CONTROL BY TSH OF A PHOSPHOLIPASE A_2 ACTIVITY, A LIMITING FACTOR IN THE BIOSYNTHESIS OF PROSTAGLANDINS IN THE THYROID

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Received 27 January 1973

1. Introduction

Thyreostimulin (TSH) stimulates the thyroid adenyl-cyclase and increases the level of cyclic 3'-5'-adenosine monophosphate (c-AMP). The model of Sutherland, in which c-AMP is the second messenger, explains many effects of TSH [1].

On the other hand, the prostaglandins (PGE_2 and $PGF_2\alpha$) which are abondant in the thyroid [2], stimulate the thyroid adenyl-cyclase [3–5].

Recently Burke [6] has shown an increase in the intracellular concentration of thyroid prostaglandins under the influence of TSH.

The hypothesis of Kuehl [7–8], according to which the action of the luteinizing hormone (LH) on the ovary adenyl-cyclase would necessarily involve a stimulation of prostaglandins biosynthesis, has been applied to the thyroid by Burke [4–9].

On the other side, several authors have reached the conclusion that the release of prostaglandins from a tissue was due to a neo-synthesis and not to a depletion of storage material [10, 11]. The efficiency of the prostaglandin synthetase seems to depend only on the amount of substrate available, as indeed the addition of arachidonic acid causes a rapid formation of prostaglandins [12, 13]. The polyunsaturated fatty acids, precursors of the prostaglandins, exist at a low concentration as free acids, but their concentration is not negligible in the membrane phospholipids. It has been suggested that the activity of endogenous phospholipases A could be the factor regulating the biosynthesis of prostaglandins [14, 15]. It has been demonstrated that phospholipids can play the role of precursors in the biosynthesis of prostaglandins [16, 17] and that prior treatment with a venom phospholipase A increases the conversion yield [15, 17].

In this work we have studied on one hand whether the prostaglandins are obligatory intermediates in the action of TSH on the adenyl-cyclase, as suggested by Burke, and on the other hand if there is a TSH-dependent control mechanism of the production of E_2 and $F_2\alpha$ prostaglandins from thyroid phospholipids. We have obtained the following results:

i) TSH increases the biosynthesis of prostaglandins in the thyroid *in vitro*; we thus confirm the results of Burke et al. [6] using a different methodology. This effect of TSH is abolished by indomethacin and aspirin, which are inhibitors of prostaglandin synthetase [18]+.

ii) The increase in the synthesis of PGE_2 and $PGF_2\alpha$ follows the stimulation by TSH of an endogenous phospholipase A_2 which releases arachidonic acid from phosphatidyl-inositol.

iii) In the absence of prostaglandins synthesis, TSH still stimulates the adenyl-cyclase.

* With the technical assistance of Mrs. O. Legue.

+ We thus confirm results published by Burke as this manuscript was being prepared (Prostaglandins 2 (1972) 413).

2. Material and methods

2.1. *Preparation of [^{14}C]phospholipids*

Pig thyroid glands, obtained from a slaughter-house[++], are prepared and incubated as previously described [19] in the presence of 250 μCi [^{14}C]acetate ([^{14}C]acetate C.U., 91.3 mCi/mM, C.E.N. France). After extraction, the individual phospholipids [^{14}C]phosphatidyl-inositol ([^{14}C]PI) and [^{14}C]phosphatidyl-choline ([^{14}C]PC) are eluted from a preparative chromatography according to Marinetti [20]. A titration of phosphorus according to Shibuya [21] and a measure of radioactivity allow a determination of the specific activity of [^{14}C]PI (31,250 cpm/100 μgP) and of [^{14}C]PC (5,700 cpm/100 μgP).

2.2. *Experiments on the biosynthesis of prostaglandins*

2.2.1. From [^{14}C]acetate

Pig thyroid slices, incubated for 2 hr in the presence of [^{14}C]acetate (250 μCi) with or without TSH (50 mU/ml), were treated according to the technique described by Coceani et al. [22] and the extracts were analyzed by thin-layer chromatography using the solvent systems described by Green and Samuelsson [23].

