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Carbachol and sodium fluoride, but not TSH, stimulate the generation of inositol phosphates in the dog thyroid

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In dog thyroid slices prelabeled with *myo*-[2-^{3}H]inositol, carbachol (10^{-7}–10^{-4} M) and NaF (10–20 mM) stimulated IP_1, IP_2 and IP_3 generation. These effects did not require the presence of extracellular calcium. Atropine and PDBu inhibited the action of the cholinergic agonist. No effect of TSH (1–100 mU/ml) could be detected on PIP_2 hydrolysis and IP production. These results suggest that (i) IP_3 could play a role in the metabolic actions of carbachol in the thyroid; (ii) a G-protein coupling the hormone-receptor binding to phospholipase C activation exists in the thyroid membrane; (iii) the well known TSH-induced increased PI turnover does not result in IP_3 accumulation.

Inositol phosphate; Carbamylcholine; Sodium fluoride; Thyrotropin; Phorbol ester; (Thyroid)

1. INTRODUCTION

Recent work has demonstrated the presence of the IP_3-DAG second messenger system and of the regulated phospholipase C hydrolyzing their membrane precursor PIP_2 in many tissues [1–3]. Ins 1,4,5-P appears to release Ca^{2+} from non-mitochondrial intracellular sources [4–6]. DAG and its phorbol ester analogues on the other hand activate a calcium- and phospholipid-dependent protein kinase [3,7,8].

In the thyroid gland, TSH exerts many of its metabolic effects through activation of the adenylate cyclase [9–12] but also increases PI turnover independently of cAMP accumulation [13–18]. Cchol similarly stimulates PI turnover in the dog thyroid [17,19] and raises $[Ca^{2+}]_i$ in dog thyroid cells [20,21]. The precise modification of thyroid phosphoinositide metabolism occurring in these conditions is unknown. This work constitutes the first report examining the production of inositol phosphates in thyroid tissue exposed to TSH and Cchol.

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Abbreviations: Ins, *myo*-inositol; IP, inositol phosphate; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; Ins 1,4,5-P_3, inositol 1,4,5-trisphosphate; Ins 1,3,4-P_3, inositol 1,3,4-trisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; $[Ca^{2+}]_i$, free intracellular calcium concentration; Cchol, carbamylcholine; TSH, thyroid stimulating hormone; PDBu, 4β-phorbol 12,13-dibutyrate; DAG, diacylglycerol; G-protein, guanine nucleotide-binding regulatory protein

2. MATERIALS AND METHODS

2.1. *Products*

myo-[2-^{3}H]Inositol (16.5 Ci/mmol) and *myo*-[2-^{3}H]inositol phosphate (8.4 Ci/mmol) were purchased from New England Nuclear (Dupont-NEN, Haren, Belgium), *myo*-[2-^{14}C]inositol phosphate (50–60 Ci/mmol) from the Radiochemical Centre

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(Amersham, England). Cchol was provided by K and K (Plain View, NY, USA), TSH (Thytropar) by Armour Pharmaceutical (Chicago, IL, USA) and PDBu by Sigma (St. Louis, MO, USA). The Dowex AG1-X8 ion-exchange resin (formate form, 100–200 mesh) was obtained from Bio-Rad (Watford, England). Ins-1[4,5-^{32}P]-trisphosphate (spec. act. 9000 cpm/nmol) was prepared from human erythrocytes by the methods of Downes et al. [22] as modified by Irvine et al. [23]. All other reagents were of the purest grade commercially available.

2.2. *Tissue preparation and incubation*

Dog thyroid slices were incubated at 37°C under an atmosphere of O_2/CO_2 (95:5, v/v) in 2 ml of Krebs-Ringer-bicarbonate buffer supplemented with 8 mM glucose and 0.5 g/l of bovine serum albumin [9]. The slices were first preincubated for 4 h in the presence of 20 μCi/ml of [^{3}H]inositol (spec. act. 16.5 Ci/mmol), then transferred to fresh unlabeled incubation medium. After 15 min, LiCl was added to the incubation flasks (final concentration 10 mM), followed after 5 min by the experimental agent to be tested for various lengths of time.

