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Evidence that a guanine nucleotide-binding protein linked to a muscarinic receptor inhibits directly phospholipase C

(cytosolic Ca^{2+}/inositol phosphates/adrenergic receptors/thyroid/FRTL5 cells)

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ABSTRACT **The mechanism of phospholipase C regulation by inhibitory receptors was analyzed both in intact and in permeabilized rat thyroid cells (FRTL5). In this system, the muscarinic agonist carbachol inhibited phospholipase C, as indicated by the decrease in the basal levels of inositol 1,4,5-trisphosphate as well as by the reduced adrenergic stimulation of phosphoinositol accumulation, which was paralleled by a fall in the cytosolic Ca^{2+} levels. This inhibition involved an M_2 muscarinic receptor because it was abolished by atropine but not by the M_1 antagonist pirenzepine. Cells pretreated with pertussis toxin were not responsive to carbachol, indicating the involvement of a guanine nucleotide-binding protein in this inhibitory process. This possibility was further evaluated in permeabilized cells, where the carbachol inhibition was shown to be completely dependent on GTP. Known second messengers were not involved in this inhibitory process since Ca^{2+}, cAMP, and activators of protein kinases were not able to mimic or prevent the carbachol effect either in intact or in permeabilized FRTL5 cells. In this system, the phospholipases C and A2 are coupled to two classes of muscarinic receptors that display a different sensitivity to pertussis toxin. The carbachol inhibitory effect occurred under conditions that prevented activation of phospholipase A2, excluding a role of the arachidonic acid metabolism in this process. Taken together these data provide the strongest support to date that an inhibitory guanine nucleotide-binding protein sensitive to pertussis toxin can directly mediate receptor-induced inhibition of phospholipase C.**

The activation of phospholipase C (PLC), a virtually ubiquitous membrane transducing enzyme, by hormones and neurotrasmitters leads to the formation of two second messengers, inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol, involved in the hormonal regulation of several cellular functions (calcium homeostasis, secretion, cell growth and differentiation, and cell transformation) (1–5). The coupling of receptors to the activation of PLC is mediated by specific guanine nucleotide-binding proteins (G proteins) (1–5). Inhibitory receptors are also coupled to PLC (6–11). However, it is not clear whether an inhibitory counterpart (Gp_i) of the stimulatory G protein (Gp_s) may be the direct link between these receptors and the enzyme. For instance, in a recent study it has been proposed that the decrease in accumulation of inositol phosphates induced by dopamine in isolated lactotrophs is secondary to a decrease in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) caused by the hormone-dependent closure of a Ca^{2+} channel (8). Therefore, the mechanism of PLC inhibition proposed is an indirect regulation of the enzyme operated by soluble second messengers (8, 9). In a different cell system (GH3 pituitary cells), the adenosine receptor is able to decrease the inositol phosphates produced by bradykinin in a manner independent on Ca^{2+} or cAMP, indicating that a different inhibitory mechanism has to be envisaged (10, 11).

In this study, the regulation of PLC by an inhibitory muscarinic receptor has been analyzed in FRTL5 rat thyroid cells with the aim to discriminate between the two above possibilities—namely, direct and second-messenger-mediated inhibitions. Thyrotropin and adrenergic and purinergic receptors have been reported to stimulate the PLC activity in FRTL5 cells (12–17). In the same system an M_1 muscarinic receptor has been shown to be coupled to phospholipase A_2 (PLA_2) activation (unpublished work; ref. 18).

Here we report that a different muscarinic receptor (M_2 class) inhibits PLC activity in both intact and permeabilized FRTL5 cells by means of a G_{pi} protein in a manner independent of all known second messengers. Therefore, it is suggested that PLC may be regulated by inhibitory (muscarinic) and stimulatory (adrenergic) receptors that use distinct G proteins in a dual mechanism resembling the regulation of adenylyl cyclase. Part of this study has been presented previously in abstract form (19, 20).

MATERIALS AND METHODS

Hormones used in the tissue culture media, Coon's modified Ham's F-12 medium, Hepes, EGTA, pertussis toxin (PTX), and quin-2 tetrakis(acetomethoxy) ester (quin-2 AM) were from Sigma. Tissue culture materials were from GIBCO. Streptolysin O was from Wellcome, and GTP and guanosine 5'-[γ-thio]triphosphate were from Boehringer Mannheim. *myo*-[^{3}H]Inositol and cAMP RIAs were from New England Nuclear/DuPont. All chemicals were obtained from commercial sources as the highest purity material available.

Cells. The FRTL-5 cells used in this study are a continuous line of functioning epithelial cells derived from normal Fisher rat thyroids (obtained from F. S. Ambesi-Impiombato). Their growth conditions have been described (21, 22).

