Stimulation of generation of inositol phosphates by carbamoylcholine and its inhibition by phorbol esters and iodide in dog thyroid cells

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The action of carbamoylcholine (Cchol), NaF and other agonists on the generation of inositol phosphates (IPs) was studied in dog thyroid slices prelabelled with *myo*-[2-³H]inositol. The stimulation by Cchol (0.1 μ M–0.1 mM) of IPs accumulation through activation of a muscarinic receptor [Graff, Mockel, Laurent, Erneux & Dumont (1987) FEBS Lett. 210, 204–210] was pertussis- and cholera-toxin insensitive. Ins(1,4,5) P_3 , Ins(1,3,4) P_3 and Ins P_4 were generated. NaF (5–20 mM) also increased IPs generation (Graff *et al.*, 1987); this effect was potentiated by AlCl₃ (10 μ M) and unaffected by pertussis toxin. Although phorbol dibutyrate (5 μ M) abolished the cholinergic stimulation of IPs generation (Graff *et al.*, 1987), it did not affect the fluoride-induced response. Cchol and NaF did not require extracellular Ca²⁺ to exert their effect, and neither KCl-induced membrane depolarization nor ionophore A23187 (10 μ M) had any influence on basal IPs levels, or on cholinergic stimulation. However, more stringent Ca²⁺ depletion with EGTA (0.1 or 1 mM) decreased basal IPs levels as well as the amplitude of the stimulation by Cchol without abolishing it. Dibutyryl cyclic AMP, forskolin, cholera toxin and prostaglandin E₁ had no effect on basal IPs levels and did not decrease the response to Cchol. Iodide (4 or 40 μ M) also strongly decreased the cholinergic action on IPs, this inhibition being relieved by methimazole (1 mM). Our data suggest that Cchol activates a phospholipase C hydrolysing PtdIns(4,5) P_2 in the dog thyroid cell in a cyclic AMP-independent manner. This activation requires no extracellular Ca²⁺ and depends on a GTP-binding protein insensitive to both cholera toxin and pertussis toxin. The data are consistent with a rapid metabolism of Ins(1,4,5) P_3 to Ins(1,3,4) P_3 via the Ins(1,4,5) P_3 3-kinase pathway, followed by dephosphorylation by a 5-phosphomonoesterase. Indeed, a Ca²⁺-sensitive Ins P_3 3-kinase activity was demonstrated in tissue homogenate. Stimulation of protein kinase C and an organised form of iodine inhibit the Cchol-induced IPs generation. The negative feedback of activated protein kinase C could be exerted at the level of the receptor or of the receptor–G-protein interaction.

INTRODUCTION

The activation of a wide range of receptors which use Ca²⁺ as one of their intracellular signals is associated with the hydrolysis of plasma-membrane PtdIns P_2 (Michell, 1975; Berridge, 1981). A phospholipase C (PLC) hydrolyses PtdIns P_2 , yielding two second messengers, Ins(1,4,5) P_3 , which releases Ca²⁺ from microsomal stores, and diacylglycerol (DAG), which stimulates a Ca²⁺- and phospholipid-dependent protein kinase C (PKC) (Berridge & Irvine, 1984; Nishizuka, 1986). There is a large body of evidence indicating that the synergistic action of Ca²⁺ and PKC plays a central role in controlling cell function. Recent studies performed in different tissues demonstrated the existence of transducing G-proteins coupling the receptor to the PLC stimulation (for review see Casey & Gilman, 1988). In several tissues muscarinic receptors exert their metabolic actions through activation of a PLC complex (for review see Harden *et al.*, 1986). In the dog thyroid, Cchol through its muscarinic receptor

stimulates protein iodination, glucose C-1 oxidation and cyclic GMP accumulation, and antagonizes the thyrotropin (TSH)-induced cyclic AMP accumulation (Decoster *et al.*, 1980; Erneux *et al.*, 1985). All these effects are reproduced by the ionophore A23187 in the presence of extracellular Ca²⁺, are inhibited in Ca²⁺-depleted cells and are accompanied by increased ⁴⁵Ca²⁺ efflux and a rise in intracellular Ca²⁺ concentration ([Ca²⁺]_i). It has been suggested that they result from a release of Ca²⁺ from intracellular stores, consequent to Ins P_3 generation from PtdIns P_2 hydrolysis (Decoster *et al.*, 1980; Rani *et al.*, 1985; Raspe *et al.*, 1986). In a preliminary report, we have provided evidence that Cchol's initial interaction with the thyroid membrane activates PtdIns P_2 hydrolysis, leading to Ins P_3 accumulation (Graff *et al.*, 1987). A detailed study of Ins P_3 metabolism in dog thyroid, in relation to the well-known extracellular signals of cyclic AMP and Ca²⁺ as second messengers, is presented.

