attenuation of the increased generation of inositol phosphates caused by carbachol, oxytocin, and fluoroaluminates (36%, 38%, and 25% decrease, respectively). Attenuation could similarly be detected at the level of InsP$_3$, InsP$_2$, and InsP (values for InsP$_3$, InsP$_2$, and InsP

[1] Unpublished observations.

TABLE 2

Changes in the [^{3}H]inositol incorporation into phosphoinositides after stimulation of myometrium with carbachol in the absence and presence of isoproterenol and nifedipine

After a 10-min incubation in the presence of 10 mM LiCl, [^{3}H]inositol-prelabeled myometrial strips were incubated for 1 min in the absence and presence of 250 nM nifedipine or 20 nM isoproterenol and were then challenged with 15 μM carbachol. At the indicated times, the incubations were stopped with 7% trichloroacetic acid and the ^{3}H-labeled phospholipids were extracted and separated by thin layer chromatography on silica gel plates, in a solvent system containing $CHCl_3$/methanol/4 M NH_4OH (90:70:20). The lipids were located according to their migration compared with authentic standards detected with iodine vapor, cut, and counted for radioactivity as described in Materials and Methods. Incorporation of ^{3}H into PtdInsP, PtdIns4P, and PtdIns(4,5)P_2 is expressed as cpm/100 mg of tissue. Values are means for the three different experiments, and the standard error did not exceed 10% of the mean.

Addition	[^{3}H]Inositol incorporated								
	PtdInsP$_2$			PtdInsP			PtdIns		
	1 min	3 min	20 min	1 min	3 min	20 min	1 min	3 min	20 min
	cpm/100 mg of tissue								
None		8,500			7,100			101,000	
Carbachol (15 μM)	5,880	5,990	8,200	6,700	6,500	6,500	110,000	113,000	113,000
Isoproterenol (20 nM) + carbachol (15 μM)	6,850	6,750	9,920	6,800	6,500	6,800	95,000	107,000	120,000
Nifedipine (250 nM) + carbachol (15 μM)	8,200	ND[a]	9,350	6,500	ND	6,900	125,000	ND	120,000

[a] ND, not determined.