2.3. *Extraction and separation of [^{3}H]inositol phosphates*

Incubation was stopped by rapid immersion and homogenization of the slices in 2 ml of ice-cold 3% $HClO_4$. The homogenate was centrifuged, and the resulting pellet washed once with 1% $HClO_4$. The combined supernatants were neutralized to pH 7.7–7.8 by addition of 0.38 M Hepes and 0.76 M KOH. After potassium perchlorate precipitation, the supernatant was treated and applied to AG1-X8 columns (0.6 × 4 cm) [24]. Ins, GPI, IP_1, IP_2 and IP_3 were eluted in a given step procedure [25] with slight modifications, using for each fraction successive volumes of 20 ml H_2O, 12 ml of 5 mM sodium tetraborate and 60 mM ammonium formate, 28 ml of 5 mM sodium tetraborate and 0.15 M ammonium formate, 24 ml of 0.1 M formic acid and 0.4 M ammonium formate, and 24 ml of 0.1 M formic acid and 1 M ammonium formate; 4 ml of each fraction were taken for determination of radioactivity in Lumagel scintillation fluid. In each experiment, a pair of unlabeled slices was homogenized with [^{3}H]Ins, [^{14}C]IP_1 and Ins-1[4,5-^{32}P]-trisphosphate and treated as the experimental samples to check the accuracy of inositol phosphate separation. Results were expressed as cpm/100 mg tissue wet wt (mean ± SE) of triplicate samples. Significance of differences between groups was calculated by the paired *t*-test. Each experimental condition was reproduced in at least three different experiments with identical results. In each experiment, the reac-

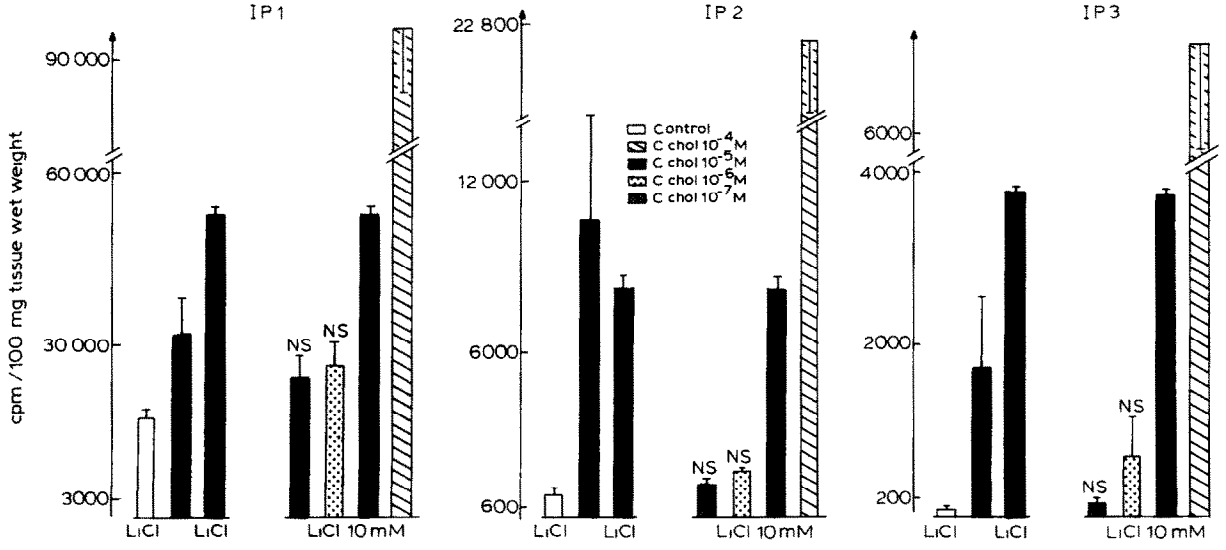

Fig.1. Effect of increasing concentrations of Cchol on IP accumulation over 20 min. The second column of the lefthand panel shows the action of Cchol 10^{-5} M in the absence of 10 mM LiCl.