Abbreviations used: Cchol, carbamoylcholine (carbachol); [Ca²⁺]_i, intracellular free calcium concentration; DAG, diacylglycerol; IP(s), inositol phosphate(s); PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; PLC, phospholipase C; TSH, thyrotropin (thyroid-stimulating hormone).
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MATERIALS AND METHODS

Materials

myo-[2-³H]Inositol (sp. radioactivity 16.5 Ci/mmol) and *myo*-[2-³H]inositol 1-phosphate, 1,4-bisphosphate, 1,4,5-trisphosphate and 1,3,4,5-tetrakisphosphate were purchased from New England Nuclear (Dupont-NEN, Haren, Belgium). *myo*-[2-¹⁴C]Inositol monophosphate (50–60 Ci/mmol) was from The Radiochemical Centre (Amersham, Bucks., U.K.). Cchol was provided by K and K (Plain View, NY, U.S.A.), TSH as Thytropar by Armour Pharmaceutical Co. (Chicago, IL, U.S.A.), forskolin by Hoechst Pharmaceuticals (Bombay, India), bovine serum albumin and noradrenaline by Calbiochem (Lucerne, Switzerland). Phorbol dibutyrate (PDBu), dibutyryl cyclic AMP and ionophore A23187 were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), cholera toxin was from Schwarz-Mann (Division of Beckton-Dickinson and Co., Orangeburg, NY, U.S.A.), prostaglandins E, F₁ and F_{2α} were from the Upjohn Co. (Kalamazoo, MI, U.S.A.), and *myo*-inositol, NaF, AlCl₃ and LiCl were from Merck (Darmstadt, Germany). The Dowex AG1-X8 ion-exchange resin (formate form, 100–200 mesh) was obtained from Bio-Rad (Watford, Herts., U.K.). [³²P]Ins(1,4,5)P₃ was prepared from human erythrocytes by the method of Downes *et al.* (1982). All other reagents were of the purest grade commercially available.

Tissue preparation and incubation

Animal studies were conducted in accordance with the highest standards of human care. On the day of the experiment, dogs were anaesthetized with pentobarbital, and the thyroid lobes were resected and cut into thin slices with a Stadie-Riggs microtome (Arthur Thomas, Philadelphia, PA, U.S.A.) (Dumont *et al.*, 1971). Within 30 min after resection, portions of about 50 mg wet wt. were distributed in flasks and incubated at 37 °C under an atmosphere of O₂/CO₂ (19:1) in 2 ml of Krebs-Ringer bicarbonate buffer (Rodesch *et al.*, 1969), pH 7.4, supplemented with 8 mM-glucose and 0.5 g of bovine serum albumin/l. The experimental procedure involved a pre-incubation for 4 h in the presence of 20 µCi of *myo*-[2-³H]inositol/ml. Slices were then transferred to fresh unlabelled incubation medium. LiCl was added after 15 min to a final concentration of 10 mM, followed 5 min later by the experimental agents to be tested for various lengths of time. In each experiment the reactivity of the slices was checked by measuring protein iodination in control slices (Rodesch *et al.*, 1969). For the study of NaF-concentration effects, a Ca²⁺-free Krebs-Ringer bicarbonate medium was used to prevent CaF₂ precipitation and errors in estimated concentrations of NaF.

Extraction and separation of ³H-labelled inositol phosphates (IPs)

Incubation was stopped by rapid immersion and homogenization of the slices in 2 ml of ice-cold 3% (v/v) HClO₄. After centrifugation (2000 g for 10 min), the pellet was washed once with 1 ml of 1% HClO₄. Combined supernatants were titrated on ice to pH 7.8 by addition of 0.76 M-KOH and 1 ml of 0.38 M-Hepes buffer. The resulting KClO₄ precipitate was removed by centrifugation (2000 g for 10 min). After addition of 12 ml of 5 mM-Na₂B₄O₇/0.5 mM-EDTA solution, the