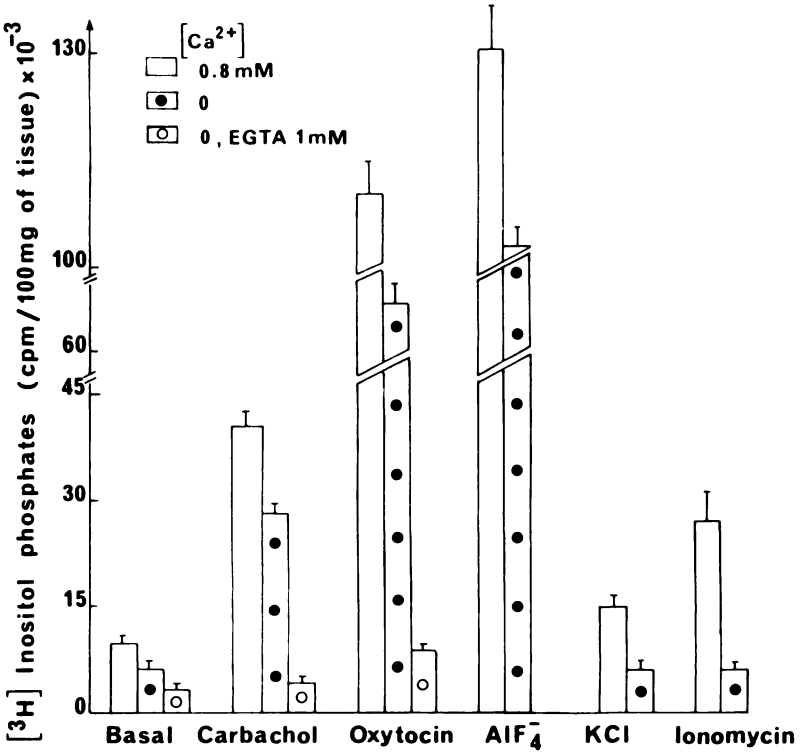

Fig. 5. Effects of ionomycin and KCl on inositol phosphate accumulation in the guinea pig myometrium. Role of Ca^{2+} in carbachol; oxytocin; and fluoroaluminate-mediated stimulations. After the [^{3}H]inositol prelabeling step, myometrial strips were transferred to a fresh (0.8 mM Ca^{2+}) Krebs bicarbonate buffer or to a Ca^{2+}-depleted medium supplemented with (○) or without (●) 1 mM EGTA and were allowed to equilibrate for 5 min before the 10-min further incubation with 10 mM LiCl. Incubations were then continued for 15 min in the absence and presence of 15 μM carbachol, 20 nM oxytocin, or 20 mM NaF plus 10 μM $AlCl_3$, 1 μM ionomycin, or 30 mM KCl. Total inositol phosphates were estimated as described in Materials and Methods. Values are means ± standard errors of four or five experiments, each done in duplicate.

were 24,000 ± 2,350, 12,000 ± 1,350, and 1,450 ± 122 cpm/100 mg of tissue, respectively, in the presence of Ca^{2+} and 18,000 ± 1,840, 8,400 ± 720, and 971 ± 102 cpm/100 mg of tissue, respectively, in the absence of Ca^{2+}). Basal levels of inositol phosphates were also reduced under these conditions. Although in the absence of extracellular Ca^{2+} total inositol phosphate accumulation was attenuated, the degree of stimulability by each agonist remained virtually unchanged (260 and 280% stimulation for carbachol and 995 and 890% stimulation for oxytocin in the presence and absence of Ca^{2+}, respectively). These findings indicated that the cascade of the signaling system involving receptor-G protein-phospholipase C interactions was not regulated by an influx of extracellular Ca^{2+}. Nevertheless, substantial depletion of Ca^{2+}, by treatment of the myometrium with 1 mM EGTA in the absence of Ca^{2+}, reduced basal levels of inositol phosphates and greatly altered the accumulation of inositol phosphates due to carbachol and oxytocin. A minimum content of Ca^{2+} was required for an optimal expression of phospholipase C activity (13).

It remained to assess to what extent the Ca^{2+} entry-dependent process, which was revealed with ionomycin, might also contribute to the attenuation of the overall agonist-induced increases in inositol phosphates detected in myometrial strips when Ca^{2+} was withdrawn from the incubation medium. Experiments were thus conducted in the presence of inhibitors of the voltage-gated Ca^{2+} channels, i.e., nifedipine and verapamil (24). As shown in Table 4, both nifedipine (250 nM) and verapamil (30 μM) caused an attenuation of the accumulation of inositol phosphates promoted by carbachol (38% and 33% decrease, respectively) and oxytocin (32% and 30% decrease,

TABLE 3

Time-dependent accumulation of inositol phosphates induced by ionomycin

[^{3}H]Inositol-labeled myometrial strips were incubated in the presence of 10 mM LiCl for 10 min and challenged with 1 μM ionomycin. At the indicated times, tissues were extracted with 7% trichloroacetic acid. The extracts were then divided into two parts; one part was applied to AG1-X8 columns for the separation of InsP, $InsP_2$, and $InsP_3$, as described in the legend to Fig. 3, and the second part was processed for HPLC analysis for the separation of $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$, as described in the legend to Fig. 4. Data are from one of three experiments, giving similar results.

	Inositol phosphate accumulation				
	0 min	0.5 min	1 min	3 min	20 min
	cpm/100 mg of tissue				
$InsP_3$	580	765	870	1,320	1,850
$Ins(1,3,4)P_3$	166	219	326	896	1,540
$Ins(1,4,5)P_3$	414	546	544	424	310
$InsP_2$	2,010	2,050	2,300	5,200	7,200
InsP	4,020	4,150	4,400	7,260	18,900

respectively) in a normal Ca^{2+}-containing medium. Nifedipine similarly decreased (24%) the inositol phosphate stimulations evoked by fluoroaluminates, which bypass receptor interaction. In all cases, the degree of inhibition was similar to that elicited by Ca^{2+} withdrawal from the medium. Both Ca^{2+} channel antagonists were without effect on basal inositol phosphate levels. Nifedipine, as expected, markedly attenuated (75% inhibition) the increased generation of inositol phosphates due to high K^+. As shown in Fig. 4, analysis of the $InsP_3$ fraction from the tissue treated with carbachol and nifedipine indicated that the nifedipine attenuating effect coincided with a decrease in both $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$. Furthermore, nifedipine totally prevented the decrease in $PtdIns(4,5)P_2$ caused by carbachol at the earliest time point tested (Table 2). Therefore, similar to isoproterenol, the inhibitory effect of nifedipine on inositol phosphate accumulation appears to be located at the level of phospholipase C, degrading $PtdIns(4,5)P_2$.