Cytosolic Ca^{2+}. $[Ca^{2+}]_i$ was measured with the fluorescent probe quin-2 as described (12). Cells suspended in Coon's modified Ham's F-12 medium containing 0.1% bovine serum albumin were labeled with 10 μM quin-2 AM (12). The incubation was at 37°C for 30 min. $[Ca^{2+}]_i$ was calculated from the fluorescence recordings as described (23).

Inositol Phosphates. Cells were labeled for 67 hr in 199 medium containing *myo*-[^{3}H]inositol (2.5 μCi/ml; 1 μCi = 37 kBq), the six-hormone mixture, and glutamine (pH = 7.4). The separation of inositol phosphates was performed as described by Berridge *et al.* (24). Briefly, cells were washed and preincubated for 15 min in Hanks' balanced salt solution

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Abbreviations: PLC, phospholipase C; PLA_2, phospholipase A_2; G protein, guanine nucleotide-binding protein; G_{pi}, inhibitory G protein coupled to PLC; G_{ps}, stimulatory G protein coupled to PLC; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentrations; NE, norepinephrine; PMA, phorbol myristate acetate; $InsP_3$, inositol 1,4,5-trisphosphate; PTX, pertussis toxin.

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(HBSS) containing 10 mM LiCl in 10 mM Hepes (pH 7.4) at 37°C. Stimulants were added, and the incubation was carried on for 30 min. Cells (six-well plates) were collected in 2 ml of methanol and combined with 2.5 ml of chloroform. Each well was washed with 0.5 ml of methanol and 1.2 ml of water, which were added to the cell extract. Inositol phosphates were separated from the aqueous phase by anion-exchange chromatography on Dowex 1 columns (100–200 mesh) with stepwise elution with ammonium formate buffers as described (25). The results are presented as the total amount of labeled inositol phosphates and expressed in cpm per well.

Inositol Phospholipids. The inositol phospholipid separation was performed by a described procedure (8). The organic phase of the cell extract (see above) was dried under nitrogen, dissolved in 20 μl of chloroform, and separated by TLC on Kieselgel 60 F_{254} (Merck), the solvent system being chloroform/acetone/methanol/acetic acid/water, 40:15:13:12:8 (vol/vol). The inositol phospholipid identification was based on the comigration with commercially available standards after exposure to iodine vapor. Quantitative determinations were obtained by evaluating the labeled material present in the silica. Data are presented as cpm per well.

Cell Permeabilization. A modification of the method by Howell and Gomperts was used (26). Cells labeled with *myo*-[^{3}H]inositol were washed twice with 20 mM Hepes (pH 7.2)-buffered HBSS and permeabilized with 125 mM NaCl/10 mM Hepes/1 mM EGTA/2 mM $MgCl_2$/5 mM KCl/5 mM glucose/2 mM NaATP/0.7–1.5 units of Streptolysin O per ml, pH 7. The percent of permeabilized cells was evaluated in parallel samples by trypan blue exclusion (>90%). The permeabilizing solution was replaced after 15 min with the incubation buffer (110 mM potassium glutamate/20 mM potassium acetate/10 mM NaCl/3 mM $MgCl_2$/2 mM EGTA/0.5 mM $CaCl_2$/2 mM NaATP/7 mM phosphocreatine/10 units of creatine phosphokinase per ml/1 mM 2,3-diphosphoglycerate/10 mM LiCl/20 mM Hepes, pH 7/GTP at concentrations indicated in tables). The incubation was carried for 30 min at 37°C unless otherwise specified.

cAMP Assay. The cAMP intracellular content was measured in cell monolayers by using a commercial RIA as reported (22, 27). All assays were performed in the presence of 0.5 mM isobutylmethylxanthine unless otherwise stated.

Pertussis Toxin (PTX) Pretreatment. PTX (10–100 ng/ml) was added to the tissue culture dish during the last 4 or 20 hr of the cell labeling with *myo*-[^{3}H]inositol. The experimental procedure was carried on as specified without further additions of the toxin. The PTX-catalyzed ADP-ribosylation of a 40-kDa protein has been documented in FRTL5 cells (27). This effect, which is time and dose dependent, was evident within 1 min from the addition of PTX both in permeabilized cells and in membrane preparations (27).

Statistical analysis of all data was either by the Student *t* test or by Kruskal–Wallis analysis of variance with Sachs multiple comparison tests.