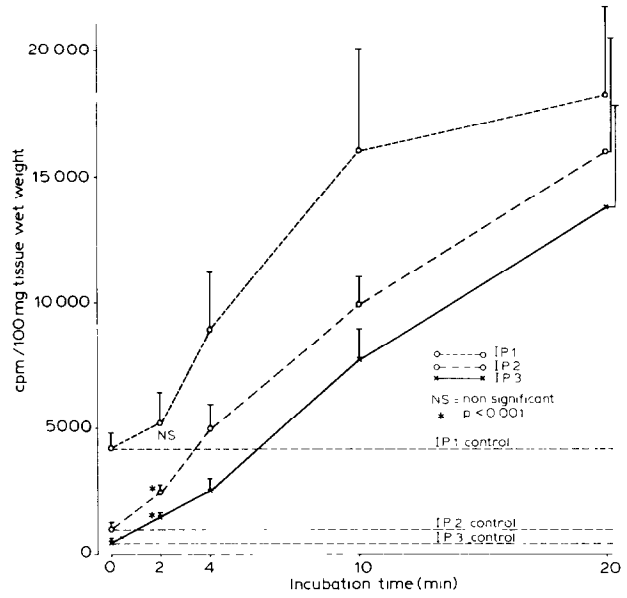

Fig.2. Time course of IP accumulation in the presence of Cchol (10^{-5} M) and 10 mM LiCl.

tivity of the slices to the agonists tested was checked by measuring protein iodination in control slices [26].

3. RESULTS

In dog thyroid slices, 10 mM LiCl had no discernible effect on the basal levels of IP, but increased significantly the accumulation of IP_3, IP_2 and IP_1 induced by 10^{-5} M Cchol (fig.1). Cchol 10^{-4} M and 10^{-5} M stimulated the generation of all three IPs (fig.1). Quantitatively, the accumulation was in the order: $IP_3 < IP_2 < IP_1$, but the factors of stimulation were in the reverse order. When the pooled IP fractions were considered, the Cchol stimulation was significant at the lowest concentration tested (10^{-7} M) and showed a log linear increase with the agonist concentration.

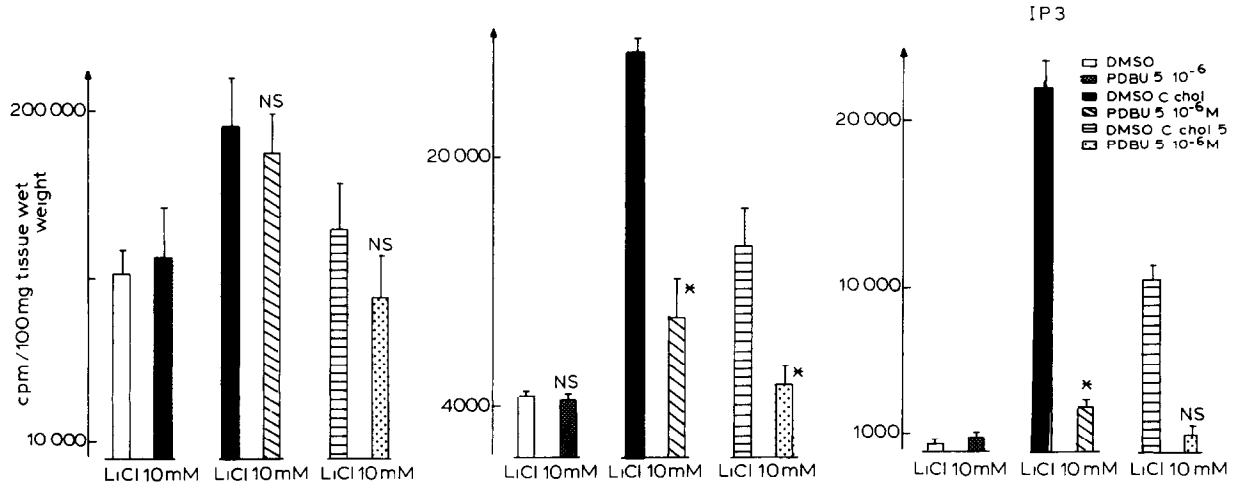

Fig.3. Inhibition by 5×10^{-6} M PDBu of the IP accumulation induced by Cchol, 5×10^{-6} and 10^{-5} M. (Incubation time 25 min, PDBu being added 5 min before Cchol.)