samples were loaded on to Dowex AG1-X8 resin columns (formate form; 1.2 ml of resin in 0.6 cm × 20 cm columns). The columns were then eluted with solutions of increasing ionic strength as described by Downes & Michell (1981), with slight modifications. The solutions used for each sequential elution were: 20 ml of water for inositol, 12 ml of 5 mM-Na₂B₄O₇/60 mM-ammonium formate for glycerophosphatidylinositol, 28 ml of 5 mM-Na₂B₄O₇/150 mM-ammonium formate for InsP₁, 24 ml of 0.1 M-formic acid/0.4 M-ammonium formate for InsP₂, 16 ml of 0.1 M-formic acid/0.8 M-ammonium formate for InsP₃, and 24 ml of 0.1 M-formic acid/1.2 M-ammonium formate for InsP₄. The validity of elution was checked in parallel by the elution profiles of standards of [¹⁴C]InsP₁, [³H]Ins(1,4)P₂, [³²P]Ins(1,4,5)P₃ and [³H]Ins(1,3,4,5)P₄. Under these conditions, elution of purified [³²P]Ins(1,4,5)P₃ did not give rise to radioactivity in the InsP₄ eluate. Columns were regenerated with 20 ml of 0.1 M-formic acid/2.0 M-ammonium formate. A 4 ml portion of each fraction was taken for determination of radioactivity in Luma-Gel scintillation fluid (Lumac, Landgraaf, The Netherlands). Ins(1,4,5)P₃ 3-kinase activity was measured as previously described (Takazawa *et al.*, 1988).

For measurements of ³²P incorporation into lipids, slices were incubated for 1 h with [³²P]P_i in the presence of the agonists. Lipids were then extracted, dried and plated for two-dimensional chromatography. PtdIns spots were scraped for phosphorus determination (Mockel *et al.*, 1987).

Presentation of results

Each experimental point consists of triplicates within each experiment and is expressed as the mean ± S.E.M. Each experimental condition was reproduced in at least three different experiments. In each case results of a representative experiment are shown. Statistical analyses were performed by Student's *t* test, with *P* < 0.05 as the level of significance.

RESULTS

Effects of Cchol on generation of inositol phosphates (IPs)

When added during 20 min, Cchol induced a concentration-dependent accumulation of IPs for concentrations in the range 1 µM–0.1 mM, without reaching a clear plateau phase. The InsP₃ response was more sensitive to low agonist concentrations (Graff *et al.*, 1987). In order to dissociate better the kinetics of the three IPs, experiments were performed at 25 °C (Fig. 1). Under such conditions, InsP₃ showed a rapid increase, whereas InsP₂ and InsP₁ accumulations lagged behind. Later (30–60 min) InsP₃ production tended to reach a plateau. In contrast, InsP₁ accumulation did not reach a plateau, and its relative increases exceeded that of InsP₂ and InsP₃ at this time. Basal levels of IPs in the slices were not affected by the incubation time. InsP₄ could be measured in experiments using slices incubated for 20–60 min in the presence of Cchol (0.1 mM). Cchol's effects were abolished by atropine (Graff *et al.*, 1987), but they were not modified by a preincubation for 2 h with cholera toxin (1 and 10 µg/ml). In one typical experiment, InsP₃ radioactivities in the slices (c.p.m.; means ± S.E.M.) were 686 ± 180 for controls, 7504 ± 1476 for Cchol (5 µM),

740 ± 39 for cholera toxin (10 µg/ml) and 6893 ± 755 for cholera toxin plus Cchol. About 90% of the total radioactivity incorporated in control slices was PtdIns, and 10% was IPs, of which InsP₁ represented the major part.

After carbachol stimulation, in the 12 min InsP₃ fraction, anion-exchange h.p.l.c. demonstrated the presence

Fig. 1. Time course of the action of Cchol (10 µM) on IP generation at 25 °C

Results are expressed as means ± S.E.M. for triplicates of ratios of radioactivity in ³H-labelled IPs of stimulated slices to that of control slices. 'IPs contr.' indicates basal levels of IPs.

of Ins(1,3,4)P₃ as the major InsP₃ isomer, and a trace amount of Ins(1,4,5)P₃. In the 60 min sample, only the 1,3,4-isomer was detected (Fig. 2).