RESULTS

Muscarinic Agonist-Induced Decrease in $[Ca^{2+}]_i$. Fig. 1 shows the increase in $[Ca^{2+}]_i$ induced by the adrenergic agonist NE (12, 17). The addition of the muscarinic agonist carbachol caused a 30% decrease in the $[Ca^{2+}]_i$ levels that had been induced by NE (Fig. 1*A*) but did not affect resting $[Ca^{2+}]_i$ (data not shown; ref. 28). This inhibitory effect was concentration dependent (Fig. 1*A Inset*) and involved a muscarinic receptor, since it could be abolished by 10–25 μM atropine (Fig. 1*B*). Since the M_1 antagonist pirenzepine (1–400 μM) did not affect this phenomenon, this receptor is likely to be of the M_2 class (Fig. 1*B*).

In this cell system, two G proteins with different sensitivity to PTX are involved in the adrenergic activation of PLA_2 and

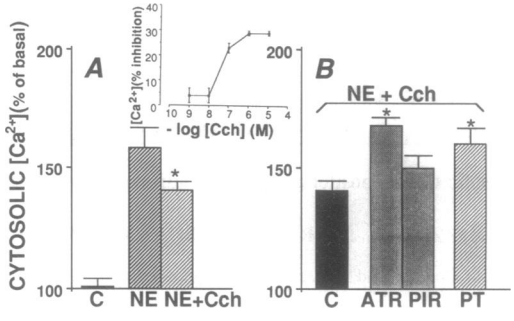

FIG. 1. Muscarinic-induced decrease in $[Ca^{2+}]_i$ in FRTL5 cells. (*A*) Increase in $[Ca^{2+}]_i$ induced upon the addition of 10 μM norepinephrine (NE) and the inhibition caused by 1 μM carbachol (Cch). (*Inset*) Dose–response curve of this inhibitory effect. (*B*) Effects of the muscarinic antagonists atropine (ATR) and pirenzepine (PIR) (both at 10 μM). Both compounds were used at concentrations ranging from 1 to 400 μM. The control bar in *B* represents the $[Ca^{2+}]_i$ induced by 1 μM carbachol and 10 μM NE (same as in *A*). The effect of a 4-hr incubation with PTX at 100 ng/ml is also shown. The basal $[Ca^{2+}]_i$ was 115 ± 10 nM (>40 observations). The quin-2-labeled cells were analyzed for 20–30 min; the data in the figure refer to the maximal NE stimulation (3 min after addition). The data are means ± SEM of 9–20 determinations obtained in at least four different cell preparations. ★, $P \leq 0.05$ (vs. NE in *A* and vs. control in *B*).

PLC, the G protein coupled to PLC being less sensitive to the toxin (17, 29, 30). PTX, an agent that is known to inactivate several G proteins by inducing their ADP-ribosylation, was used to examine the possible involvement of a G_{pi} protein in the muscarinic regulation of PLC. As in other cell types, in FRTL5 cells PTX catalyzes the ADP-ribosylation of a 40-kDa protein (27). We have previously shown that the toxin is also able to catalyze the ADP-ribosylation in intact FRTL5 cells (i.e., under the experimental conditions used in this study), since pretreatment of intact cells with 0.1–100 ng of toxin per ml prevented the ADP-ribosylation of the 40-kDa protein in membrane preparations (27).

The inhibitory effect of carbachol was completely abolished upon a 4-hr pretreatment with PTX at 100 ng/ml (Fig. 1*B*). Similar data were obtained upon 20-hr pretreatment of the cells with PTX at 10 ng/ml (data not shown). Under these experimental conditions, PTX does not affect the adrenergic increase in $[Ca^{2+}]_i$ (which requires a more prolonged incubation with a higher concentration of the toxin) (17, 29).

These results support the conclusion that at least two classes of muscarinic receptors are present in FRTL5 cells, distinguishable by their sensitivity to PTX and to pirenzepine, the M_2 receptor linked to the inhibition of PLC (this report) and the M_1 receptor coupled to the activation of PLA_2 (unpublished work; ref. 18).

Inhibition of the Accumulation of Inositol Phosphates. The inositol phospholipid breakdown and the accumulation of inositol phosphates were evaluated to ascertain whether the M_2 receptor was acting at the level of the PLC enzymatic activity. In intact cells carbachol decreased both basal and NE-produced inositol phosphates by ≈30% (Fig. 2*A*). When the accumulation of $InsP_3$ only was taken into account, the inhibition was more pronounced (≈50% of the NE-induced levels). Fig. 2*B* shows the time course of the inhibitory effect of carbachol on the basal levels of total inositol phosphates. The very rapid response to carbachol is suggestive of a direct mechanism of interaction between the receptor and the enzyme (9, 11).