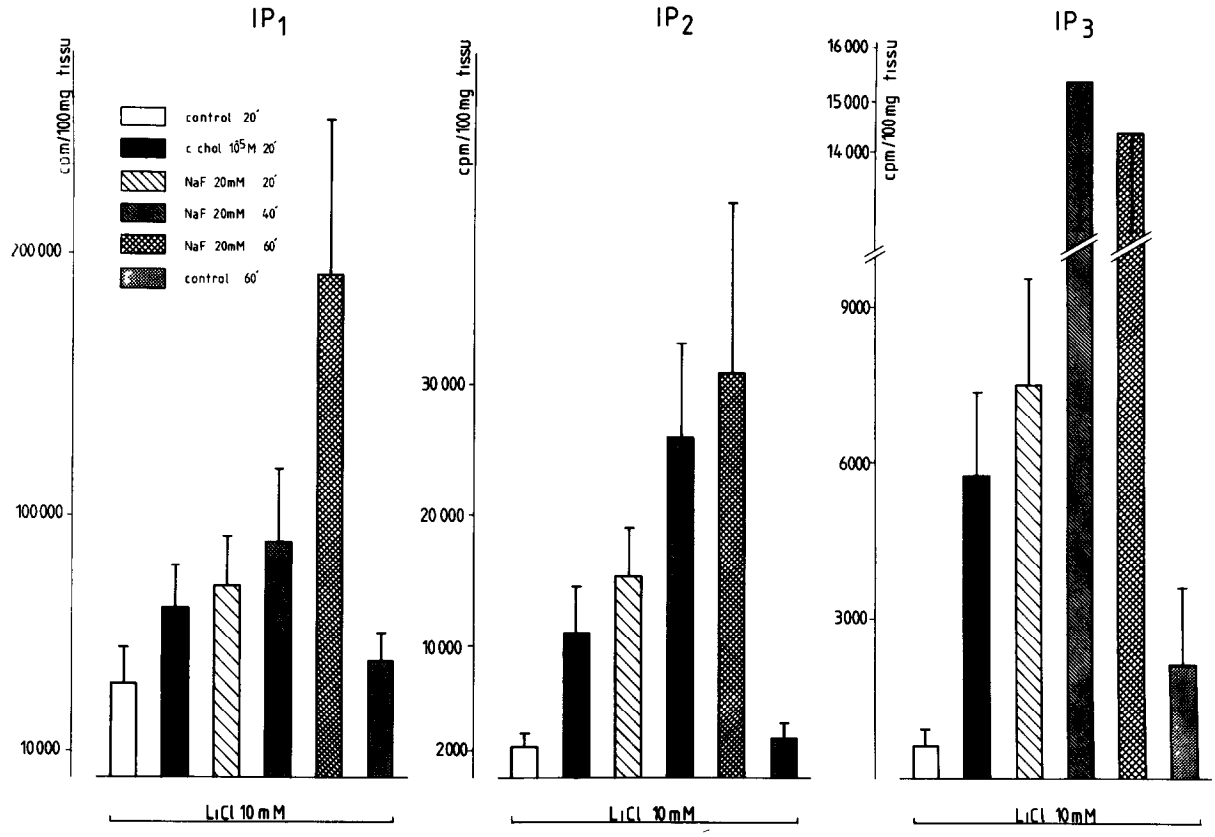

Fig.4. Effects of 20 mM NaF on IP accumulation (incubation time as indicated).

