Polarization of Thyroid Cells in Culture: Evidence for the Basolateral Localization of the Iodide "Pump" and of the Thyroid-stimulating Hormone Receptor–Adenyl Cyclase Complex

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ABSTRACT When cultured in collagen gel-coated dishes, thyroid cells organized into polarized monolayers. The basal poles of the cells were in contact with the collagen gel, whereas the apical surfaces were facing the culture medium. Under these culture conditions, thyroid cells do not concentrate iodide nor respond to acute stimulation by thyroid-stimulating hormone (TSH). To allow the free access of medium components to the basal poles, the gel was detached from the plastic dish and allowed to float in the culture medium. After release of the gel, the iodide concentration and acute response to TSH stimulation were restored. Increased cAMP levels, iodide efflux, and formation of apical pseudopods were observed.

When the thyroid cells are cultured on collagen-coated Millipore filters glued to glass rings, the cell layer separates the medium in contact with the apical domain of the plasma membrane (inside the ring) from that bathing the basolateral domain (outside the ring). Iodide present in the basal medium was concentrated in the cells, whereas no transport was observed when iodide was added to the luminal side. Similarly, an acute effect of TSH was observed only when the hormone was added to the basal medium. These results show that the iodide concentration mechanism and the TSH receptor-adenylate cyclase complex are present only on the basolateral domain of thyroid cell plasma membranes.

In vivo, thyroid epithelial cells are organized into follicles. They concentrate and organify circulating iodide and respond within minutes to acute thyrotropin stimulation by increased cAMP synthesis, iodide efflux, and formation of apical pseudopods (10). As both iodide and thyroid-stimulating hormone (TSH) present in the blood have access to the basal surface of follicular cells, it is likely that the iodide concentration mechanism and the TSH receptor–adenyl cyclase complex are present on the basolateral domain of the plasma membrane of the thyroid epithelial cell. No direct evidence of an asymmetrical distribution of these components between the apical and basolateral domain of the plasma membrane has yet been reported.

In vitro, when cultured at high cell density on glass or polystyrene substrates, isolated porcine thyroid cells form polarized monolayers (14, 20, 26). As in other epithelial systems, the basal surface of the cell layer is in contact with the substrate, whereas the apical pole of the cells is oriented towards the culture medium. Under these culture conditions, cells are unable to concentrate iodide and the intracellular cAMP level is not changed upon addition of TSH to the culture medium. This loss of iodide concentration activity and of responsiveness to acute thyrotropin stimulation might be due, at least partly, to the inaccessibility of the basolateral domain of the plasma membrane to molecules present in the culture medium.

We used two different experimental procedures to overcome the inaccessibility of the basal surface of the cell layer. (a) Thyroid cells were cultured in petri dishes coated with a thick layer of collagen gel on which they form a monolayer (4). The gel was subsequently detached from the plastic dish and maintained floating in the culture medium. Such hydrated collagen rafts have been used for culturing various types of epithelial cells (7, 11, 28). When the gel stayed attached to the plastic dish, the components of the culture medium had a direct access

only to the apical surface of the cell layer, whereas with floating collagen rafts the accessibility of the basal surface was restored. However, in this system, the apical and basal compartments were not separated from each other as they are in the thyroid follicles. (b) Thyroid cells were cultured on a collagen-coated Millipore filter glued to a glass ring (24) as described for toad bladder cells (17). When the monolayer was confluent the apical compartment, inside the ring, was isolated from the basal compartment by the cell layer. With this experimental system the apical and basolateral membrane domains are accessible separately.

We report that concentration of iodide in the cell layer and acute response to TSH is seen only when iodide or TSH had access to the basolateral surface of the cell layer. This occurs either with cells cultured on floating collagen rafts or with cells cultured on mounted filters after I⁻ or TSH addition to the basal compartment. This result shows that the iodide concentration mechanism and the TSH receptor–adenylate cyclase complex are present only on the basolateral domain of the thyroid cell plasma membrane. In addition we observed a good correlation between pseudopod formation and increase of intracellular cAMP levels. Some of these results have been presented in a preliminary form (5).