The decrease in accumulation of inositol phosphates could result from a decreased activity of PLC as well as from a decrease in the substrate available to the enzyme. This second possibility has been shown to occur in pituitary cells (31). In the FRTL5 cells, the amount of inositol phospholipids

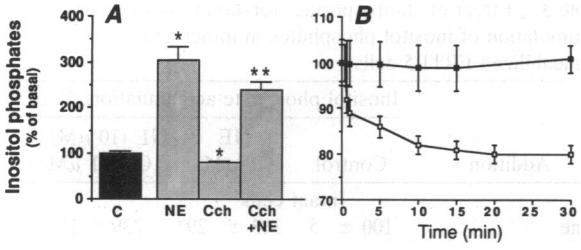

FIG. 2. Muscarinic inhibition of the accumulation of inositol phosphates in monolayers of FRTL5 cells. (*A*) Increase in inositol phosphates induced by the addition of 10 μM NE and the inhibition caused by 1 μM carbachol (Cch) on both stimulated and basal levels. (*B*) Time course of the decrease (□) in basal inositol phosphates (■) induced by 1 μM carbachol. The basal level amounted to 2899 ± 170 cpm per well (>40 observations). The data are means ± SEM of four to eight experiments performed in duplicate. ★, Significantly different ($P \leq 0.05$) from control; ★★, significantly different ($P \leq 0.05$) from values with addition of NE or carbachol alone.

did not vary upon incubation with carbachol (Table 1), which excludes a variation in substrate content as an explanation of the observed decrease in accumulation of inositol phosphates and suggests that the muscarinic effect was exerted at the level of the PLC enzymatic activity.

As in the Ca^{2+} experiments, a treatment for 4 hr with PTX at 100 ng/ml counteracted the carbachol-induced inhibition of the accumulation of inositol phosphates (data not shown). The adrenergic increase in inositol phosphates was not affected by PTX under these conditions (13, 17, 29, 30). These data indicate that G proteins sensitive to PTX play a role in the negative control of PLC.

Muscarinic Inhibition in Permeabilized Cells. Further support to this conclusion was obtained by using permeabilized cells. As in other cell systems, the accumulation of InsP_3 in streptolysin O-permeabilized cells was dependent on GTP and required concentrations of GTP ≥ 1 μM (Table 2 and Fig. 3). GTP at 1–100 μM increased the PLC activity, probably by acting on the G_{ps} protein coupled to the enzyme; NE significantly potentiated this effect (Table 2 and Fig. 3). Carbachol was able to inhibit almost completely the effect of NE and also to decrease the basal InsP_3 levels (Fig. 3). Neither the carbachol inhibition of the basal level nor that of the stimulated levels of InsP_3 occurred in the absence of GTP (Table 2). A 4-hr pretreatment with PTX at 100 ng/ml prior to cell permeabilization prevented the carbachol-induced inhibition of InsP_3 accumulation. (The InsP_3 levels were as follows: basal level, 100 ± 9, with 1 μM carbachol, 73 ± 6; basal level of cells treated with PTX, 100 ± 7; with 1 μM carbachol, 98 ± 3. The experimental procedure was as described in the legend to Table 2 and included 10 μM GTP.)

The basal level of InsP_3 was slightly affected by the ATP in the permeabilizing solution, since these cells possess a purinergic receptor coupled to the activation of PLC (16). This was not the case in intact cells where the experiments were performed in the absence of extracellularly added ATP.

Table 2. The effect of GTP on the InsP_3 accumulation in permeabilized FRTL5 cells

Addition	InsP_3 accumulation, % of basal		
	No GTP	1 μM GTP	10 μM GTP
None	100 ± 5	108 ± 5	189 ± 20
NE (10 μM)	133 ± 9	162 ± 6*	250 ± 17*
Cch (1 μM)	100 ± 5	121 ± 10	147 ± 11*
NE + Cch	130 ± 9	149 ± 14	161 ± 26†

Cells were permeabilized with 0.7 unit of streptolysin O, and the InsP_3 accumulation was evaluated as described. Data are expressed as the percentage of the basal level of InsP_3, which amounted to 754 ± 71 cpm per well. The addition of 100 μM GTP caused an InsP_3 increase of 167 ± 5%. The data presented are means ± SEM of four to eight experiments performed in duplicate. Cch, carbachol.
*Significantly different from no addition of NE or Cch at the same concentration of GTP.
†Significantly different from addition of NE and 10 μM GTP.

When the stimulation of the purinergic receptor was tested in intact cells, it was 30–40% over the basal levels.

Role of Ca^{2+}. The coupling between the G_{pi} protein and PLC could be direct or mediated by known second messengers such as Ca^{2+}, cAMP, arachidonic acid, and activators of protein kinases. The contribution of Ca^{2+} in the carbachol-induced inhibition of PLC activity was excluded because (*i*) in intact cell suspensions, carbachol did not affect the resting $[Ca^{2+}]_i$ (data not shown; ref. 28); and (*ii*) in permeabilized cells, under conditions where the Ca^{2+} concentration was kept at 100 nM, carbachol was still able to inhibit PLC activity (Fig. 3). Moreover, in the presence of the Ca^{2+} ionophore A23187, which markedly augments $[Ca^{2+}]_i$, the basal levels of inositol phosphates as well as the levels induced by the adrenergic stimulus and the carbachol inhibition remained unchanged (Fig. 4). This is in agreement with the observation by Bone *et al.* that the PLC activity in FRTL5 cells is not stimulated by Ca^{2+} (13).