Taken together, these findings would imply that, in addition to the direct coupling of receptors to phospholipase C activation via a G protein, a process that contributes predominantly to the increased generation of inositol phosphates, there is also a coupling of cell surface receptors to voltage-operated Ca^{2+} channels. The resulting increase in Ca^{2+} influx would then be responsible for the additional, although modest (35%), receptor-induced Ca^{2+}-mediated phospholipase C activation.

Involvement of a dihydropyridine-sensitive Ca^{2+} channel in the inhibitory effect of isoproterenol. As shown above, the increased generation of inositol phosphates caused by oxytocin and carbachol could similarly be attenuated either by removal of Ca^{2+} from the medium (Fig. 5) or by the addition of isoproterenol (Fig. 1). Of importance were the findings (Fig. 6) that isoproterenol could no longer exert any attenuation of the inositol phosphate response when incubations were carried in a Ca^{2+}-free medium, suggesting that a common mechanism may operate under both conditions. The interpretation was further supported by the findings obtained in the presence of nifedipine; the simultaneous addition of both nifedipine and isoproterenol did not result in an inhibition greater than that obtained with either agent alone.

Data in Table 5 illustrate the failure of both isoproterenol and nifedipine to attenuate the increased generation of inositol phosphates evoked by ionomycin. Of further importance was the ability of the Ca^{2+} ionophore to counteract the inhibitory effect normally exerted by isoproterenol and nifedipine. When carbachol was added together with ionomycin, a further significant stimulation of inositol phosphate production was observed, but under these conditions the inhibitory effect of isoproterenol, as well as that of nifedipine, was no longer seen. In the simultaneous presence of ionomycin, isoproterenol, similarly, could no longer attenuate the increased generation of inositol phosphates caused by oxytocin. It is also worth noting that isoproterenol failed to inhibit the increased generation of isoproterenol phosphates due to high K^+ (values for [^{3}H]inositol phosphates with 30 mM KCl were 14,235 ± 1,231 and 15,863 ± 1,184 cpm/100 mg of tissue in the absence and presence of isoproterenol, respectively). Conversely, high K^+ was able to prevent the inhibitory effect of isoproterenol versus carbachol-induced inositol phosphate accumulation (in the presence of 30 mM KCl, values for [^{3}H]inositol phosphates due to carbachol were 39,572 ± 3,393 and 41,370 ± 4,106 cpm/100 mg of tissue without and with isoproterenol, respectively). These findings would tend to suggest that the attenuated effect exerted by isoproterenol was related to an inhibition of Ca^{2+} influx and operated at the level of the Ca^{2+} component contributing to the inositol phosphate response triggered by receptor and fluoroaluminate activation. Additionally, the inhibitory effect of isoproterenol on the increased generation of inositol phosphates due to carbachol was antagonized by Bay K 8644 (25), similar to the antagonistic effect exerted by the Ca^{2+} channel agonist on the nifedipine-induced attenuated inositol phosphate responses (Table 5). These data would imply that the action of isoproterenol to attenuate the generation of inositol phosphates resulted from an inhibition of Ca^{2+} influx through nifedipine-sensitive Ca^{2+} channels.

Blockade of isoproterenol inhibitory effect by pertus-

TABLE 4

Inhibitory effects of nifedipine and verapamil on the generation of inositol phosphates induced by carbachol, oxytocin, fluoroaluminates, and high K^+

After a 10-min incubation in the presence of 10 mM LiCl, [^{3}H]inositol-prelabeled myometrial strips were exposed for 1 min to 250 nM nifedipine or to 30 μM verapamil and then challenged for 20 min with 15 μM carbachol, 20 nM oxytocin, 20 mM NaF plus 10 μM $AlCl_3$, or 30 mM KCl. Total inositol phosphates were estimated as described in Materials and Methods. Values are means ± standard errors for three to six experiments, each done in duplicate.

