Deactivation of TSH receptor signaling in filter-cultured pig thyroid epithelial cells

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Ericson, Lars E., and Mikael Nilsson. Deactivation of TSH receptor signaling in filter-cultured pig thyroid epithelial cells. Am J Physiol Endocrinol Metab 278: E611–E619, 2000.—Thyrotropin [thyroid-stimulating hormone (TSH)] receptor on-off signaling was studied in polarized monolayers of pig thyrocytes cultured on permeable support. Transepithelial resistance (R) and potential difference (PD) were used as parameters to monitor the effect of altered TSH concentrations on vectorial electrolyte transport. TSH induced rapid but long-lasting changes in R (decrease) and PD (increase) that were cAMP-dependent and related to enhanced transcellular conductance of sodium and chloride. Withdrawal of TSH from cultures prestimulated with TSH (0.1 mU/ml) for 48 h resulted in restitution of R to control level within 30 min. Such deactivation was markedly accelerated by mild trypsinization, which degraded receptor-bound ligand without affecting TSH receptor responsiveness or ion transporting capacity. Small alterations in the TSH concentration (0.01–0.1 mU/ml) were followed almost instantaneously by adjustments of R. In contrast, the reversal of R after acute TSH stimulation (30 min) and subsequent TSH washout was delayed for several hours independently of cell-surface trypsinization. The observations indicate that, during continuous exposure to physiological concentrations, TSH exerts a close minute-to-minute surveillance of thyroid function and the rate-limiting step of deactivation is the dissociation of ligand from the TSH receptor at the cell surface. TSH-deprived cells briefly exposed to TSH are refractory to rapid deactivation, probably because of altered metabolism downstream of TSH receptor signal transduction.

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MATERIALS AND METHODS

Chemicals. Bovine TSH, IBMX, amiloride, forskolin, furosemide, trypsin, and trypsin inhibitor were purchased from Sigma Chemical, St Louis, MO.

Cell culture. The methods of follicle isolation and culture have been described previously (27). Briefly, pig thyroid glands were minced and repeatedly digested with collagenase and disintegrated by pipetting. The follicle fragments thus obtained were rinsed from connective tissue elements and single cells by filtrations and washings and then seeded on microporous filters of bicameral chambers (pore size 0.4 µm) coated with collagen type I (0.3 mg/ml). Culture was carried out in MEM, supplemented with 5% fetal calf serum, penicillin (200 U/ml), streptomycin (200 µg/ml), and fungizone (2.5 µg/ml) at 37°C in a CO2 (5%) incubator (all medium components from Gibco, Paisley, UK). The same medium was used in both chamber compartments (200 µl apically and 500 µl basolaterally).
basally). After 4–5 days of culture, the cells were organized into a confluent polarized monolayer on the filter. Growth to confluence occurred in the absence of TSH. Long-term TSH stimulation, as applied in the experiments presented in this paper, has no growth-stimulating effect on confluent pig thyrocytes, which thus stay in monolayer formation on the filter (13).

Experimental design. From previous studies (26–29, 32), we know that filter-cultured pig thyrocytes form a very tight epithelial barrier designated by a high transepithelial R. In such a tight epithelium, the paracellular flux of ions and molecules is negligible unless the epithelial barrier is impaired, e.g., after removal of extracellular Ca2+ (26). Under normal conditions, therefore, R reflects mainly the transcellular transfer of ions; a high resistance (or low conductance) indicates a low transfer, and a low resistance (or high conductance) depends on a high transfer of ions across the monolayer. An altered transcellular conductance is also reflected by changes in transepithelial PD (29, 32).

All experiments reported here were performed during the 2nd wk after seeding and only on cultures in which the tightness (R >6,000 Ω·cm²) was ascertained before TSH stimulation. “TSH-deprived” and “prestimulated” cultures, respectively, were kept for 48 h in serum-supplemented medium with or without 0.1 mU/ml of TSH present in the basal chamber. R and PD were measured with a Millicell ERS ohmmeter (Millipore, Bedford, MA) under sterile conditions outside the incubator, and the cultures were generally kept in the incubator between measurements. The details of each type of experiment are described in the text. Addition or removal of substances was performed by exchanging the basal (in a few instances the apical) medium. If not otherwise indicated, experimental observations were made on triplicate cultures, and a particular type of experiment was performed in at least three different seedings. Data from a single representative experiment are presented in the graphs.

RESULTS

Effects of TSH and TSH withdrawal on R and PD. Pig thyrocytes grown to confluence on filter in the absence of TSH established a high transepithelial R, often exceeding the detection limit of the ohmmeter (20 kΩ/filter or 6.7 kΩ·cm² for the type of inserts used), and a PD of ~20 mV (apical side negative) across the cell layer. TSH (0.1 mU/ml) added to the basal medium induced a rapid fall in R to about 1 kΩ·cm² and a gradual increase in PD to >30 mV (Fig. 1). This effect of TSH is known to be mimicked by forskolin (30), indicating involvement of cAMP as second messenger, and it depends on increased transcellular ion transport (29, 32) with maintained function of the tight junctions (30). In the continued presence of 0.1 mU/ml TSH, both R and PD were sustained at the new steady-state levels for >48 h without signs of desensitization (Fig. 1). A higher TSH dose (1 mU/ml) was only slightly more effective in depressing R and elevating PD, whereas a smaller dose (0.01 mU/ml) caused only minor changes compared with untreated cultures after 48 h (not shown). The response to 0.1 mU/ml was thus close to maximal and therefore chosen for acute and long-term stimulation (the latter referred to as “prestimulated cultures”) throughout the experiments unless otherwise stated.