Role of Arachidonic Acid. The activation of PLA_2 and the metabolites of arachidonic acid have been proposed to play a role in the regulation of PLC in various systems (2). In FRTL5 cells a muscarinic receptor of the M_1 class activates PLA_2 (unpublished work; ref. 18). The antagonist pirenzepine at 0.1–10 μM blocked the PLA_2 activation, whereas it did not affect the muscarinic inhibition of PLC even at a 40-fold higher concentration, thus excluding a role of the arachidonic acid metabolites in this inhibitory process (Fig. 1).

Role of cAMP. The possibility that cAMP could play a role in this inhibitory event is unlikely because, under our experimental conditions, NE and carbachol did not affect the formation of cAMP in FRTL5 cells (control, 12 ± 2 pmol of cAMP per ml; with carbachol, 11 ± 1; with NE, 12 ± 1). Support of this hypothesis came from the experiments performed in the presence of forskolin, which is known to cause a pronounced increase in the cAMP levels in FRTL5 cells (27). Under these conditions, carbachol was still able to inhibit the accumulation of inositol phosphates (Table 3). Moreover, forskolin was slightly increasing the levels of

Table 1. Effect of carbachol on the inositol phospholipid levels of FRTL5 cells

Time, min	Inositol phospholipid, cpm × 10^{-3} per well					
	PtdIns		PtdIns4*P*		PtdIns(4,5)P_2	
	Control	Carbachol	Control	Carbachol	Control	Carbachol
1	25.6 ± 3	27.5 ± 3	0.5 ± 0.1	0.4 ± 0.1	0.2 ± 0.01	0.2 ± 0.02
10	30.8 ± 2	30.6 ± 2	0.9 ± 0.2	0.8 ± 0.2	0.2 ± 0.03	0.2 ± 0.03
30	49.1 ± 3	55.2 ± 7	1.2 ± 0.1	1.3 ± 0.2	0.3 ± 0.02	0.3 ± 0.03

Carbachol was at 1 μM. The levels of inositol phospholipids were evaluated as described. Determinations were at three different times of incubation. Data are means ± SEM of three experiments. PtdIns, phosphatidylinositol; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate.

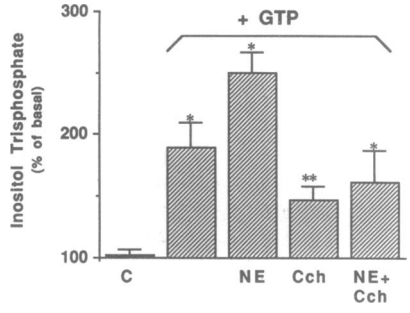

FIG. 3. Muscarinic inhibition of InsP_3 accumulation in permeabilized FRTL5 cells. Carbachol (Cch) inhibited the increase in InsP_3 produced by NE in cells permeabilized by 0.7 unit of streptolysin O. The concentrations of NE and carbachol were 10 μM and 1 μM, respectively; 10 μM GTP was used in all experiments. The basal level of InsP_3 was 754 ± 71 cpm per well (>35 observations). The data are means ± SEM of four to eight experiments performed in duplicate. *, Significantly different ($P \leq 0.05$) from control; **, significantly different ($P \leq 0.05$) from values with GTP addition.

inositol phosphates, suggesting that cAMP could represent a positive regulator for this enzyme.

In permeabilized cells, exogenously added cAMP (1–50 μM) did not affect the basal or NE-stimulated InsP_3 accumulation (Table 3). Under these conditions, the carbachol inhibition was ≈30%, indicating that fixed cAMP concentration would not interfere with the muscarinic regulation of PLC activity (Table 3). A similar carbachol-induced inhibition was observed when cAMP was tested in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (0.1–0.5 mM) (data not shown).