Additions	Total [^{3}H]inositol phosphates		
	Control	Nifedipine	Verapamil
	cpm/100 mg of tissue		
None	8,791 ± 975	8,212 ± 811	7,612 ± 800
Carbachol (15 μM)	39,802 ± 3,102	27,200 ± 1,860	27,634 ± 2,500
Oxytocin (20 nM)	111,034 ± 11,250	78,218 ± 6,919	81,041 ± 7,100
NaF (20 mM) + $AlCl_3$ (10 μM)	143,040 ± 12,119	110,957 ± 10,698	ND[a]
KCl (30 mM)	18,060 ± 1,602	11,350 ± 947	ND

[a] ND, not determined.

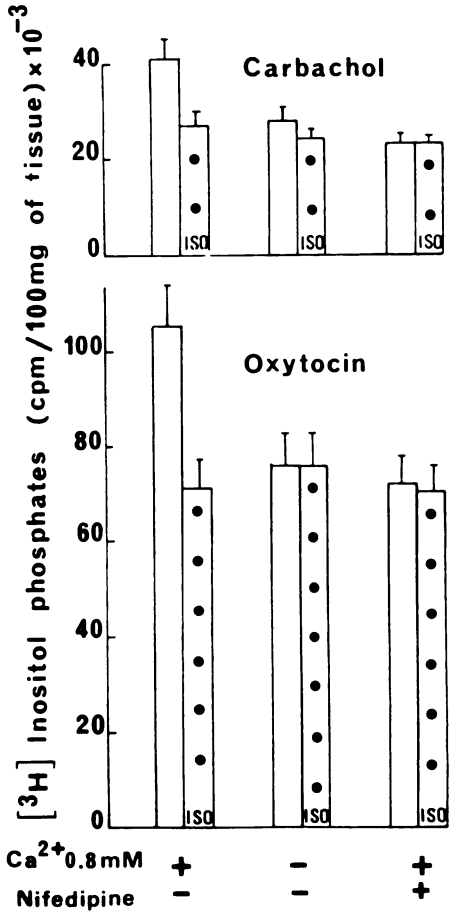

Fig. 6. Loss of the inhibitory effect of isoproterenol on the accumulation of inositol phosphates by removal of extracellular Ca^{2+} and by nifedipine. After the [^{3}H]inositol prelabeling step, myometrial strips were transferred to a fresh (0.8 mM Ca^{2+}) Krebs bicarbonate buffer or to a Ca^{2+}-depleted medium and were allowed to equilibrate for 5 min before the 10-min further incubation in the presence of 10 mM LiCl. When used, nifedipine (250 nM) was added in a normal Ca^{2+}-containing medium during the last minute of LiCl treatment. Tissues were then incubated without (□) or with (⊡) 20 nM isoproterenol for 1 min, followed by the addition of 15 μM carbachol or 20 nM oxytocin. Incubations were stopped 20 min later. Total [^{3}H]inositol phosphates were determined as described in Materials and Methods. Values are means ± standard errors of five or six experiments, each done in duplicate.

sis toxin. In additional experiments, the inhibitory effect of isoproterenol was analyzed in myometrial strips that had been pretreated with pertussis toxin (400 ng/ml for 6 hr). These conditions have been reported to abolish muscarinic receptor-mediated activation of G_i in the myometrium (6). Data in Table 6 show that, as previously reported (6), pertussis toxin treatment did not affect carbachol- and oxytocin-mediated stimulations of inositol phosphate accumulation. In contrast, the inhibitory effect of isoproterenol on the increased generation of inositol phosphates elicited by carbachol and oxytocin was markedly reduced. These data imply that a G protein of the G_i or G_o family (5) was involved in the β-adrenergic-mediated attenuation of inositol phosphate accumulation.

Discussion

The reported experiments demonstrate that, in the intact guinea pig myometrium, two distinct mechanisms underly the activation of phospholipase C degrading (PtdIns(4,5)P_2, with the sequential generation of $InsP_3$, $InsP_2$, and InsP (6, 13). One mechanism concerns the well accepted agonist-induced receptor-G protein-phospholipase C cascade activation, which appears to be insensitive to elevations in intracellular Ca^{2+}, and a second, Ca^{2+}-dependent, pathway, which involves activation of phospholipase C through an increased influx of Ca^{2+}.