2.2.2. From [^{14}C]phospholipids

The prostaglandin synthetase activity of thyroid homogenates was measured according to the technique described by Pace Asciak and Wolfe [13]. Prior to the preparation of the homogenate, the slices were incubated for 30 min in KRB under a carbogen atmosphere. Without preincubation there is no prostaglandin synthetase activity. The thyroid homogenate (Dounce homogenizer) (900 μg of protein as measured by the method of Lowry [24]) is added at zero time to the [^{14}C]phospholipid emulsified according to Nachbaur et al. [25], in the presence or absence of TSH, in the presence or absence of inhibitors of prostaglandin synthetase [18]. (The volume of the reaction mixture is 500 μl). At the end of the incubation, the total lipid extract is chromatographed according to the technique described by Lands and Samuelsson [16]. The lipids are visualized by spraying a solution of 1% iodine in methanol. The areas which correspond to the R_f of the control prostaglandins E_2 and $F_{2\alpha}$ are scraped, transferred into scintillation vials and counted.

2.3. *Measure of phospholipase A activity*

The phospholipase A activity of a thyroid homogenate is determined by measuring the amount of labelled fatty acids released from the phospholipids [^{14}C]phosphatidyl-inositol or [^{14}C]phosphatidyl-choline. The reaction mixture contains the emulsified [^{14}C]phospholipid, Tris-maleate buffer pH 7.4, 20 mM and an amount of homogenate identical to that used for the assay of prostaglandin synthetase. The total volume of the reaction mixture is 500 μl.

At the end of the incubation, the free fatty acids are extracted according to the method of Dole [26]. An aliquot is counted (liquid scintillation) to determine the total amount of released material. The fatty acids are methylated by the method of Metcalf [27]. The fatty acids methyl esters are analyzed by thin-layer chromatography on a silica gel G plate (0.25 mm thick) impregnated with 12% $AgNO_3$ (solvent: heptane–ethyl ether–acetic acid, 70:30:0.4, v/v). The methyl esters are fractionated according to the number of double bonds and are identified by comparison with control substances (Applied Science Laboratories); the spots are scraped, transferred into scintillation vials and counted.

2.4. *Determination of c-AMP*

The effects of indomethacin and of aspirin (in the presence or absence of TSH), and the action of PGE_2, arachidonic acid and TSH are measured *in vitro* on pig thyroid slices. Assays are performed according to the technique of Gilman [28].

The comparisons between assays and control experiments have been examined using the Student's *t*-test [29].

3. Results

After incubation of the thyroid slices in the presence of [^{14}C]acetate, the prostaglandins are extracted [22] and analyzed by thin-layer chromatography

[++] We thank the slaughter-house of the city of Reims for providing us with the glands.

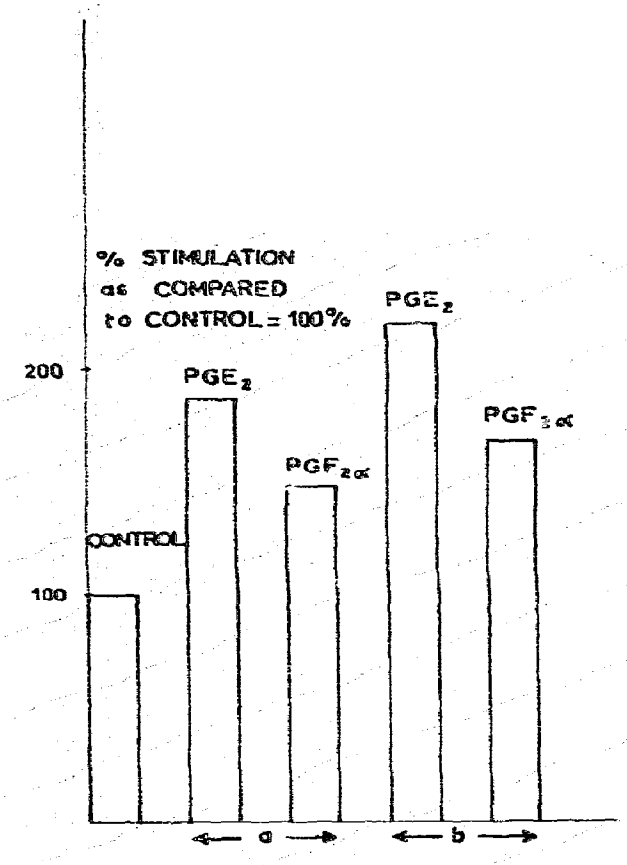

Fig. 1. Biosynthesis of prostaglandins in thyroid slices incubated with [^{14}C]acetate (see Material and methods). Thin-layer chromatography-solvent system A II [23]. a) 2 hr preincubation, then TSH for 15 min (50 mU/ml). b) 2 hr preincubation, then TSH for 30 min (50 mU/ml). The experiments were repeated three times with identical results.