Role of Protein Kinases. Enzymes involved in the transduction cascade such as cAMP-dependent protein kinase A and protein kinase C could play a role in the inhibition of PLC activity. The data obtained in the presence of forskolin in intact cells and of cAMP in permeabilized cells do not support a possible role of the protein kinase A, since it is shown that the carbachol inhibition was evident under conditions where the protein kinase A was activated (see above and Table 3). The possible role of protein kinase C was analyzed by using its specific activator phorbol myristate acetate (PMA). PMA did not affect the basal PLC activity. [The total inositol phosphates (cpm per well) in intact cells were: control, 2014 ± 798; with 10 nM PMA 2193 ± 451. InsP_3 values (cpm per well) in permeabilized cells were: control, 690 ± 20; with PMA 622 ± 31. Similar data were obtained in the presence of 100 nM PMA.] The NE-stimulated PLC could not be studied because PMA abolishes the adrenergic activation of FRTL5

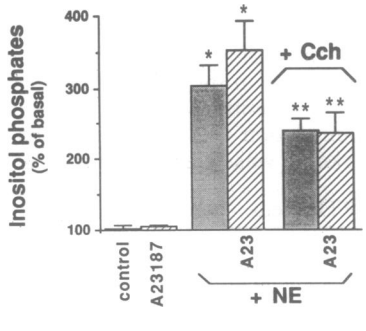

FIG. 4. The Ca^{2+} ionophore A23187 does not affect the accumulation of inositol phosphates in FRTL5 cells. The concentrations of the Ca^{2+} ionophore A23187, NE, and carbachol (Cch) were 100 nM, 10 μM, and 1 μM, respectively. Similar results were obtained with 0.1–1 μM A23187. The data presented are the means ± SEM of four experiments performed in duplicate. ★, Significantly different ($P \leq 0.05$) from control; ★★, significantly different ($P \leq 0.05$) from values with NE addition.

Table 3. Effect of staurosporine, forskolin, and cAMP on the accumulation of inositol phosphates in intact and permeabilized FRTL5 cells

Addition	Inositol phosphate accumulation, % of basal: Control	NE (10 μM)	NE (10 μM) + Cch (1 μM)	n
	Intact cells			
None	100 ± 5	303 ± 29*	239 ± 17†	33
Staurosporine (1 μM)	119 ± 7	531 ± 138*	327 ± 87†	4–6
Forskolin (10 μM)	141 ± 9*	301 ± 6*	270 ± 12†	5–6
	Permeabilized cells			
GTP (10 μM)	100 ± 17	144 ± 11*	92 ± 4†	7–10
+ cAMP (1 μM)	110 ± 8	156 ± 9*	102 ± 12†	7–10

Inositol phosphates were evaluated as described. Data are expressed as percent of basal inositol phosphates, which amounted to 2899 ± 170 cpm per well in intact cells and 1229 ± 208 cpm per well in permeabilized cells. In the absence of GTP, the InsP_3 value was 754 ± 71 (see the legends to Fig. 3 and Table 2). Cch, carbachol; n, number of separate determinations.
*Significantly different from control.
†Significantly different from the NE column.

cells, as has been shown (14). Therefore, the protein kinase inhibitor staurosporine was used. At a concentration of 1 μM, staurosporine increased the activity of PLC (≈20%) and synergized with the NE stimulation (Table 3). Similar data were obtained in the presence of 0.1 μM inhibitor (data not shown). Under these conditions, carbachol was still able to decrease the levels of inositol phosphates, suggesting that the muscarinic inhibition is neither mediated nor prevented by protein kinase C or by other kinases (Table 3). Similar results were obtained in permeabilized cells (data not shown).

DISCUSSION

This report analyzes the mechanism of negative regulation of PLC by an M_2 inhibitory receptor in thyroid cells. In this system the adrenergic stimulation of PLC, which has been well documented, functions as the positive counterpart of the muscarinic inhibition (12, 14, 17). Both the adrenergic and the muscarinic receptors are coupled to G proteins sensitive to PTX, since the stimulatory as well as the inhibitory pathways were inactivated by toxin pretreatment (this report and ref. 29). However, the sensitivity of the two pathways to the toxin is very different, indicating that distinct G proteins are involved. The role of G proteins in the stimulation and inhibition of PLC activity is further supported by data obtained in permeabilized cells, where the effect of NE was potentiated by the addition of GTP and the effect of carbachol required the presence of GTP (this report). Therefore, it appears that PLC is regulated in a dual manner by receptors interacting with either G_{ps} or G_{pi} proteins. We propose, based on several lines of evidence, that the G_{pi} protein coupled to PLC regulation is directly acting on the enzyme—namely, that no other second messenger or transducing enzyme mediates the muscarinic inhibition here reported. The supporting evidence is 4-fold. (*i*) Soluble messengers such as Ca^{2+} or cAMP or other unknown cytosolic cofactors are not involved in this phenomenon, as shown by the ability of carbachol and GTP to induce the PLC inhibition in permeabilized cells (i.e., under the condition of extreme dilution of any soluble factor). (*ii*) High $[Ca^{2+}]_i$ induced by a specific ionophore in intact cells or fixed Ca^{2+} concentrations in permeabilized cells did not affect the muscarinic inhibition, which excludes a dependence of this phenomenon on $[Ca^{2+}]_i$. (*iii*) Carbachol was still able to decrease the PLC activity in the presence of saturating levels of cAMP in permeabilized cells and in the presence of forskolin in intact cells (32). Under the experimental conditions used, carbachol did not