Some evidence was previously reported that suggested that PtdIns(4,5)P_2 was the target substrate for the enhanced phospholipase C activity induced by carbachol, oxytocin, and fluoroaluminates (6, 13). It was shown that, upon addition of the stimulatory agonists to myometrial strips, exemplified in the present paper with carbachol (Fig. 3), there was a rapid increase in $InsP_3$, which preceded that of $InsP_2$ and InsP. Also, using a receptor-blocking approach (6), it was found that addition of atropine after a 20-min stimulation of the tissue with carbachol in the presence of LiCl resulted in a progressive return to the basal values for both $InsP_3$ and $InsP_2$, with an early change detected for $InsP_3$, whereas the rate of InsP was maintained during the first 3–5 min of atropine treatment. These observations were taken as indirect evidence that, for the generation of inositol phosphates, there was a sequential precursor-product relationship in the order of $InsP_3$, $InsP_2$, and InsP. The present study extends these findings. The HPLC analysis of the $InsP_3$ fraction showed that, at all time points tested, carbachol stimulation resulted in the formation of both Ins(1,4,5)P_3 and Ins(1,3,4)P_3 isomers. At the earliest time tested, Ins(1,4,5)P_3 accumulated to a greater extent than Ins(1,3,4)P_3, accounting for 65% of the total $InsP_3$ pool; as incubation proceeded, Ins(1,3,4)P_3 predominated gradually and became the major isomer. These results were taken as indirect evidence that sustained muscarinic receptor-stimulated accumulation of Ins(1,3,4)P_3 arises from the persistent stimulated hydrolysis of PtdIns(4,5)P_2, which produces Ins(1,4,5)P_3, acting as a precursor for the generation of Ins(1,3,4)P_3, probably through the intermediate Ins(1,3,4,5)P_4. Although an isomeric heterogeneity of polyphosphoinositides has been described recently (26), it seems unlikely that the accumulation of Ins(1,3,4)P_3 could be ascribed predominately to the degradation of PtdIns(3,4)P_2 (27). Furthermore, PtdIns(3,4)P_2 has been demonstrated to be a poor substrate for purified phospholipase C (28). Additional evidence confirming that the inositol lipid hydrolyzed by receptor-mediated phospholipase C was PtdIns(4,5)P_2 was provided by a decrease in the level of PtdIns(4,5)P_2 at a time point corresponding to the earliest rise in Ins(1,4,5)P_3 formation due to carbachol.

Support for a Ca^{2+} entry-mediated regulation of phospholipase C was provided by the ability of agents such as high K^+ and the Ca^{2+} ionophore ionomycin to cause, independently of receptor activation, an increased breakdown of PtdIns(4,5)P_2. Analogous to the pattern of inositol phosphate accumulation induced by carbachol, ionomycin caused an early production of $InsP_3$, followed by that of $InsP_2$ and InsP. The HPLC analysis of the $InsP_3$ fraction revealed a concomitant increase of both Ins(1,4,5)P_3 and Ins(1,3,4)P_3, with a predominant accumulation of Ins(1,4,5)P_3 at the earliest time (0.5–1 min) and accumulation of Ins(1,3,4)P_3 at the latest time (20 min). The responses to both high K^+ and ionomycin were prevented when Ca^{2+} was withdrawn from the incubation medium, indicating that an increase in Ca^{2+} entry was responsible for the observed stimulations. A Ca^{2+}-induced generation of endogeneous modulators, more specifically arachidonic acid metabolites, that could have

TABLE 5

Reversal by Bay K 8644 and ionomycin of both isoproterenol and nifedipine inhibitory effects on inositol phosphate accumulation

[^{3}H]Inositol-labeled myometrial strips were treated for 10 min with 10 mM LiCl and incubated further for 1 min in the absence and presence of isoproterenol (20 nM) or nifedipine (250 nM) before the 20-min exposure to carbachol (15 μM) and ionomycin (1 μM), combined or added individually. When used, Bay K 8644 (5 μM) was added 5 min before the addition of isoproterenol and nifedipine. Total inositol phosphates were determined as described in Materials and Methods. Values are means ± standard errors for three to six independent experiments, each done in duplicate.