MATERIALS AND METHODS

Cell Isolation and Culture: Porcine thyroid epithelial cells were isolated from adult thyroid glands by a discontinuous trypsin–EGTA treatment as previously described (26). Freshly isolated cells were suspended at a concentration of 2×10^6 cells/ml in Eagle minimum essential medium (MEM) (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) supplemented with 10% newborn calf serum (Gibco Laboratories) and antibiotics. Cultures were incubated at 37°C in a 5% CO_2–95% air water-saturated atmosphere. The medium was changed 24 h after seeding and then every second or third day. The metabolic tests were performed 6–8 d after the onset of culturing, 48 h after the last medium change.

We used two different culture systems:

(a) Culture on attached or floating collagen gel. 2 ml of the cell suspension were plated in 35-mm petri dishes coated with a layer of hydrated collagen gel prepared from acetic acid-soluble rat tail tendon collagen (500 µl of gel/35-mm dish) (4). Within 2 to 3 d, the cells formed a confluent monolayer covering the collagen substrate up to the edge. The gel supporting the monolayer was detached from the plastic dish by rimming, either at the last medium change or just before the metabolic test. Labeled iodide or TSH, when added to the culture medium, was in contact only with the apical cell surface when the gel was attached or with both apical and basal surfaces when the gel was floating.

(b) Culture on mounted Millipore filters. 700 µl of the cell suspension was seeded in a chamber formed by a Millipore filter (0.45-µm pore diameter) glued to a glass ring (21-mm diameter), the filter forming the permeable bottom of the chamber (17, 24). The filter was coated with a thin layer of collagen. The chambers were placed in 35-mm petri dishes on V-shaped capillaries to allow free diffusion of the medium below the filter. 0.7 ml and 2.5 ml of culture medium were added respectively inside and outside the culture chamber. Iodide or TSH was in contact either with the apical surface of the cell layer when added inside the chamber or with the basal surface when present outside the chamber.

Iodide Metabolism: On the day indicated the medium was supplemented with ¹²⁵I-labeled iodide (0.5 µM, ~300,000 cpm/dish), and after 2-h incubation at 37°C the cells and the substrate were extensively washed with ice-cold saline solution containing 0.1 mM I⁻. Iodide concentrated in the cells was measured by scintillation counting; radioactivity retained in cell-free filters was subtracted. As a control, perchlorate ion (1 mM) which inhibits specific iodide concentration (16) was added to the medium, 1 h before labeled iodide was added. For acute TSH stimulation (bovine TSH 1 U/mg Armour Chicago, IL), the hormone, (20 mU/ml) was added 30 min before the iodide incorporation was stopped. Results are given in pmol I⁻ per dish.

Cellular cAMP Levels: After 6 d of culture, monolayers were incubated at 37°C for given times in the presence of various concentrations of TSH (0–20 mU/ml). To allow a quantitative correlation of intracellular cAMP concentration with other cellular responses to TSH stimulation (iodide efflux, pseudopod formation), the incubation was performed in the absence of a phosphodiesterase inhibitor. After incubation, the medium was removed and per-

chloric acid (1 N final) was added. Cyclic AMP was measured in the acid extract by radio-immunoassay (2) on two different dishes. Each value (pmol/dish) is the mean of closely agreeing duplicate determinations (<5% variation).

Pseudopod Formation: Pseudopod formation after acute stimulation by TSH was monitored on the living culture by direct observation through the transparent gel with a phase-contrast microscope (Wild M40) or, after fixation and processing, by scanning electron microscopy (Jeol SM35) (4). Quantitative studies were made with monolayers formed on floating collagen membranes. Pseudopod-bearing cells were counted on a series of light micrographs taken at random all over the dish after fixation of the cells (1 h, 2% glutaraldehyde in sodium cacodylate 0.1 M, pH 7.2). A minimum of 700 cells was counted for each assay and the percentage of responding cells was plotted versus stimulation time or hormone concentration.