[23]. Several radioactive spots have been detected and among them we have identified PGE_2 and $PGF_2\alpha$ which have been shown to exist in human thyroid [2]. This result shows that, under the conditions of *in vitro* incubation that we have used, a synthesis of endogenous prostaglandins really takes place. It can also be seen that, after 2 hr of pre-labelling, the incubation in the presence of TSH (15 min and 30 min) increases the radioactivity of prostaglandins by 50 to 100% (fig. 1). We are not in a position to interpret this observation, as the determination of the specific radioactivity of the direct precursors of the prostaglandins is not possible. Arachidonic acid can be regarded as the precursor of PGE_2 and $PGF_2\alpha$ [30–32]. This acid is present in the phospholipids and the neutral lipids. We have no information on their nature or on their intracellular localization, we do not know whether the specific radioactivity is identical for all the fatty acids in the lipids and whether it is modified during the hormonal stimulation. As a matter of fact it is known that TSH stimulates the turnover of the fatty acids, in the neutral lipids and the phospholipids [33]. It therefore appeared important to us to study the biosynthesis of prostaglandins, and its stimulation by TSH, starting from precursors whose specific activity is known and not modified during the experiment.

Thyroid homogenates were incubated with [^{14}C]-phosphatidyl-inositol obtained by biosynthesis from [^{14}C]acetate, in the presence or absence of TSH (50 mU/ml) and of inhibitors of prostaglandin synthetase (indomethacin 10 μg/ml and aspirin 100 μg/ml). Phosphatidyl-inositol was chosen, because in the thyroid this phospholipid contains high amounts of arachidonic acid: 14.3% in the sheep, according to Scott and Trikojus [34]. We have checked that in the pig, the percentage is similar (17.5%). The results are summarized in fig. 2. Incubation in the presence of TSH increases by approx. 100% the accumulation of a radioactive material which has the R_f of the prostaglandins. The nature of these substances is confirmed by inhibition of their formation upon addition of indomethacin and aspirin.

We then turned our attention to the titration of the endogenous phospholipase A activity in the thyroid, in the presence or absence of TSH and in the presence of indomethacin in order to block the further conversion of arachidonic acid into prostaglandins. A comparative study was performed with two radioactive substrates: [^{14}C]phosphatidyl-choline and [^{14}C]phosphatidyl-inositol. The radioactivity present in the fatty acids released during the incubation with a thyroid homogenate was determined (fig. 3). It can be seen that TSH stimulates a type A phospholipase activity, but that the stimulation is stronger with phosphatidyl-inositol as a substrate. Moreover the evolution of the levels of released fatty acids as a function of time varies according to the nature of the radioactive substrate. In order to specify the nature of these differences, we have analyzed the fatty acids by thin-layer chromatography in a system which separates them according to the number of double-bonds. The