Additions	Total [^{3}H]inositol phosphates		
	Control	Isoproterenol	Nifedipine
	cpm/100 mg of tissue		
None	8,672 ± 712	8,791 ± 745	8,821 ± 773
Carbachol (15 μM)	39,546 ± 3,126	27,642 ± 2,259	28,842 ± 2,586
Ionomycin (1 μM)	28,636 ± 1,529	29,818 ± 2,789	27,900 ± 2,750
Carbachol (15 μM) + ionomycin (1 μM)	66,713 ± 5,251	69,758 ± 6,779	62,325 ± 5,986
Oxytocin (20 nM)	115,527 ± 9,246	80,038 ± 6,459	ND[a]
Oxytocin (20 nM) + ionomycin (1 μM)	150,870 ± 13,560	154,459 ± 13,320	ND
Bay K 8644 (5 μM)	7,671 ± 634	7,637 ± 728	8,318 ± 719
Bay K 8644 (5 μM) + carbachol (15 μM)	38,826 ± 3,260	42,500 ± 3,860	37,920 ± 3,521

[a] ND, not determined.

TABLE 6

Reversal by pertussis toxin of isoproterenol inhibitory effects on inositol phosphate accumulation

Myometrial strips were incubated for 6 hr in the absence and presence of 400 ng/ml pertussis toxin; [^{3}H]inositol was added during the last 4 hr of the pertussis toxin treatment. Tissues were then washed with fresh buffer. Rechallenge incubations were conducted with 10 mM LiCl for 10 min, followed by 1-min incubation with and without isoproterenol (20 nM). Carbachol (15 μM) and oxytocin (20 nM) were then added and incubations were further continued for 20 min. Total inositol phosphates were determined as described in Materials and Methods. Values are means ± standard errors for three experiments, each done in duplicate.

Additions	Total [^{3}H]inositol phosphates	
	−Pertussis toxin treatment	+Pertussis toxin treatment
	cpm/100 mg of tissue	
Carbachol (15 μM)	40,826 ± 3,613	40,681 ± 3,457
Isoproterenol (20 nM) + carbachol (15 μM)	28,567 ± 2,306	40,588 ± 3,641
Oxytocin (20 nM)	128,565 ± 10,961	132,828 ± 10,926
Isoproterenol (20 nM) + oxytocin (20 nM)	87,466 ± 8,335	131,072 ± 11,075

activated the generation of inositol phosphates via the receptor-G protein-phospholipase C cascade did not seem to contribute predominantly to the high K^+- and ionomycin-mediated stimulations. Our conclusion that an increase in cytoplasmic Ca^{2+} can activate PtdIns(4,5)P_2 cleavage by phospholipase C in the myometrium is consistent with previous observations made in many excitable cells (29).

Of interest were the findings that the Ca^{2+}-dependent component of phospholipase C regulation also contributed, although to a minor (35%) extent, to the overall inositol phosphate responses evoked by carbachol and oxytocin (receptor activation), as well as by AlF_4^- (direct G activation). Biochemical evidence that part of the receptor-mediated generation of inositol phosphates was dependent on the influx of Ca^{2+} through voltage-gated Ca^{2+} channels was further provided. Indeed, the production of inositol phosphates triggered by carbachol, oxytocin, and AlF_4^- was similarly reduced (30–35%) by removal of extracellular Ca^{2+} or by the Ca^{2+} channel blockers nifedipine and verapamil, in the same range of concentration as those required to prevent the increase in inositol phosphates evoked by high K^+. The inhibitory effect of nifedipine towards carbachol-induced stimulations was revealed at the level of the individual inositol phosphates, i.e., InsP, InsP_2, and both Ins(1,4,5)P_3 and Ins(1,3,4)P_3. The early decrease in PtdIns(4,5)P_2 normally evoked by carbachol was also prevented in the simultaneous presence of nifedipine. It was thus reasonable to consider that the inhibitory effect of nifedipine, by blocking Ca^{2+} entry, was operating at the level of phospholipase C degrading PtIns(4,5)P_2. It is worth noting that the Ca^{2+} channel agonist Bay K 8644 was able to reverse inhibition caused by nifedipine at the level of the inositol phosphate responses induced by carbachol. The data indirectly imply a functional coupling of oxytocin and muscarinic receptors to the activation of dihydropyridine-sensitive Ca^{2+} channels, whose presence, based on biochemical and functional observations, has been described in the myometrium (24, 30). The similarities in the observations made with AlF_4^- support the contention that a G protein is contributing to the activation of a voltage-operated Ca^{2+} channel in the myometrium. It is becoming clear that hormones and neurotransmitters can modulate voltage-operated Ca^{2+} channels through the intervention of G proteins in many excitable cells (31–33). Two G protein-involving mechanisms are proposed. G proteins may participate distantly and indirectly in channel modulation because they mediate hormonal regulation of intracellular messengers that ultimately modulate, mostly via phosphorylation reactions, the activity of the channel. It is possible that, in the myometrium, the two prospective second messengers, Ins(1,4,5)P_3 and diacylglycerol, that are generated through muscarinic and oxytocin receptor-mediated activation of phospholipase C may serve such a regulatory function (34, 35). On the other hand, there is supportive evidence that G proteins also couple membrane receptors to ionic channels, including voltage-gated Ca^{2+} channels, by a membrane-confined mechanism that does not involve cytosolic signal molecules (31–33, 36). Our present data do not allow us to discriminate between a direct and an indirect G protein pathway for the increased transmembrane Ca^{2+} influx, sensitive to nifedipine, that contributes to the agonist-induced, Ca^{2+}-dependent production of inositol phosphates.