modify the cAMP levels in these cells, confirming that a role of the cAMP-dependent protein kinase could reasonably be excluded. (*iv*) Protein kinase C does not seem to play a role in the muscarinic inhibition because its direct activator, PMA, and the kinase inhibitor staurosporine did not interfere with the carbachol effect. Although both PMA and staurosporine may have nonspecific effects, they are potent modulators of protein kinase C. Therefore, their lack of effect on carbachol inhibition clearly supports our point. Staurosporine potentiated the adrenergic-induced stimulation of PLC. This effect could be exerted either at the enzyme level (i.e., the potentiation of PLC activity could derive from the removal of an inhibitory control exerted by an endogenous phosphorylation) or at the receptor level. The α_1-adrenergic receptor in these cells is in fact completely inhibited by PMA (14). Thus, by inhibiting protein kinase C, staurosporine could potentiate the adrenergic stimulation. Accordingly, the effect of staurosporine was more pronounced in NE-stimulated cells than under basal conditions (Table 3).

At the moment, we can only speculate about the molecular mechanism of action of the G_{pi} protein coupled to the muscarinic receptor; we do not have evidence to favor a role of the G_{pi} α subunit versus the $\beta\gamma$ subunit in this process. A recent report has shown that exogenously added $\beta\gamma$ subunit was able to decrease PLC activity, indicating that this subunit may operate by a mechanism of mass action (33). However, this does not exclude the possibly direct role of a G_{pi} α subunit on the enzyme. We have identified two different G α subunits in FRTL5 cells, both of which are substrates of the PTX-induced ADP-ribosylation (M.D.G., unpublished data).

As mentioned before, it has been proposed that in lactotrophs the inhibition of PLC activity by dopamine is secondary to a decrease in $[Ca^{2+}]_i$ (8, 9). This is not necessarily in conflict with our proposal of a direct Ca^{2+}-independent regulation of PLC. Different receptors could act via a Ca^{2+}-dependent mechanism (9, 11). The two mechanisms could be related to different receptors in different cell systems or could coexist under the control of the same receptor. In lactotrophs the inhibition caused by Ca^{2+} depletion was always less pronounced than the dopamine effect, so that a contribution by the direct mechanism was not excluded by the authors (6–8). Note that the activity of PLC in FRTL5 cells, unlike PLC activity in other systems, is not dependent on and does not require Ca^{2+} (13). This might have facilitated our demonstration of a purely Ca^{2+}-independent mechanism of inhibition.

Five muscarinic receptors have recently been cloned and expressed in different cell lines so that their coupling to the transducing enzymes could be analyzed (34–36). None of these receptors has been reported to inhibit PLC activity, whereas three of the clones were positively coupled to the enzyme (34–36). We have not yet identified the muscarinic receptors present in FRTL5 cells. However, we have shown that under the experimental conditions used in this study, carbachol did not affect the cAMP levels, although under different conditions it was able to potentiate the thyrotropin-induced stimulation of the adenylyl cyclase (M.D.G., unpublished observations). It remains to be clarified which of the muscarinic receptors present in FRTL5 cells is able to interact with the adenylyl cyclase. We think that the two characteristics mentioned above (i.e., inhibition of PLC under conditions of noninteraction with the adenylyl cyclase) could make of this inhibitory receptor a candidate for a new subclass of muscarinic receptors that could represent either a different protein (i.e., with a different sequence) or one of the already known receptors coupled to transducing enzymes in a manner not yet described in other systems.

In summary, our analysis of the modulation of PLC activity strongly supports the idea that this enzyme can be controlled by the direct interaction with two different G proteins—G_{ps} linked to the adrenergic receptor and G_{pi} linked to the muscarinic receptor. Therefore, this dual regulation, which has been well documented for the adenylyl cyclase, could represent a more general mechanism to control the activity of other transducing enzymes.