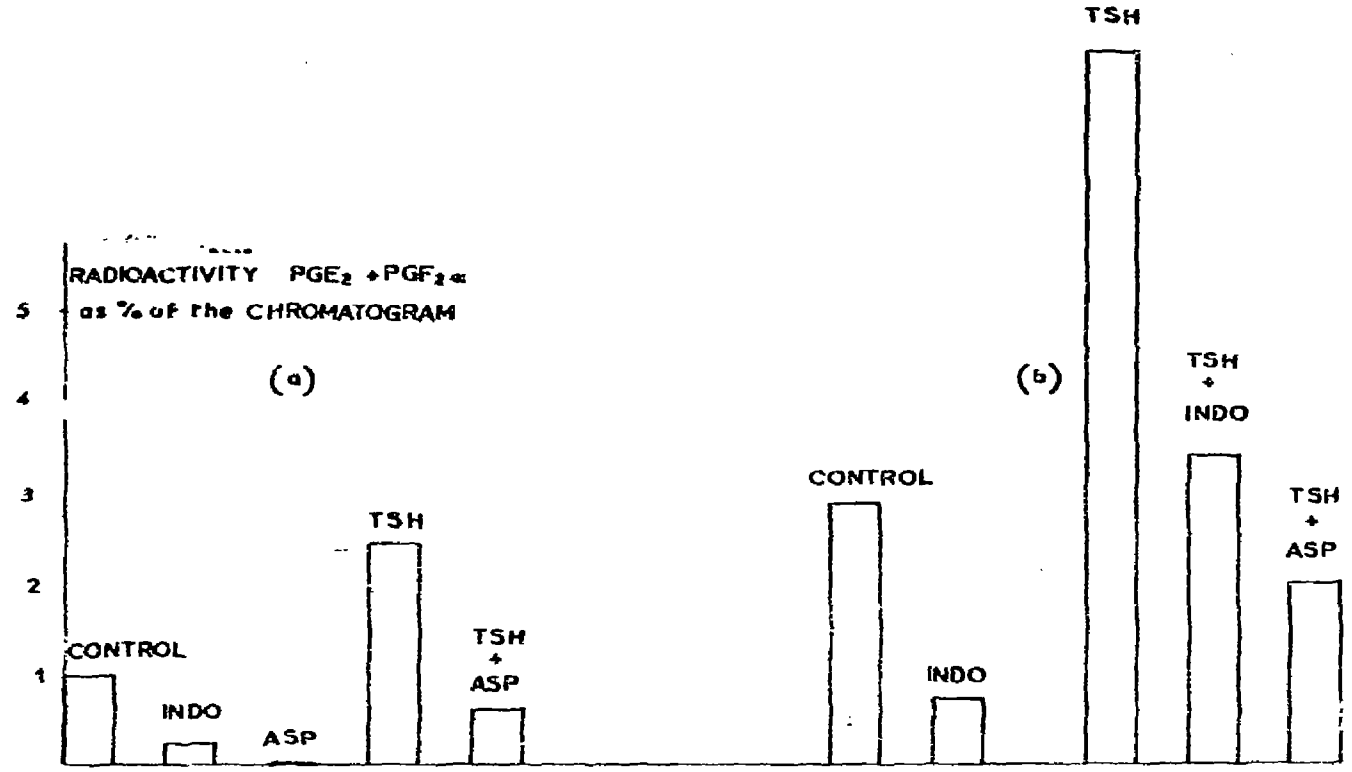

Fig. 2. Biosynthesis of prostaglandins in thyroid homogenates incubated in the presence of [^{14}C]phosphatidyl-inositol. The incubation mixture contains the phospholipid (whose lysophosphatide content is checked before the experiment), 56 μg/ml reduced glutathione, 0.57 μg/ml hydroquinone. The buffer is either a phosphate buffer 50 mM KH_2PO_4–NaOH pH 7.4, or a Tris-maleate 20 mM pH 7.4, without EDTA. The total volume of the reaction mixture is 500 μl. At the end of the incubation the total lipid extract is analyzed [16]. INDO = Indomethacin, 10 μg/ml; ASP = aspirin, 100 μg/ml; TSH = 50 mU/ml. a) 2 min incubation time. b) 15 min incubation time. The experiment was repeated three times with identical results.

hormonal effect is exerted on a type A_2 phospholipase: only the release of unsaturated fatty acids is increased. Phosphatidyl-choline and phosphatidyl-inositol are substrates. However, only PI allows, under the action of TSH, an important release of arachidonic acid (fig. 4) which accumulates, whereas the fatty acids released from PC are mainly mono-unsaturated and do not remain free in the medium. The different behaviour of these two phospholipids is perhaps related to their different fatty acid compositions. In the thyroid, phosphatidyl-inositol seems to play an important role in the biosynthesis of prostaglandins. If it is true that free arachidonic acid is the limiting factor of this biosynthesis, increasing its concentration should stimulate their production. A stimulation of the adenyl-cyclase should follow. Such a result has been reported by Kuehl et al. [7] on mouse ovary. We have repeated this experiment and have observed a 50% stimulation of c-AMP formation in thyroid slices preincubated for 5 min with 10^{-4} M arachidonate (control = 0.20 ± 0.015 pmoles c-AMP/5 mg tissue, arachidonate = 0.29 ± 0.04 pmoles c-AMP/5 mg tissue, $P < 0.10$).