Cchol-induced InsP₁ and InsP₃ accumulations were potentiated by LiCl (10 mM) (Graff *et al.*, 1987). To a lesser extent, this effect was also observed for InsP₄ [InsP₄ radioactivities (c.p.m.; mean ± S.E.M.) were 161 ± 12 and 222 ± 34 for control + LiCl (10 mM) and without LiCl, 532 ± 168 for Cchol (0.1 mM) without LiCl, 920 ± 142 for Cchol (0.1 mM) + LiCl (10 mM), for an incubation of 20 min].

Effects of NaF and AlCl₃ on IP production

NaF (20 mM) stimulated IP generation at 20 min (Graff *et al.*, 1987), but a significant effect at 5 mM-NaF was obtained only for InsP₁ (Fig. 3). A marked potentiation was observed in the presence of AlCl₃ (10 µM), which by itself had little effect (Table 1). Preincubation for 18 h with pertussis toxin (250 ng/ml) (Cochaux *et al.*, 1985) did not modify the responses to Cchol or NaF. In one typical experiment, InsP₃ radioactivities in the slices (c.p.m.; mean ± S.E.M.) were 30 300 ± 4311, 197 937 ± 30 524 and 122 056 ± 31 552 versus 31 808 ± 2884, 191 945 ± 49 059 and 112 916 ± 15 763 for respectively controls, Cchol (5 µM) and NaF (20 mM) in the absence versus in the presence (18 h) of pertussis toxin.

Effects of phorbol esters and Ca²⁺ on the Cchol- and NaF-induced IP responses

Phorbol 12,13-dibutyrate (PDBu) (5 µM) had little effect by itself on IP basal levels, but, when added 5 min before Cchol, it inhibited nearly completely the effect of equimolar Cchol and partially that of Cchol (10 µM) (Graff *et al.*, 1987). However, PDBu (5 µM) had no effect on NaF (10 and 20 mM)-induced accumulations of IPs. Fig. 4 shows the results for InsP₃.

Fig. 2. Ins(1,4,5)P₃ and Ins(1,3,4)P₃ characterization by h.p.l.c.

Slices were incubated for 12 or 60 min in the presence of Cchol (0.1 mM). After stepwise elution of glycerophosphatidylinositol, InsP₁ and InsP₂, the [³H]inositol-labelled InsP₃ fraction was eluted with 0.1 M-formic acid/1.2 M-ammonium formate and collected in 4 ml fractions. The sample was desalted on a Sephadex G-10 column. After addition of [³²P]Ins(1,4,5)P₃, it was injected on a Partisil SAX column and eluted as described by Piroton *et al.* (1987). The column was calibrated by injected standards of the two isomers Ins(1,4,5)P₃ and Ins(1,3,4)P₃ (Piroton *et al.*, 1987).

Fig. 3. Concentration/response curve of NaF on IP₃ generation for a 20 min incubation

Special care was taken to avoid aluminium contamination of the solutions (overnight treatment of glass vials with 0.1 M-HNO₃). Experiments were performed in the presence of 10 mM-LiCl. Symbols: □, control; ▨, NaF (1 mM); ▩, NaF (5 mM); ▤, NaF (10 mM); ▥, NaF (20 mM); * *P* < 0.05; ** *P* < 0.01. Results are means ± S.E.M. of triplicates.

Table 1. Potentiation of the NaF-induced IP₃ generation by AlCl₃

The various concentrations of NaF and AlCl₃ (10 μM) were added simultaneously for 20 min. The same precautions as explained in Fig. 3 legend were taken. Results are means ± S.E.M., and are expressed as total radioactivity (c.p.m.) detected in the supernatant after addition of HClO₄ and centrifugation (see the Materials and Methods section). N.S., not significant.

[NaF]	Addition...	IP ₃ (c.p.m./100 mg wet wt.)		<i>P</i>
		None	AlCl ₃ (10 μM)	
0		63 626 ± 5870	75 563 ± 7670	N.S.
5 mM		87 263 ± 4630	106 029 ± 5603	< 0.05
20 mM		102 552 ± 8980	213 405 ± 33 138	< 0.01

Increasingly severe Ca²⁺-depletion conditions were obtained either by simple omission of added Ca²⁺ from the Krebs-Ringer bicarbonate preincubation and incubation media, or by concomitant addition of EGTA (0.1 or 1 mM). Cchol-stimulated InsP₃ accumulation was not abolished by any of these conditions. However, drastic Ca²⁺ deprivation decreased basal levels of InsP₃ and the relative Cchol stimulation (Fig. 5), as well as the incorporation of [³H]inositol or [³²P]P_i into PtdIns (results not shown). Absence of extracellular Ca²⁺ did not inhibit the NaF-induced IP response.