The present experiments have further revealed the ability of isoproterenol to exert a negative modulation at the level of the generation of inositol phosphates triggered by carbachol, oxytocin, and AlF_4^-. Inhibition by isoproterenol reflected a β-adrenergic receptor-mediated event and could be noted at the level of both InsP_3 isomers, Ins(1,4,5)P_3 and Ins(1,3,4)P_3, as well as at the level of InsP_2 and InsP, with no change in the rate of the sequential appearance of InsP_3, InsP_2, and InsP

upon carbachol stimulation. These data, added to the observation that addition of isoproterenol also resulted in an attenuation of the decrease in PtdIns(4,5)P_2 levels caused by carbachol at the early points of stimulation, supported the interpretation of an inhibitory effect of isoproterenol on PtdIns(4,5)P_2 hydrolysis. The findings that isoproterenol similarly reduced inositol phosphate formation when the receptor was bypassed with AlF_4^- suggested a post-receptor site for the β-adrenergic inhibitory effect. In a number of cell types, elevated cAMP levels were found to be associated with an attenuation of agonist-induced phosphoinositide hydrolysis (37, 38). In most cases, this attenuation appeared to reflect cAMP-dependent inhibition of G protein-phospholipase C coupling. Such a mechanism did not seem to contribute to the β-adrenergic-mediated inhibition of inositol phosphate formation in the guinea pig myometrium. Indeed, no clear-cut rises in cAMP could be detected with isoproterenol at concentrations (20–100 nM) that were already supramaximal for inducing inhibition of agonist-mediated inositol phosphate accumulation. Additionally, two agents, namely forskolin and choleratoxin, that caused marked elevations of cAMP (6, 15) failed to attenuate the generation of inositol phosphates elicited by oxytocin. Finally, and more importantly, the finding that isoproterenol-mediated inhibition of inositol phosphate accumulation was prevented by pretreatment of the myometrium with pertussis toxin indicated that a G protein of the G_i or G_o family was involved in the coupling of the β-adrenergic receptor to the attenuated phospholipase C activity. This further excluded the participation of G_s and the stimulatory pathway of the adenylate cyclase in the β-adrenergic inhibition. Taken together, the data favor, rather, the interpretation that the β-adrenergic inhibitory effect on the breakdown of PtdIns(4,5)P_2 in the guinea pig myometrium is a cAMP-independent, pertussis toxin-sensitive event.