The results that we have just reported (stimulation by TSH of the biosynthesis of thyroid prostaglandins) and these obtained by other authors [3–5] (stimulation by prostaglandins of thyroid adenyl-cyclase) prompted us to specify the role of the endogenous prostaglandins in the effect of TSH on adenyl-cyclase. We have found that it is always possible to stimulate the formation of c-AMP by TSH (40 mU/ml) when the synthesis of prostaglandins is inhibited (fig. 5).

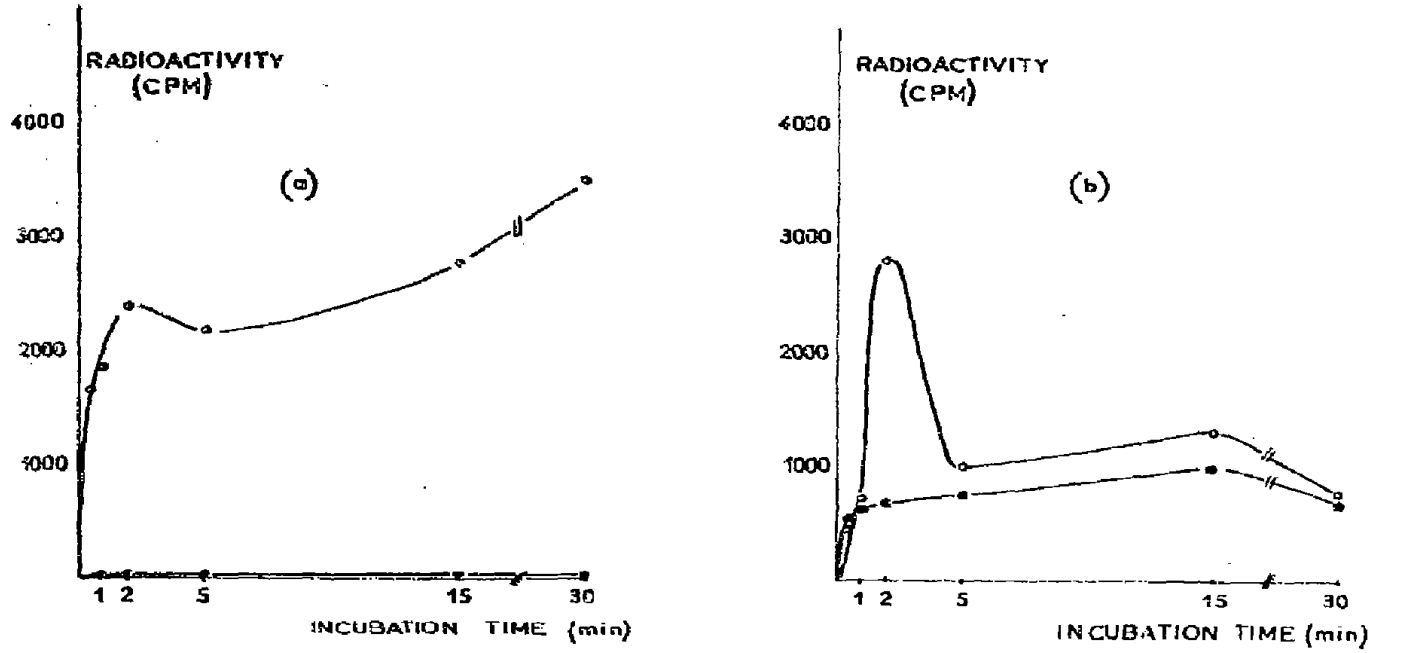

Fig. 3. Phospholipase A activity of a thyroid homogenate (see Material and methods). Total fatty acids released: a) [^{14}C]phosphatidyl-inositol as a substrate. b) [^{14}C]phosphatidyl-choline as a substrate. Control (●—●—●). TSH, 50 mU/ml (○—○—○). The experiment was repeated three times with identical results.