Ionophore A23187 (1 and 10 μM), replacement of NaCl by KCl in the incubation medium up to 130 mM-KCl, prostaglandins F₁ (25 μg/ml) and F_{2x} (2.5 and 25 μg/ml), noradrenaline and phenylephrine (both at 10 μM and 0.1 mM) failed to induce any significant IP effect (Table 2). Cchol (10 μM-0.1 M)-induced IP accumulations were not potentiated either by addition of ionophore A23187 (1 μM) or in the presence of KCl-induced membrane depolarization. In one typical experiment, InsP₃ radioactivities (c.p.m.; mean ± S.E.M.) in the slices were 867 ± 69 for controls, 5623 ± 276 for Cchol (1 μM), 40476 ± 758 for Cchol (10 μM), 843 ± 132 for A23187 (1 μM), 5973 ± 407 for A23187 (1 μM) plus Cchol (1 μM), and 45944 ± 4775 for A23187 (1 μM) plus Cchol (10 μM).

Fig. 4. Effects of PDBu (5 μM) on Cchol (10 and 5 μM)- and NaF (10 and 20 mM)-induced InsP₃ stimulations in the presence of LiCl (10 mM)

Reaction was stopped at 20 min. Numbers above the bars refer to the c.p.m. ratios of stimulated to control slices. Symbols: □, control; ▨, Cchol (5 μM); ▩, Cchol (10 μM); ▤, NaF (10 mM); ▥, NaF (20 mM); (↓) indicates that PDBu (5 μM) is added. Results are expressed as means ± S.E.M. for triplicates; ** *P* < 0.01.

Effect of activators of the cyclic AMP system, iodide and other modulators

Prostaglandin E₁ (10 and 25 μg/ml), cholera toxin (1 and 10 μg/ml), dibutyryl cyclic AMP (1 mM) and forskolin (10 μM) did not increase the generation of IP at 20 min, although increased protein iodination in slices in the same experiments demonstrated the biological activity of the agonists used (results not shown).

KI (4 and 40 μM) abolished the Cchol-induced InsP₃ generation. When added simultaneously, methimazole

Fig. 5. InsP_3 responses to Cchol ($10 \mu\text{M}$) with preincubation and/or incubation in Ca^{2+} -deprived buffers

When EGTA was added in the preincubation, it was for 1 h (the 1 h before the beginning of the incubation). If present, EGTA was supplemented at the beginning of the 20 min incubation period. Key: □, control; ▨, Cchol ($10 \mu\text{M}$); 1, KRB (Krebs-Ringer bicarbonate) + Ca^{2+} in preincubation and KRB + Ca^{2+} in incubation; 2, KRB + 0 Ca^{2+} in preincubation and KRB + 0 Ca^{2+} in incubation; 3, KRB + Ca^{2+} in preincubation and KRB + 0 Ca^{2+} , EGTA (0.1 mM) in incubation; 4, KRB + 0 Ca^{2+} , EGTA (1 mM) in preincubation and KRB + 0 Ca^{2+} , EGTA (0.1 mM) in incubation. Results are expressed as means $\pm$ S.E.M. for triplicate determinations.

Table 2. Effects of ionophore A23187 and of 'Ca²⁺-pathway'-activating agents on InsP_3 generation in comparison with the Cchol ($10 \mu\text{M}$) response

Results are means $\pm$ S.E.M.

	InsP_3 (c.p.m./100 mg wet wt.)
Control	732 ± 129
Cchol ($10 \mu\text{M}$)	14921 ± 2258 ($P < 0.01$)
Prostaglandin F_1	994 ± 213
Prostaglandin $\text{F}_{2\alpha}$	742 ± 203
Noradrenaline ($10 \mu\text{M}$)	680 ± 230
($0.1 \mu\text{M}$)	728 ± 279
A23187 ($1 \mu\text{M}$)	632 ± 191
($10 \mu\text{M}$)	814 ± 134

(1 mM) fully reversed this inhibition (Fig. 6). The same results were also observed when ^{32}P incorporation into PtdIns was measured (results not shown).

Fig. 6. Effect of $40 \mu\text{M}$ -KI [with or without 1 mM -methimazole (MMI)] on the Cchol-induced InsP_3 response

MMI and KI were added 2 h before the end of the preincubation and during the whole incubation period. Key: □, MMI (1 mM); ▤, KI ($40 \mu\text{M}$); ▥, MMI (1 mM) and KI ($40 \mu\text{M}$). Results are expressed as means $\pm$ S.E.M. of triplicates.