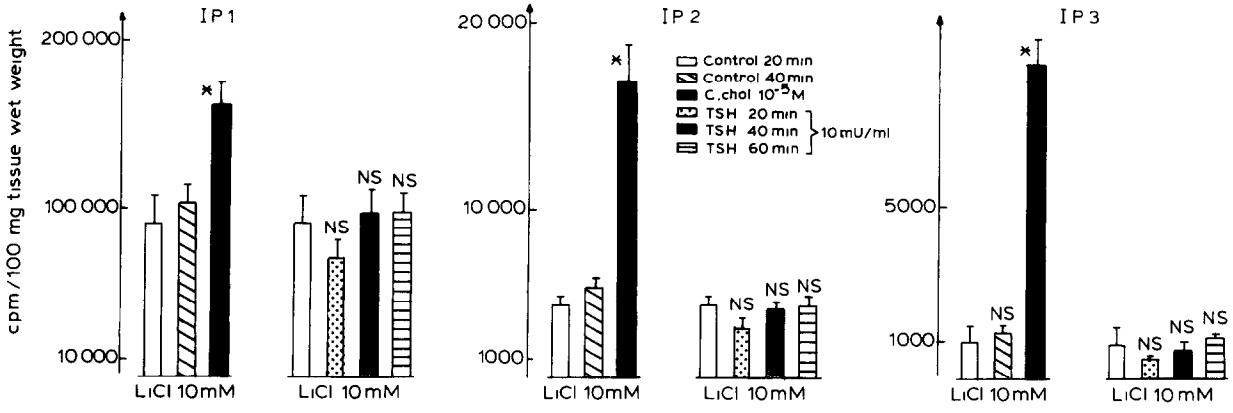

Fig.5. Lack of effects of 10 mU/ml TSH on IP accumulation over a 60 min time course.

The time course of a 10^{-5} M Cchol stimulation demonstrated a significant accumulation of IP_3 and IP_2 after 2 min, and of IP_1 after 4 min (fig.2). For the next 20 min, the generation of the three IPs was parallel with no obvious precursor-product relationship. Atropine completely inhibited the effects of Cchol (not shown).

PDBu (5×10^{-6} M) had no effect by itself on the basal levels of IP, but, when added 5 min before the agonist, it inhibited nearly completely the ef-

fect of equimolar 5×10^{-6} M Cchol, and partially the action of 10^{-5} M Cchol (fig.3).

Sodium fluoride (NaF, 20 mM) increased the generation of the three IP fractions, its effect being equivalent to that of 10^{-5} M Cchol at 20 min, and with a time course sustained for at least 60 min (fig.4). The stimulation exerted by 10^{-5} M Cchol as well as 20 mM NaF remained unchanged in the absence of extracellular Ca^{2+} (not shown).

No effect of 10 mU/ml TSH on IP production could be detected over an hour incubation (fig.5). Varying TSH concentrations from 1 to 100 mU/ml and incubation times from 2 to 60 min could not reveal any significant change in any of the three IP fractions (not shown).

4. DISCUSSION

In the dog thyroid, Cchol through a muscarinic receptor stimulates protein iodination, glucose C_1 oxidation, cGMP accumulation, and antagonizes the TSH-induced cAMP accumulation and hormone secretion [12,17,19,27–29]. As these effects are reproduced by the ionophore A23187 in the presence of Ca^{2+}, are inhibited in Ca^{2+} depleted cells and are accompanied by accelerated PI turnover, increased $^{45}Ca^{2+}$ efflux and a raise in $[Ca^{2+}]_i$, it has been suggested that they result from a release of Ca^{2+} from intracellular stores consequent to PI hydrolysis [20,21,29,30]. The present data provide the first direct evidence that Cchol's initial interaction with the thyroid membrane activates PIP_2 hydrolysis, leading to IP_3 accumulation and DAG release. As could be expected the increased IP production was blocked by atropine. The relationship between Cchol's concentration and its effect on PIP_2 hydrolysis is similar to that observed for the metabolic action of the cholinergic agonist [28,29]. The time course of IP generation in the presence of 10^{-5} M Cchol showed no early peak nor plateau phase for any of the IP fractions. This is compatible with the generally admitted sequential generation of IP in the order IP_3, IP_2, IP_1 [2,24], as well as with a direct generation of IP_2 and IP_1 respectively from PIP and PI [31,32]. Considering the short half-life of Ins 1,4,5-P_3, the linear increase of IP_3 over at least 20 min could suggest the generation of an unknown proportion of Ins 1,3,4-P_3 as in the parotid gland [6] or cerebral cortex [33].

NaF has long been known to exert various metabolic effects in the thyroid, some of them being Ca^{2+} dependent like increased cGMP concentrations, stimulated protein iodination and glucose C_1 oxidation, while others are presumably related to inhibition of glycolysis: e.g. inhibition of TSH-induced cAMP accumulation and hormone secretion [9,27,28,34–36]. The recent demonstration of a transducing G-protein coupling the hormonal receptor to the activation of phospholipase C [37,38] and of its stimulation by NaF in hepatic cells [39], neutrophils [40] and WRK_1 cell membranes [41], might explain the previous findings in the thyroid. The present data indeed show that NaF stimulates IP production in the thyroid over a prolonged time course, and without requiring the presence of extracellular Ca^{2+}, which strongly suggest the validity of this model for the action of NaF on this tissue.