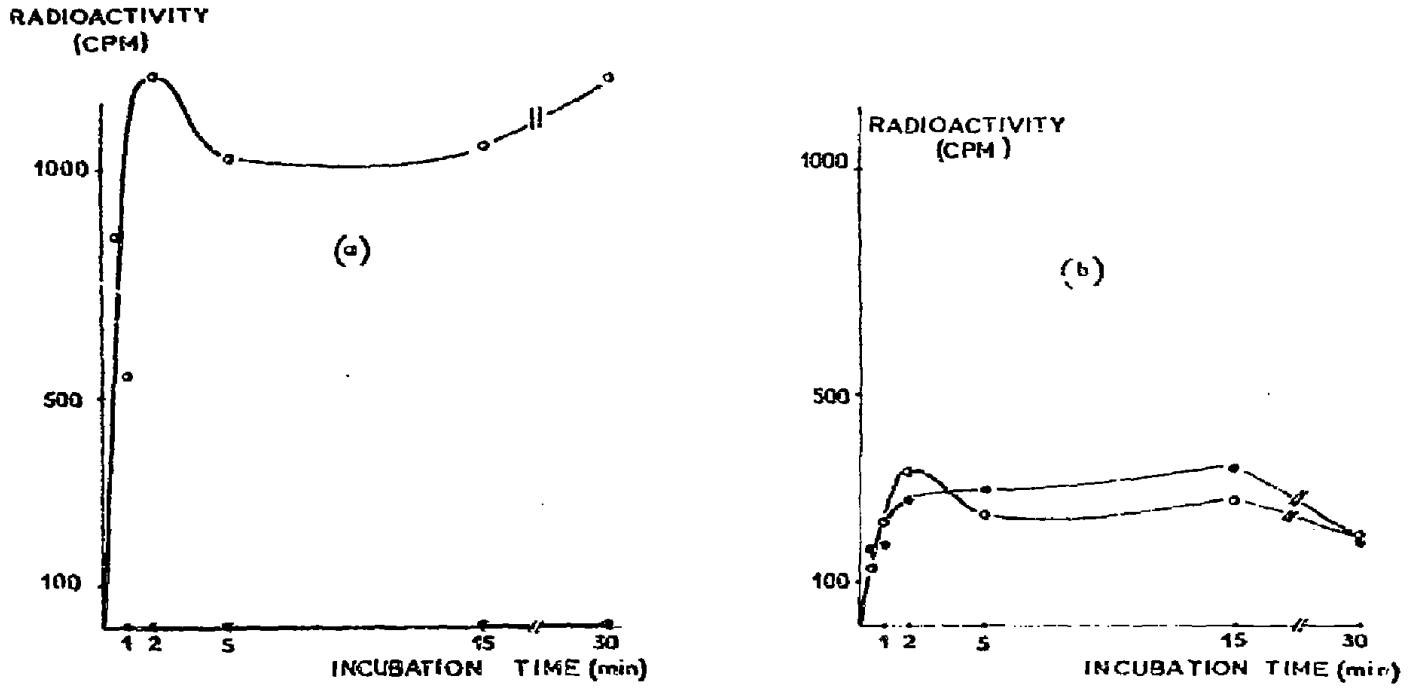

Fig. 4. Arachidonic acid liberated in a thyroid homogenate (see Material and methods). a) [^{14}C]phosphatidyl-inositol as a substrate. b) [^{14}C]phosphatidyl-choline as a substrate. Control (●—●—●). TSH, 50 mU/ml (○—○—○). The experiment was repeated three times with identical results.