RESULTS

Iodide Concentration

When labeled iodide (0.5 µM) was added to the culture medium of cells cultured in monolayer on attached collagen gel or to the medium present inside the culture chamber, no concentration of iodide in the cell layer was observed (Tables I and II). In contrast, when the gel covered by the cell layer was released and allowed to float in the medium, or when

TABLE I

Concentration of Iodide by Thyroid Cells Cultured on Collagen Gels

Monolayer on collagen gel	TSH, 20 mU/ml, 30 min	Iodide uptake pmol/dish
Attached	−	0
	+	0
Floating, 2 h	−	18.3
	+	1.5
Floating, 48 h	−	18
	−*	2.4
	+	1.7
	+*	2

After 6 d in culture, the medium was supplemented with ¹²⁵I-labeled iodide (INa, 0.5 µM, 300,000 cpm/dish) and cells were incubated for 2 h. The collagen gel was either left attached to the bottom of the dish or released and allowed to float at the time when ¹²⁵I was added or 48 h prior to the addition of label.
* When present, sodium perchlorate (1 mM) was added 1 h before ¹²⁵I⁻. The sensitivity to TSH was evaluated by adding the hormone (20 mU/ml) 30 min before the end of the incubation in the presence of ¹²⁵I⁻. Cells and collagen were extensively washed with cold saline solution containing 0.1 mM NaI. Results are given in pmol I⁻/dish (mean of closely agreeing duplicates).

TABLE II

Concentration of Iodide by Thyroid Cells Cultured on Mounted Filters

Compartment containing ¹²⁵I⁻ (0.5 µM)	TSH, 20 mU/ml 30 min, basal compartment	Iodide uptake, pmol/chamber
Apical	−	0.22
Apical*	−	0.17
Basal	−	2.53
Basal*	−	0.20
Basal	+	0.33

After 8 d in culture on Millipore filters, the medium inside (0.7 ml) or outside (2.5 ml) the culture chamber was supplemented with ¹²⁵I⁻ labeled INa (0.5 µM, final concentration). Cells were incubated for 2 h. Iodide taken up by the cells was measured after extensive washing of cells and substrate with a cold saline solution containing 0.1 mM INa.
* When present, sodium perchlorate (1 mM) was added in the basal compartment one hour before ¹²⁵I⁻. The effect of TSH (20 mU/ml) was measured by adding the hormone in the basal compartment 30 min before the end of the incubation in the presence of ¹²⁵I⁻. Results given in pmol I⁻/chamber are the mean of closely agreeing duplicates.

labeled iodide was added in the petri dish outside the culture chamber, the halogen was concentrated in the cells (Tables I and II). Iodide equilibrium between cells and medium was reached after 30-min incubation at 37°C (result not shown). Perchlorate (1 mM) abolished halogen concentration. Based on the assumption that, in the presence of perchlorate, the ratio of iodide concentration between cells and medium (C/M) is 1 (16), concentration ratios of 7–14 were obtained in the absence of perchlorate. This ratio was not modified when the collagen raft supporting the monolayer was allowed to float for 48 h before testing the iodide concentrating activity (Table I). Under these incubation conditions, radioactivity present in the cells is free iodide, since it is not precipitated by 10% trichloracetic acid. When cells, cultured on floating collagen rafts or on mounted filters and preloaded with labeled iodide during 90 min, were basally stimulated by TSH (20 mU/ml, 30 min), the intracellular iodide level dropped to 10% of its initial value (Tables I and II). The efflux of iodide was already maximal within 5 min after TSH addition (not shown).

The lack of iodide concentration observed when ^{125}I-labeled iodide was added in the medium in contact only with the apical surface of the thyroid cell monolayer suggests that the iodide concentration mechanism is localized on the basolateral domain of the thyroid cell plasma membrane.