Thyrotropin-releasing hormone (thyroliberin; $1 \text{ ng-}1 \mu\text{g/ml}$), epidermal growth factor ($10\text{--}30 \text{ ng/ml}$) and human serum (1 and 10%) had no effect at 20 min. The stable ATP analogue adenosine 5'-[γ -thio]triphosphate ($10\text{--}300 \mu\text{M}$) and bradykinin ($0.1\text{--}10 \mu\text{g/ml}$) (for $10\text{--}20 \text{ min}$) were also ineffective.

Demonstration of $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase activity

With [^3H] $\text{Ins}(1,4,5)\text{P}_3$ ($10 \mu\text{M}$) as substrate, we measured $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase activity in dog thyroid extract. This activity was always lower when measured in the presence of EGTA (1 mM - Ca^{2+}) as compared with the activity in the presence of both Ca^{2+} and calmodulin. In one typical extract, activities were $7.6 \pm 0.5 \text{ nmol/min per g}$ of tissue when assayed in the presence of EGTA (1 mM), and 19.0 ± 0.4 when assayed in the presence of $1 \mu\text{M}$ -calmodulin and $10 \mu\text{M}$ free Ca^{2+} .

DISCUSSION

Cchol through a muscarinic receptor enhances the accumulation of IPs (Graff *et al.*, 1987) and the concentration of intracellular Ca^{2+} (Rani *et al.*, 1985; Raspe *et al.*, 1986) in dog thyroid cells. Through Ca^{2+} as intracellular signal, it stimulates the accumulation of cyclic GMP, the hydrolysis of cyclic AMP and the synthesis of thyroid hormones (Decoster *et al.*, 1980; Erneux *et al.*, 1985). In the present study we have analysed the mechanisms and control of this IPs generation. On cooling at 25°C , the kinetics of Cchol action showed a more distinct separation of IP accumulation:

there was a rapid increase in InsP_3 , whereas the accumulation of InsP_2 and InsP_1 was delayed. This sequence indicates that PtdInsP_2 is possibly the first substrate of Cchol-mediated activation of PLC. The mean quantitative accumulation of InsP_1 is 10–30 times that of InsP_3 , which might be explained by the rapid turnover of InsP_3 to InsP_1 , but also raises the possibility of alternative pathways leading to InsP_1 formation, as suggested in polymorphonuclear leucocytes (Dillon *et al.*, 1987) and GH_3 cells (Imai & Gershengorn, 1986).

In our thyroid slices, a small h.p.l.c. peak of $\text{Ins}(1,4,5)\text{P}_3$ was present at 12 min, whereas at later times $\text{Ins}(1,3,4)\text{P}_3$ continued to accumulate and was the only isomer still present. The presence of the small amount of InsP_4 detected at high Cchol concentrations (0.1 mM) was a clue for the existence of a $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase in the thyroid. This InsP_3 3-kinase activity was indeed detected in our dog thyroid homogenates and was also Ca^{2+} -sensitive, as in rat and bovine brain (Erneux *et al.*, 1987; Takazawa *et al.*, 1988). Thus, by all the criteria, the action of Cchol through muscarinic receptors in dog thyroid cells corresponds to the classical model of the PtdInsP_2 - Ca^{2+} cascade in which $\text{Ins}(1,4,5)\text{P}_3$ is rapidly phosphorylated to $\text{Ins}(1,3,4,5)\text{P}_4$ (Batty *et al.*, 1985), which is then hydrolysed to $\text{Ins}(1,3,4)\text{P}_3$ (Irvine *et al.*, 1985).

Receptor activation of PLC and of the consequent cascade is mediated by a specific set of G-proteins (Straub & Gershengorn, 1986). Our data in intact cells suggest that this concept applies to Cchol stimulation in dog thyroid, since NaF increased InsP_3 generation (Graff *et al.*, 1987), and that this effect was enhanced by AlF_4^- . Potentiation of fluoride activation of PLC by AlCl_3 has been documented in some systems (Blackmore *et al.*, 1985; Guillon *et al.*, 1986), AlF_4^- substituting for the γ -phosphate of GTP (Bigay *et al.*, 1985). NaF indeed exerts the same metabolic effects as Cchol on dog thyroid (Dumont *et al.*, 1971; Van Sande *et al.*, 1979; Decoster *et al.*, 1980).