Phorbol esters, probably through protein kinase C activation, exert some of the metabolic effects of Cchol in the dog thyroid (protein iodination, secretion), but they inhibit the initial steps of the Cchol-stimulated PI-Ca^{2+} cascade [19]. The present data extend these observations by showing that PDBu also inhibited the IP generation induced by Cchol. Similar observations have been reported with other tissues [42–44] and it has recently been suggested that phorbol ester action could result from increased IP_3 degradation [45]. Such a mechanism of action is compatible with our data, but is insufficient to explain the inhibition of IP_1 accumulation and of the increased PI turnover. In our dog thyroid system, other possible feedback targets of activated protein kinase C could thus be the muscarinic receptor, G-protein or phospholipase C complex [46–50].

We were unable to detect any effect of TSH on IP production in our system. Such a negative result must be considered with caution. Indeed cAMP-independent increased PI turnover and $^{45}Ca^{2+}$ efflux induced by TSH have repeatedly been documented by a number of authors over the last twenty years in the dog and pig thyroid [13–16,19]. These effects have been called the A effects of TSH [18] by contrast to the B, cAMP-dependent effects. Moreover, TSH has been reported to increase $[Ca^{2+}]_i$ in FRTL-5 thyroid cells [51], but not in dog thyroid cells [20,21]. Alternative hypotheses on

mechanisms compatible with such data will have to be investigated.

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REFERENCES

[1] Sekar, M.C. and Hokin, L.E. (1986) J. Membrane Biol. 89, 193–210.
[2] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
[3] Nishizuka, Y. (1984) Nature 308, 693–698.
[4] Berridge, M.J., Heslop, J.P., Irvine, R.F. and Brown, K.D. (1984) Biochem. J. 222, 195–201.
[5] Irvine, R.F., Brown, K.D. and Berridge, M.J. (1984) Biochem. J. 221, 269–272.
[6] Irvine, R.F., Anggard, E.E., Letcher, A.J. and Downes, C.P. (1985) Biochem. J. 229, 505–511.
[7] Rasmussen, H. (1986) New Engl. J. Med. 314, 1094–1101; 1164–1170.
[8] Tanabe, A., Nielsen, T.B., Rani, C.S.S. and Field, J.B. (1985) Arch. Biochem. Biophys. 243, 92–99.
[9] Dumont, J.E., Willems, C., Van Sande, J. and Nève, P. (1971) Ann. NY Acad. Sci. 185, 291–316.
[10] Dumont, J.E. (1976) Vitam. Horm. 29, 287–412.
[11] Dumont, J.E., Boeynaems, J.M., Decoster, C., Lamy, F., Lecocq, R., Mockel, J., Unger, J. and Van Sande, J. (1978) Adv. Cyclic Nucleotide Res. 9, 723.
[12] Dumont, J.E., Miot, F., Erneux, C., Couchie, D., Cochaux, P., Gervy-Decoster, C., Van Sande, J. and Wells, J.N. (1984) Adv. Cyclic Nucleotide Res. 16, 325–336.
[13] Scott, T.W., Jay, S.M. and Freinkel, N. (1966) Endocrinology 79, 591–600.
[14] Jacquemin, C. and Haye, B. (1970) Bull. Soc. Chim. Biol. 52, 153–165.
[15] Scott, T.N., Freinkel, N., Klein, J.H. and Nitzan, M. (1970) Endocrinology 87, 854–863.
[16] Gerard, C., Haye, B., Jacquemin, C. and Mauchamp, J. (1982) Biochim. Biophys. Acta 710, 359–369.
[17] Takeuchi, A. and Mockel, J. (1983) Ann. Endocrinol. 44, 54A.
[18] Dumont, J.E., Takeuchi, A., Lamy, F., Gervy-Decoster, C., Cochaux, P., Roger, P., Van Sande, J., Lecocq, R. and Mockel, J. (1981) Adv. Cyclic Nucleotide Res. 14, 479–489.
[19] Mockel, J., Van Sande, J., Decoster, C. and Dumont, J.E. (1986) Metabolism, in press.
[20] Rani, C.S.S., Boyd, A.E. and Field, J.B. (1985) Biochem. Biophys. Res. Commun. 131, 1041–1047.
[21] Raspe, E., Roger, P. and Dumont, J.E. (1986) Biochem. Biophys. Res. Commun., in press.
[22] Downes, C.P., Mussat, M.C. and Michell, R.H. (1982) Biochem. J. 203, 169–177.
[23] Irvine, R.F., Letcher, A.J. and Dawson, R.M. (1984) Biochem. J. 218, 177–185.
[24] Berridge, M.J. (1983) Biochem. J. 212, 849–858.
[25] Downes, C.P. and Michell, R.H. (1981) Biochem. J. 198, 133–140.
[26] Rodesch, F., Nève, P., Willems, C. and Dumont, J.E. (1969) Eur. J. Biochem. 8, 26–32.
[27] Yamashita, K. and Field, J.B. (1972) J. Biol. Chem. 247, 7062–7066.
[28] Van Sande, J., Decoster, C. and Dumont, J.E. (1975) Biochem. Biophys. Res. Commun. 62, 168–175.
[29] Decoster, C., Mockel, J., Van Sande, J., Unger, J. and Dumont, J.E. (1980) Eur. J. Biochem. 104, 199–208.
[30] Arem, R., Chayoth, R., Shenrenberg, T.D., Miller, S.I., Chou, M.C.Y. and Field, J.B. (1983) Arch. Biochem. Biophys. 225, 66–74.
[31] Fain, J.N. (1984) Vitam. Horm. 41, 117–160.
[32] Dixon, J.F. and Hokin, L.E. (1985) J. Biol. Chem. 260, 16068–16071.
[33] Batty, I.R., Nahorsky, S.R. and Irvine, R.F. (1985) Biochem. J. 232, 215–221.
[34] Dumont, J.E. (1966) Bull. Soc. Chim. Biol. 48, 419–439.
[35] Willems, C., Berberof-Van Sande, J. and Dumont, J.E. (1972) Biochim. Biophys. Acta 264, 197–204.
[36] Van Sande, J., Decoster, C. and Dumont, J.E. (1979) Mol. Cell. Endocrinol. 14, 45–57.
[37] Straub, R.E. and Gershengorn, M.C. (1986) J. Biol. Chem. 261, 1158–1162.
[38] Kikuchi, A., Kozawa, O., Kaibuchi, K., Katada, T., Ui, M. and Takai, Y. (1986) J. Biol. Chem. 261, 11558–11562.
[39] Blackmore, P.F. and Exton, J.H. (1986) J. Biol. Chem. 261, 11056–11063.
[40] Strand, C.F., Parente, J.E. and Wong, K. (1985) FEBS Lett. 206, 20–24.
[41] Guillon, G., Mouillac, B. and Balestre, M.N. (1986) FEBS Lett. 204, 183–188.