Acute TSH Stimulation: cAMP Levels

To specify the localization of the TSH receptor–adenyl cyclase complex, the cAMP response was investigated. As shown in Tables III and IV, at 30 min after adding TSH no change of intracellular cAMP level was observed either in cells cultured on attached collagen gel or in monolayers on Millipore filters and apically stimulated. In contrast, when the gel supporting the monolayer was released just before adding TSH or when the cells cultured on mounted filters were basally stimulated, the intracellular cAMP levels were increased threefold, when assayed 30 min after the addition of the hormone. The cAMP response was not much greater when the gel was floating

TABLE III

Acute Effect of TSH on Intracellular cAMP Levels and on the Formation of Pseudopods

Monolayers on collagen gel	TSH, 20 mU/ml, 30 min	cAMP, pmol/dish	Pseudopod-bearing cells (%)
Attached	−	12	0
		11, 4	
	+	12, 9	2–3
		14	
Floating (30 min)	−	12, 4	0
		10, 8	
	+	31, 3	22
		31, 6	
Floating (48 h)	−	11, 3	0
		15, 8	
	+	40, 6	42
		46, 4	

Cells were cultured for 6 d on collagen gel. TSH (20 mU/ml) was added 30 min before the end of the culture period. The gel was attached to the plastic dish or was released just before adding TSH or 48 h before. The culture medium was removed and 1 N perchloric acid was added. cAMP content was measured by radioimmunoassay, on two different dishes. Each value (pmol/dish) is the mean of two closely agreeing duplicate determinations (<5% variation). Pseudopod-bearing cells were counted on micrographs. Around 700 cells were counted for each assay in areas of cell density of 3.7 × 10⁵ cells/cm².

TABLE IV

Acute Effect of TSH on Intracellular cAMP Levels and on Pseudopod Formation in Thyroid Cells Cultured on Mounted Filters

Compartment with TSH (20 mU/ml)	cAMP, pmol/ chamber	Pseudopod formation
−	1.5	−
	1.6	
Apical	1.7	−
	1.8	
Basal	6.4	+++
	5.0	

After 6 d in culture on Millipore filters, cells were stimulated by TSH (20 mU/ml, 30 min). The hormone was added to the apical or basal compartment separated by the cell layer. After 30 min the medium was removed and 1 N perchloric acid was added. cAMP was measured in the PCA extract by radioimmunoassay on two different dishes. Each value (pmol/dish) is the mean of two closely agreeing duplicate determinations (<5% variation). Pseudopods were observed by scanning electron microscopy (−, no pseudopods; +++, 50–90% responding cells).

48 h before TSH stimulation (Table III). From these results we can conclude that the TSH receptor–adenyl cyclase complex is present only on the abluminal surface of the thyroid epithelium.

Acute TSH Stimulation: Pseudopod Formation

When TSH was added in the medium of a monolayer cultured on attached collagen gel or inside a culture chamber, no important modification of the apical surface of the cell was observed, by light or scanning electron microscopy. The apical poles of the cells were more convex, as a result of a slight cell swelling. When the gel was released from the plastic dish before adding TSH, or when the hormone was added in the petri dish outside the culture chamber, large membrane ruffles appeared on the apical surface within 15–30 min after addition of the stimulator (Fig. 1). These membrane protrusions can be observed, after fixation, by electron microscope (transmission or scanning) or on the living culture by phase-contrast optics when cells are cultured on the transparent gel (Fig. 2). They look like the pseudopods described after stimulation of rat thyroid or of dog thyroid slices (12, 21). Pseudopods were principally localized at the margin of the apical poles, close to the junctions, as observed in the rat thyroid after stimulation in vivo (12). The response was not uniform. Some cells bear several pseudopods while others do not show any. In addition, the cell density was nonuniform along the monolayer and the number of responding cells was greater in areas of higher cell density, particularly in the central area of the dish. When TSH was removed, pseudopods disappeared within 2 h.