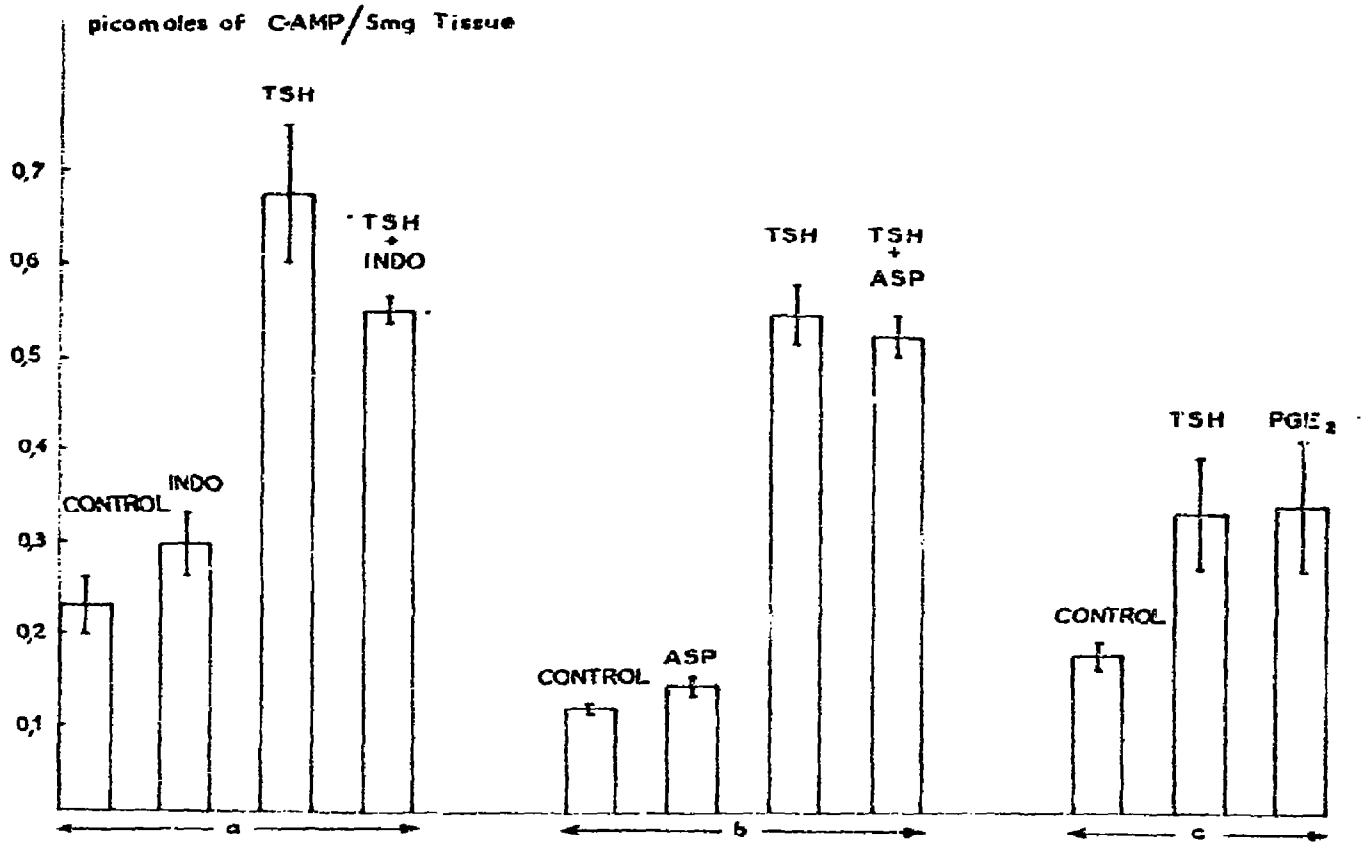

Fig. 5. Assay of c-AMP. 150 mg pig thyroid slices are incubated in 2 ml of glucose-containing KRB (2 mg/ml) (pH 7.4) at 37°; the slices are transferred in 1 ml of glucose-containing KRB and incubated in the presence or absence of indomethacin or aspirin. After 4 min at 37°, theophyllin (10^{-2} M) and TSH dissolved in glucose-containing KRB are added (final volume 2 ml). After 15 min at 37° the slices are frozen in liquid nitrogen and homogenized in 2 ml of 5% TCA at 0°. The assay of c-AMP is performed according to Gilman [28] using [^3H]c-AMP (20.7 Ci/mM, C.E.N. France). a) TSH = 40 mU/ml, indomethacin = 5 μg/ml. INDO as compared to control, $P < 0.20$. TSH as compared to control, $P < 0.01$. TSH + INDO as compared to control, $P < 0.001$. TSH + INDO as compared to TSH, $P < 0.20$. b) TSH = 40 mU/ml, aspirin = 100 μg/ml. ASP as compared to control, $P < 0.10$. TSH as compared to control, $P < 0.001$. TSH + ASP as compared to control, $P < 0.001$. TSH + ASP not significantly different from TSH. c) TSH = 20 mU/ml, $PGE_2 = 3 \times 10^{-5}$ M. TSH and PGE_2 as compared to control, $P < 0.005$.