[42] Labarca, R., Janowsky, A., Patel, J. and Paul, S.M. (1984) Biochem. Biophys. Res. Commun. 123, 703–709.
[43] Schimmel, R.J., Dzierzanowski, D., Elliott, M.E. and Honeyman, T. (1986) Biochem. J. 236, 757–764.
[44] Monaco, M.E. and Mufson, R.A. (1986) Biochem. J. 236, 171–175.
[45] Molina, Y., Vedia, L.M. and Lapetina, E.G. (1986) J. Biol. Chem. 261, 10493–10495.
[46] Watson, S.P. and Lapetina, E.G. (1985) Proc. Natl. Acad. Sci. USA 82, 2623–2626.
[47] Orellana, S.A., Solski, P.A. and Brown, J.H. (1985) J. Biol. Chem. 260, 5236–5239.
[48] Hunter, T., Ling, N. and Cooper, S.A. (1984) Nature 311, 480–483.
[49] Sibley, D.R., Nambi, P., Peters, J.R. and Lefkowitz, R.J. (1984) Biochem. Biophys. Res. Commun. 121, 973–979.
[50] Leeb-Lundberg, L.M.F., Cotecchia, S., Lomasney, J.W., De Bernardis, J.F., Lefkowitz, R.J. and Caron, M.G. (1985) Proc. Natl. Acad. Sci. USA 82, 5651–5655.
[51] Corda, D., Marcocci, C., Kohn, D.L., Axelrod, J. and Luini, A. (1985) J. Biol. Chem. 260, 9230–9236.