Accumulation of InsP_1 induced by Cchol was insensitive to pertussis toxin, as were all the other metabolic effects of Cchol (Cochaux *et al.*, 1985). We conclude that in the dog thyroid muscarinic stimulation of PLC is transduced by a pertussis-toxin-insensitive Gp coupling protein.

The activation of the PtdInsP_2 cascade releases two general intracellular signals in the cell, Ca^{2+} and DAG. The effects of those signals on the first step of the cascade have been investigated. Using phorbol esters as probes of PKC, we have previously demonstrated in dog thyroid cells a drastic inhibition by PDBu of the Cchol-induced IPs accumulation (Graff *et al.*, 1987), as well as of the Cchol effects that they do not elicit by themselves: the enhancement of cyclic GMP accumulation and of intracellular Ca^{2+} level (Mockel *et al.*, 1987). This suggests that, as in other systems, the activation of PKC exerts a negative feedback on PLC activation by its agonists (Hunter *et al.*, 1984; Schimmel *et al.*, 1986). In hamster fibroblasts, phorbol esters inhibit the effect of AlF_4^- (Paris & Pouyssegur, 1987), whereas in our dog thyroid cells stimulation of IPs generation was completely unaffected by these agents. These data suggest that in our system the negative feedback of PKC is exerted at the level of the receptor or receptor-G-protein interaction, leaving intact the fluoride-G-protein and the G-protein-PLC interactions.

In dog thyroid cells, Ca^{2+} omission from the medium

or even stringent Ca^{2+} depletion by EGTA never suppressed the Cchol-mediated IPs increase, but at most decreased the amplitude of the response, whereas A23187 or depolarization by KCl did not activate or potentiate the activation of PLC. This confirms in this system that IPs generation induced by Cchol is the primary, Ca^{2+} -independent, signal of receptor activation.

The relation in the dog thyroid cell of the PtdIns cascade to the other major signalling pathway, the cyclic AMP system, is simple. All agonists that increase cyclic AMP or cyclic AMP analogues do not affect basal IPs concentrations. Moreover, general activators of the system (forskolin or cholera toxin) do not inhibit, as in platelets (Nishizuka, 1984), or potentiate Cchol- or NaF-induced IPs generation. On the other hand, we have previously shown that Cchol decreased TSH- and forskolin-dependent cyclic AMP accumulation by activating a calmodulin-sensitive cyclic nucleotide phosphodiesterase (Van Sande *et al.*, 1979; Erneux *et al.*, 1985), whereas phorbol esters do not inhibit the TSH-induced cyclic AMP accumulation (Mockel *et al.*, 1987).

Iodide inhibits several steps of thyroid metabolism, including adenylate cyclase and TSH-induced cyclic AMP accumulation (Van Sande & Dumont, 1973; Cochaux *et al.*, 1987; Corvilain *et al.*, 1988). As these effects are relieved by drugs that inhibit iodide trapping and iodide oxidation, they have been related to a postulated organified intermediate $\text{X} \sim \text{I}$ (Van Sande & Dumont, 1973). In the present work, iodide also inhibited the Cchol-mediated IPs generation. As for the inhibition of cyclic AMP accumulation, this effect was relieved by methimazole, which blocks iodide organification. Thus iodide by a similar mechanism inhibits both the main signalling pathways of the thyroid cell: the cyclic AMP and the PtdIns cascades. Several other metabolic steps appear to be independently inhibited by a $\text{X} \sim \text{I}$ type of effect: glucose transport, uridine incorporation, H_2O_2 generation, iodide transport etc. (Corvilain *et al.*, 1988; for review see Pisarev, 1985). It is remarkable that the inhibited reactions take place at the level of the plasma membrane. This large panoply of effects of iodide suggests the existence of either a non-specific general negative feedback of organified iodine on different thyroid metabolic pathways or the existence of several specific inhibitors.

Our previous data concerning incorporation of $[\text{}^{32}\text{P}]\text{P}_i$ into PtdIns in the presence of Cchol, phorbol esters (Mockel *et al.*, 1987) or iodide show parallelism with the effects obtained with these agents on IPs generation in the present work. This demonstrates that the 'PtdIns effect' measured in the 1970s (Michell, 1975) was a good, albeit indirect, index of the activation of the PtdInsP_2 - Ca^{2+} pathway.

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