Correlation between cAMP Level and Pseudopod Formation

Since pseudopods were not present on all cells, a quantitative estimation of the pseudopod response can be made by counting the percentage of responding cells. The correlation between the pseudopod response and the increase of intracellular cAMP levels was studied using cells cultured on floating collagen rafts. Measurements were made in areas with equal cell density, around 4 × 10⁵ cells/cm². As shown in Fig. 3, after stimulation with a maximal concentration of TSH (20 mU/ml), the number of responding cells increased with time; a plateau was reached after 1 h and maintained for several hours. A good correlation
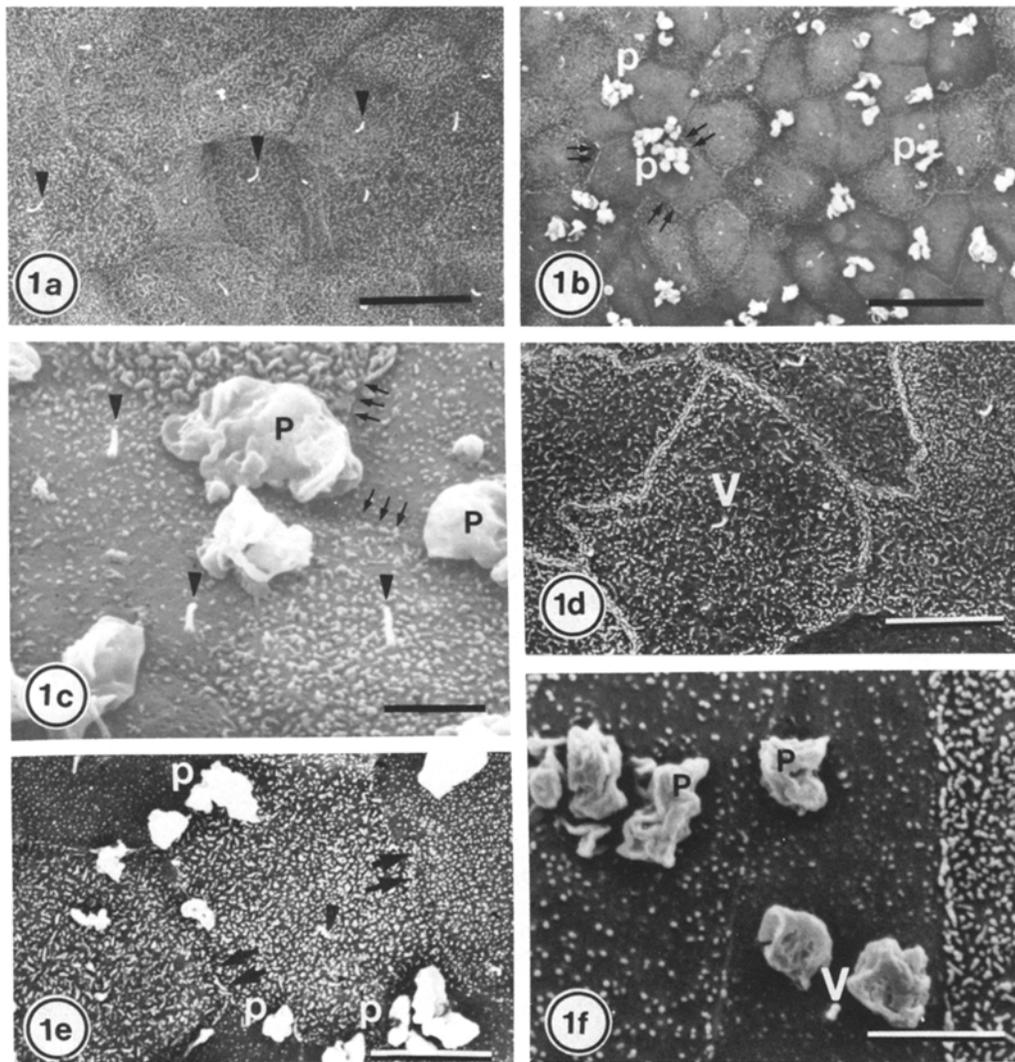
FIGURE 1 Formation of pseudopods on the apical surface of thyroid cell monolayers after stimulation by TSH. (Scanning electron microscopy.) (a, b, and c) Cells were cultured on hydrated collagen rafts. The gel supporting the monolayer was released and allowed to float 48 h before the end of the culture period (6 d). (a) Unstimulated cells. The apical pole bears numerous microvilli and a cilium per cell (arrowhead). (b and c) TSH (20 mU/ml) was added to the medium 30 min before fixation. Pseudopods (p) appear on the apical poles, frequently at the cell margin close to the intercellular junction (double arrows). Pseudopod-bearing cells have often shorter microvilli. Bars: a, 10 μm; b, 10 μm; c, 3 μm. a, × 1,600; b, × 1,500; c, × 4,500. (d, e, and f) Cells were cultured on collagen-coated Millipore filters glued to a glass ring. (d) Unstimulated cells. Microvilli and cilium (arrowhead) are present on the apical poles. (e and f) TSH (20 mU/ml) was added to the basolateral compartment outside the ring 30 min before fixation. Pseudopods (p) appear near cell margin (double arrows). Bars: d, 6 μm; e, 6 μm; f, 3 μm. d, × 2,600; e, × 2,600; f, × 6,000.