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1. Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56,** 159–193.
2. Rhee, S. G., Suh, P., Ryu, S. & Lee, S. Y. (1989) *Science* **244,** 546–550.
3. Fain, J. N., Wallace, M. A. & Wojcikiewicz, J. H. (1988) *FASEB J.* **2,** 2569–2574.
4. Cockcroft, S. (1987) *Trends Pharmacol. Sci.* **12,** 75–78.
5. Berridge, M. J. & Irvine, R. F. (1989) *Nature (London)* **341,** 197–205.
6. Enjalbert, A., Sladeczek, F., Guillon, G., Bertrand, P., Shu, C., Epelbaum, J., Garcia-Sainz, A., Jard, S., Lombard, C., Kordon, C. & Bockaert, J. (1986) *J. Biol. Chem.* **261,** 4071–4075.
7. Journot, L., Homburger, V., Pantaloni, C., Priam, M., Bockaert, J. & Enjalbert, A. (1987) *J. Biol. Chem.* **262,** 15106–15110.
8. Vallar, L., Vicentini, L. M. & Meldolesi, J. (1988) *J. Biol. Chem.* **263,** 10127–10134.
9. Vallar, L. & Meldolesi, J. (1989) *Trends Pharmacol. Sci.* **10,** 74–77.
10. Delahunty, T. M., Cronin, M. J. & Linden, J. (1988) *Biochem. J.* **255,** 69–77.
11. Linden, J. & Delahunty, T. M. (1989) *Trends Pharmacol. Sci.* **10,** 114–120.
12. Corda, D., Marcocci, C., Kohn, L. D., Axelrod, J. & Luini, A. (1985) *J. Biol. Chem.* **260,** 9230–9236.
13. Bone, E. A., Alling, D. W. & Grollman, E. F. (1986) *Endocrinology* **219,** 2193–2200.
14. Corda, D. & Kohn, L. D. (1987) *Endocrinology* **120,** 1152–1160.
15. Field, J. B., Ealey, P. A., Marshall, N. J. & Cockcroft, S. (1987) *Biochem. J.* **247,** 519–524.
16. Okajima, F., Sho, K. & Kondo, Y. (1988) *Endocrinology* **123,** 1035–1043.
17. Corda, D., Iacovelli, L. & Di Girolamo, M. (1988) in *Horizons in Endocrinology*, eds. Maggi, M. & Johnston, C. A. (Raven, New York) pp. 169–180.
18. Di Girolamo, M., Bizzarri, C. & Corda, D. (1989) in *FRTL-5 Today*, eds. Ambesi-Impiombato, F. S. & Perrild, H. (Elsevier, Amsterdam) pp. 195–196.
19. Bizzarri, C., Di Girolamo, M. & Corda, D. (1989) *J. Cell Biol.* **109,** 212 (abstr.).
20. Bizzarri, C., Di Girolamo, M. & Corda, D. (1989) *Eur. J. Cell Biol.* **49,** Suppl. 28, 9 (abstr.).
21. Ambesi-Impiombato, F. S., Picone, R. & Tramontano, D. (1982) in *Cold Spring Harbor Symposia*, eds. Serbasku, D. A., Sato, G. H. & Pardee, A. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), Vol. 9, pp. 483–492.
22. Valente, W. A., Vitti, P., Kohn, L. D., Brandi, M. L., Rotella, C. M., Toccafondi, R., Tramontano, D., Aloj, S. M. & Ambesi-Impiombato, F. S. (1983) *Endocrinology* **112,** 71–79.
23. Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *J. Cell Biol.* **94,** 325–334.
24. Berridge, M. J., Downes, C. P. & Hanley, M. R. (1982) *Biochem. J.* **206,** 587–595.
25. Batty, I. R., Nahorski, R. & Irvine, R. F. (1985) *Biochem. J.* **232,** 211–215.
26. Howell, T. W. & Gomperts, B. D. (1987) *Biochim. Biophys. Acta* **927,** 177–183.
27. Corda, D., Sekura, R. D. & Kohn, L. D. (1987) *Eur. J. Biochem.* **166,** 475–481.
28. Bizzarri, C., Di Girolamo, M. & Corda, D. (1989) in *FRTL-5 Today*, eds. Ambesi-Impiombato, F. S. & Perrild, H. (Elsevier, Amsterdam), pp. 193–194.
29. Corda, D. & Kohn, L. D. (1986) *Biochem. Biophys. Res. Commun.* **141,** 1000–1006.
30. Burch, R. M., Luini, A. & Axelrod, J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 7201–7205.
31. Jarvis, W. D., Judd, A. M. & MacLeod, R. M. (1988) *Endocrinology* **123,** 2793–2799.
32. Miyazaki, O., Reisine, T. & Kebabian, J. W. (1984) *Endocrinology* **115,** 1933–1945.
33. Moriarty, T. M., Gillo, B., Carty, D. J., Premont, R. T., Landau, E. M. & Iyengar, R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8865–8869.
34. Bonner, T. I. (1989) *Trends Neurosci.* **12,** 148–151.
35. Conklin, B. R., Brann, M. R., Buckley, N. J., Ma, A. L., Bonner, T. I. & Axelrod, J. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8698–8702.
36. Ashkenazi, A., Peralta, E. G., Winslow, J. W., Ramachandran, J. & Capon, D. J. (1989) *Cell* **56,** 487–493.