As recently reviewed (9), activation of a number of receptors can lead to an inhibition of phosphoinositide metabolism. In some (11, 12), although not all (8–10), cases, it has been reported that this effect could be a direct result of receptor-mediated inhibition of phospholipase C and that an inhibitory counterpart, G_{pi}, of the stimulatory protein G_{ps} may be the direct link between these receptors and phospholipase C. Our present findings in the myometrium seem to exclude a direct link between β-adrenergic receptors and phospholipase C. Supportive evidence was provided, rather, for a β-adrenergic interference with the Ca^{2+} entry-dependent process that contributes to phospholipase C activation. 1) The extent of inhibition of agonist-induced generation of inositol phosphates, including the active Ins(1,4,5)P_3 isomer, by isoproterenol was of the same order of magnitude as that reached by omission of Ca^{2+} from the medium and that caused by the Ca^{2+} channel blocker nifedipine in the absence of isoproterenol. No additional β-adrenergic inhibitory effect could be demonstrated in a Ca^{2+}-depleted medium and, similarly, inhibitions elicited by isoproterenol and by nifedipine were not additive, suggesting a common inhibitory pathway. 2) Stimulations of inositol phosphate accumulation triggered by agents that elevate intracellular Ca^{2+}, namely high K^+ and ionomycin, were totally insensitive to the inhibitory action of isoproterenol. 3) Furthermore, high K^+ as well as ionomycin were able to counteract the β-adrenergic receptor-mediated inhibition at the level of inositol phosphate accumulation induced by receptor and G protein activation. 4) The Ca^{2+} channel agonist Bay K 8644, which antagonized nifedipine-mediated inhibitory effects, similarly antagonized inhibition triggered by isoproterenol. These data strongly indicated that isoproterenol was affecting the portion of the agonist-induced increase in the production of inositol phosphates that apparently depends on nifedipine-sensitive Ca^{2+} influx. We propose, based on the lines of evidence discussed above, that β-adrenergic receptor-mediated inhibition of PtdIns(4,5)P_2 breakdown is most probably secondary to inhibition of Ca^{2+} entry through voltage-gated Ca^{2+} channels, which in turn is responsible for the attenuated phospholipase C activity. This proposal fits with recent observations in isolated lactograph cells (9), demonstrating that the decrease in the accumulation of inositol phosphates induced by dopamine is secondary to a decrease in cytosolic Ca^{2+} concentrations caused by the hormone-dependent closure of a Ca^{2+} channel. Finally, it is worth recalling that, similar to high K^+, carbachol was demonstrated to cause depolarization of the uterine plasma membrane (39). The failure of isoproterenol to counteract the rises in inositol phosphates caused by high K^+ may clearly imply that the β-adrenergic agonist would interfere with the function of the L-type channel only if its activation was triggered by a G protein-mediated event. Of relevance are our present observations of a consistently weaker inhibitory effect of isoproterenol and nifedipine towards fluoroaluminate-mediated than towards carbachol-mediated stimulations (25% and 35% inhibition, respectively). This may possibly be due to the differential activated states of the G protein generated by the liganded receptor or by a direct interaction with fluoroaluminates (5).

Our interpretations indirectly imply that activation of β-adrenergic receptors in the myometrium might be linked directly, via a pertussis toxin-sensitive G protein, to an inhibition of voltage-gated Ca^{2+} channels. This would be analogous to the direct receptor-mediated inhibition of Ca^{2+} channels, involving pertussis toxin-sensitive G proteins, in neuronal and endocrine cells (31, 33). Our results could, however, also be accounted for by the coupling of β-adrenergic receptors to a K^+ channel, similar to the recently demonstrated dopamine- and somatostatin-induced increases in K^+ conductance in clonal pituitary cells (9). Hyperpolarization due to the activation of this channel would inhibit Ca^{2+} influx, thus indirectly affecting phosphoinositide hydrolysis. Along this line, it is worth mentioning a recent report describing the ability of norepinephrine to activate myometrial K^+ channels incorporated into lipid bilayers and the dependency of stimulation upon the activation of G proteins coupled to β-adrenergic receptors (40). However, the susceptibility to the action of pertussis toxin was not analyzed in the latter study.

In most myometrial preparations so far analyzed (for review, see Ref. 16), the coupling of β-adrenergic receptors through G_s to the stimulatory pathway of adenylate cyclase has been shown (14–16) to contribute to the β-adrenergic relaxation. The present findings imply that, in the guinea pig myometrium, β-adrenergic receptors are additionally coupled to a distinct effector, i.e., an ionic channel, via a pertussis toxin-sensitive G protein (distinct from G_s). An important question that remains to be clarified is whether both pathways involve the same or distinct subpopulations of β-adrenergic receptors. In any case, the present work provides a further example of divergence in β-adrenergic receptor-G protein-effector coupling (32, 41). Additional experiments are also needed to define the relative

contribution of the cAMP-independent pathway in the β-adrenergic-mediated relaxation of the myometrium.

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