was observed with the time-dependent increase of cellular cAMP level. When cells were stimulated with graded concentrations of TSH for 2 h, we observed (Fig. 4) that the maximal pseudopod response was obtained with 100 μU/ml TSH, whereas higher concentrations of hormone were needed to obtain the maximal cAMP response.

DISCUSSION

Freshly isolated porcine thyroid cells cultured in 10% serum-containing medium, without addition of TSH, reorganize into monolayers on classical impermeable tissue culture substrates (glass, treated polystyrene) (14, 20, 26), or into vesicles when maintained in suspension (18, 26, 29). In the two polarized multicellular structures, only the apical domain of the cell plasma membrane is in contact with the culture medium. Under these conditions, the cells neither concentrate and organify iodide nor respond to acute stimulation by TSH (22, 25). They synthesize thyroglobulin at a low rate (3). We report here that, when cultured in monolayer on hydrated collagen gels, thyroid cells concentrate iodide and respond to acute TSH stimulation only when the gel is floating in the culture medium. In addition, we show that, when cultured on a Millipore filter glued to a glass ring, the thyroid cells concentrate iodide and respond to TSH when these compounds are added to the medium outside the ring.

It appears, therefore, that the iodide concentration mechanism and the TSH receptor–adenyl cyclase complex are localized on the basolateral domain of the thyroid cell plasma membrane. Moreover, as cells cultured on floating collagen or on mounted filters have similar properties, the separation of the apical and basal compartments is not required for the expression of iodide concentration and of sensitivity to TSH. The accessibility of iodide and TSH to the basal pole of cultured cells, through the permeable substrate, is sufficient.

A specific role of collagen in the maintenance of the functional state of membrane structures associated with specific functions was suggested for hepatocytes (28), mammary cells (11), and transitional epithelium (7). Both permeability, elasticity, and fibrous structure of collagen gels might be important in this respect. In the present state of our knowledge, the permeability of the culture substrate seems to be the determining factor since thyroid cells cultured on collagen-coated or uncoated Millipore filters have the same metabolic properties (results not shown).

Three observations made with thyroid cells cultured in monolayer on permeable substrates deserve additional discussion:

(a) Cells concentrate iodide only by their basolateral membrane.

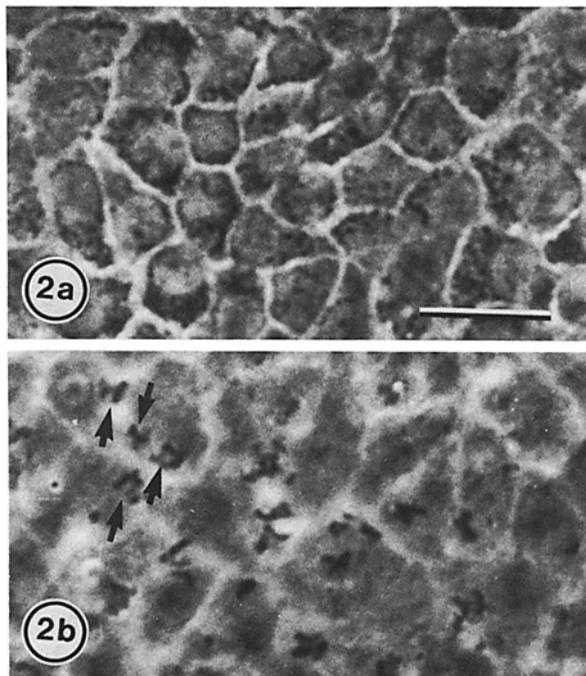(b) An acute stimulation by TSH induced a rise of intracel-