4. Discussion

The results obtained show that thyroid tissue incubated *in vitro* with [^{14}C]acetate synthesizes radioactive substances whose chromatographic criteria are those of prostaglandins, particularly PGE_2 and $PGF_{2\alpha}$, and this is in agreement with the data of the literature [2]. This synthesis of prostaglandins can be obtained in a cell-free medium, under conditions similar to those described by Lands and Samuelsson [16], from ^{14}C-labelled phospholipids. Phosphatidyl-inositol of the thyroid is a better precursor than phosphatidyl-choline, probably because of its high arachidonic acid content. Thyreostimulin stimulates the biosynthesis of prostaglandins in a cell-free system. This increase in the biosynthesis seems to be a direct consequence of the release in the medium of arachidonate from phosphatidyl-inositol after activation of an endogenous type A_2 phospholipase. This interpretation is confirmed by the fact that arachidonic acid reproduces the stimulatory effect of prostaglandins on thyroid adenyl-cyclase. The role of prostaglandins in the stimulation of thyroid adenyl-cyclase by TSH has been considered. Kuehl et al.

[7,8] have proposed, for the ovary, a scheme according to which the prostaglandins were obligatory intermediates of hormonal action (LH) on c-AMP production. The experiments that we have performed with inhibitors of prostaglandin synthetase [18] show that in the thyroid this hypothesis is not verified, as, under conditions where the biosynthesis of prostaglandins is inhibited, adenyl-cyclase can still be stimulated by a high dose of TSH. This observation, together with the results of Burke et al. [4], describing the additive effects of low doses of TSH and prostaglandins and the antagonist effects of high doses, prompted us to suggest the following provisory scheme where prostaglandins action occurs in a loop allowing a modulation of the effect of TSH: an amplification would only take place in the presence of low doses of TSH (scheme 1).

We are presently not in a position to make this scheme more precise as we do not know the intracellular localization of the TSH-dependent phospholipase A and the mechanism of its stimulation. We are continuing our studies on these problems. On the other hand, we are studying the possible relationship between this effect and the preferential increase in the turnover rate of PI under the influence of TSH, which is not mediated by c-AMP [19, 35].

Acknowledgements

This work was supported by a grant from the C.N.R.S. (A.T.P. no 4212). We warmly thank Dr. J.E. Pike (Upjohn Company, Kalamazoo, Michigan) and Dr. D.A. van Dorp (Unilever) who provided us with the prostaglandins, Merck Sharp and Dohme Laboratories who provided us with indomethacin, the NIH (NIH-TSH B_6 2.54 U/mg) and Endopancrine Laboratories who provided us with the TSH.

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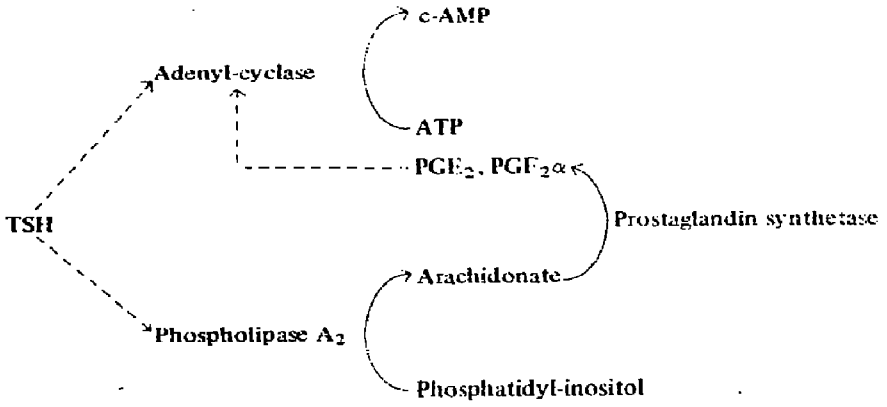
Scheme 1



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