FIGURE 2 Thyroid cell monolayers on floating collagen membrane: stimulation by thyrotropin (phase-contrast microscopy). The collagen gel was allowed to float 48 h before adding TSH (20 mU/ml, 30 min). (a) Control cells; cells form a regular pavement. Clear spaces between cells show the zone of intercellular junctions. No irregularity appears when focusing on the apical surface. (b) TSH-stimulated cells; pseudopods appear as irregular dark spots when focusing slightly above the apical level. The cell layer is out of focus. Pseudopods are often near the cell margin (arrows). Bar, 25 μm. × 680.

lular cyclic AMP level of the whole cell population.

(c) The thyrotropin stimulation is rapidly followed by the emergence of apical pseudopods. This morphological response is not uniform among cells forming the monolayer.

Iodide Metabolism

When iodide added to the medium has access to the basal surface of the cell layer, it is concentrated into the cells. This is in agreement with autoradiographic studies of iodide uptake into mouse thyroid glands (1). The measured C/M fluctuated between 7 and 14 according to the primoculture. After acute stimulation by TSH, 90% of the free intracellular iodide is discharged into the medium. Such an efflux has been previously observed in vivo (13) and in vitro with cells reorganized in follicular structures (13, 25). As shown by incubations in the presence of perchlorate, iodide remaining in the cells after acute TSH stimulation (10% of initial level) may correspond to diffusion of the halogen through the cell membrane. No uptake of iodide from the apical medium into the cells has been observed in our system. This result seems to contradict the interpretations of iodide distribution studies made on turtle thyroid suggesting a transport of iodide from lumen to cell (9). In contrast, it is consistent with a report from the same authors on rat or guinea-pig thyroid glands showing that iodide, as perchlorate, is not concentrated from lumen into cells (8).

Hormone-stimulated cAMP Levels

The intracellular cAMP concentration is not increased after an acute TSH stimulation of cells reorganized in monolayer on plastic substrate (unpublished observation) or in vesicles in

suspension (25). This absence of stimulation could depend on a decreased number of TSH receptors (23) or result from the inaccessibility of these receptors when cells are organized in monolayers or vesicles. The first possibility is not sufficient to explain the absence of response, since the adenyl cyclase of washed membrane preparations, measured under optimal conditions (presence of GTP 0.1 mM and absence of Na^+), is stimulated by TSH (33, 34). The use of cells cultured on permeable substrates and apically or basally stimulated allows the unambiguous localization of the TSH receptor–adenyl cyclase complex on the basolateral domain of the plasma membrane. An asymmetrical distribution of hormone-sensitive adenyl cyclase has been described in several transporting epithelial systems (32).
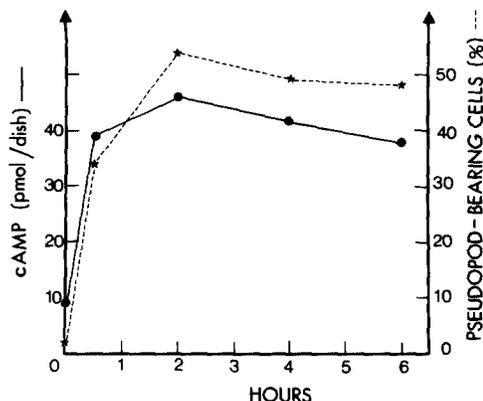

FIGURE 3 Kinetics of the acute effect of TSH on intracellular cAMP level and on apical pseudopod formation. Cells were cultured in monolayer on collagen gel. At day 4, the gel was released from the plastic dish and allowed to float in the culture medium. Two days later, TSH (20 mU/ml) was added. At various times in the presence of the hormone, the percent of pseudopod-bearing cells and the intracellular cAMP level were measured. The value of cAMP (pmol/dish) was the mean of two closely agreeing duplicate determinations. For pseudopod counting, several micrographs were taken and each value corresponds to the observation of 700 cells in areas of equivalent cell density (3.5 × 10^5 cells/cm^2).
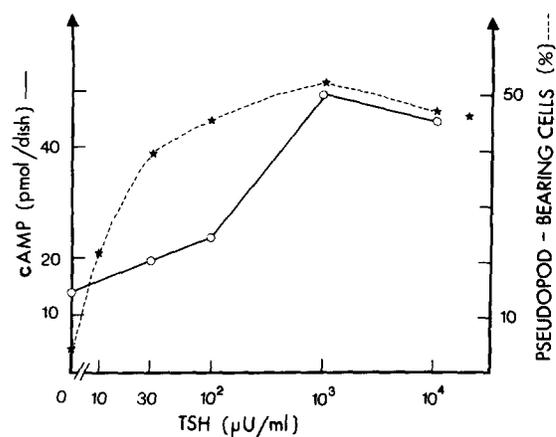

FIGURE 4 TSH dose-dependent stimulation of intracellular cAMP level and apical pseudopod formation. Cells were cultured in monolayer as in Fig. 3. Collagen was released from the plastic dish 48 h before adding graded concentrations of TSH. cAMP levels and the percent of pseudopod-bearing cells were measured 2 h later. The value of cAMP (pmol/dish) was the mean of two closely agreeing determinations. Around 700 cells were counted for each assay in areas of cell density of 4.8 × 10^6 cells/cm^2.

Pseudopod Formation

The formation of apical pseudopods has been described after TSH stimulation of the thyroid gland in vivo and of thyroid slices in vitro (12, 21). Zamora et al. (35) describe the appearance of apical membrane protrusions on sheep epithelial thyroid cells cultured in monolayer on glass coverslips, after an acute stimulation by 50 mU/ml TSH. Although the likeness between these protrusions and genuine pseudopods is poor, the time course of their formation is similar to that of pseudopod emergence in our observations. These results obtained with cells cultured in monolayer on an impermeable substrate can be explained by the leakiness of the tight junctions, involved in the maintenance of apical and basolateral domains of the cell plasma membrane (19), when cells are stretched on a glass substrate.

Pseudopods are not present on all cells as previously observed in rat and dog thyroid glands (12, 21). In our system, pseudopods appear first in areas of high cell density, and a low number of responding cells was observed with low hormone concentrations (5–50 μU/ml) or at the beginning of the response (5–10 min). A similar phenomenon has been described for the vasopressin-induced clustering of intramembrane particles of the luminal surface of amphibian urinary bladder (6).

Relation between cAMP Level and Pseudopod Formation

When monolayers on floating collagen gels are incubated with TSH, we observe a good correlation between the time course of the rise of intracellular cAMP level and the emergence of apical pseudopods. The differences observed in the pseudopod responses of various cells may result from variations either in the individual cellular cAMP content or in the sensitivity of apical membranes and associated cytoskeleton (15) to the rise of cAMP level. The TSH dose-response curves show that the maximal pseudopod response is reached for concentrations of hormone which do not elicit the maximal increase of intracellular cAMP level. Such a discrepancy between the cAMP response and a secondary response has been described in various cAMP-dependent hormone-stimulated systems (27, 30, 31). This suggests that a submaximal increase of intracellular cAMP concentration is sufficient to saturate cAMP-binding proteins, thus giving the maximal secondary response.

Thyroid cells cultured in monolayer on permeable substrates, such as hydrated collagen gel or filters, provide a suitable experimental system for the study, by morphological techniques, of the properties of individual cells within a population, such as the monolayer, and of their evolution during culture.

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