We next addressed the question of how fast the TSH-induced changes of R and PD were reversed after removal of TSH from the culture medium. Washout of TSH after 30 min of stimulation in previously TSH-deprived cultures had no immediate effect on R, and full restitution was not observed until after several hours in TSH-free medium (Fig. 2). In contrast, washout of TSH from prestimulated cultures (exposed to TSH for 48 h) caused a much more rapid recovery of R, which generally was completed within 30 min (Figs. 2 and 3). TSH withdrawal also induced a fall in PD (Fig. 3).

Effects of amiloride and furosemide on R and PD. Because the TSH-induced changes in R and PD are due to stimulation of transepithelial ion transport (29, 32), we examined whether the effect of omitting TSH was mimicked by ion transport inhibitors. Amiloride and furosemide were added to the apical and basal media, respectively. In TSH-deprived cultures, these agents caused a decrease in PD to about the same extent (Fig. 4), although the effect of furosemide was slightly delayed. At the same time, R remained at the highest detection limit (not shown), indicating that the reduction of PD was indeed due to inhibited ion transport and not associated with a loss of barrier function. The ion transport blockers might in fact be expected to increase R further, but this was not possible to register due to limitations of the ohmmeter.
In cultures prestimulated with TSH for 48 h and still exposed to the ligand, furosemide induced a gradual increase in R (Fig. 5A) and a rapid fall in PD (Fig. 5B). In contrast, amiloride had only marginal effects on R and PD (Fig. 5A and B). Thus inhibition of a furosemide-sensitive effector mechanism at the basolateral cell surface reproduced, albeit at a slower rate, the response of R and PD to TSH withdrawal in prestimulated cultures.

Effects of IBMX, forskolin, and trypsinization on R and PD. As shown in Fig. 2, the deactivation of TSH-induced ion transport was much more rapid in prestimulated cultures than after acute TSH stimulation. In principle, the rate-limiting step of deactivation might be the effector mechanism itself, i.e., the ion transporter proteins in the plasma membrane or at any level of the upstream TSH receptor signaling pathway. However, the rapid rise of R observed after removal of TSH from prestimulated cultures was completely prevented by the phosphodiesterase inhibitor IBMX (Fig. 6), indicating that deactivation involved breakdown of cAMP. Interestingly, IBMX was able to decrease R in TSH-deprived cultures (Fig. 6), supporting the notion that the TSH receptor has an intrinsic stimulatory effect on adenylate cyclase leading to the formation of cAMP also in the absence of agonist (42).

To study further the deactivation mechanism, trypsinization was used as a tool to rapidly eliminate TSH, and possibly the extracellular portion also of its receptor, from the cell surface. In prestimulated cultures, the recovery of R after washout of TSH was markedly accelerated by trypsin being added to the basal medium (Fig. 7). This occurred without impairment of the epithelial barrier, unless a high trypsin concentration (1% and in some experiments 0.1%) was applied (Fig. 7). Similar results were obtained when trypsinization was allowed in the presence of TSH (not shown). In contrast, trypsin did not influence the lack of rapid restitution of R when TSH was removed after acute stimulation (Fig. 8). Thus deactivation of TSH receptor signaling by trypsin required that the cells be long-term stimulated with TSH.

Treatment of prestimulated cultures with IBMX (Fig. 9A) or forskolin (Fig. 9B) abolished the effect of trypsin on R. This indicates that trypsin affected the ligand-receptor complex at the cell surface rather than affecting postreceptor events along the signaling pathway or ion transport molecules in the plasma membrane. To further elucidate the main target of trypsin-

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**Fig. 2.** Effect of TSH withdrawal (“washout”) on transepithelial R after acute and long-term TSH stimulation. Cultures were exposed to TSH (0.1 mU/ml) for 30 min (squares) or 48 h (circles) and then exposed to TSH-free medium (filled symbols) or continuously cultured in presence of TSH (open symbols). Arrows indicate acute addition (1) and washout (2) of TSH. Note different levels of R obtained after acute and long-term TSH stimulation, respectively. Data shown are means ± SD, n = 3.

**Fig. 3.** Effect of washout of TSH on transepithelial R (∅) and PD (•) in cultures prestimulated with TSH (0.1 mU/ml) for 48 h. Data shown are means ± SD, n = 3.

**Fig. 4.** Effects of amiloride and furosemide on transepithelial PD in TSH-deprived cultures. According to previous findings on site of action of drugs (3, 19), amiloride (0.1 mM) was added to apical medium, and furosemide (0.1 mM) was added to basal medium (arrow). ○ Untreated, □ amiloride, ▲ furosemide. Transepithelial R was maintained at highest detectable level (20 kΩ/filter) during treatments (not shown). Data shown are means ± SD, n = 3.
ization, prestimulated cultures were first exposed to trypsin during a short washout period and then stimulated with TSH again. As shown in Fig. 10, readdition of TSH caused a rapid decrease of R to almost the same level as that induced when trypsin was omitted; thus the TSH receptor was still capable of binding TSH and transmitting the signal into the cell. On the other hand, the bioactivity of TSH was lost when TSH-containing medium was trypsinized before being added to the cells (Fig. 9C). Together these observations indicate that trypsin affected mainly the ligand rather than the receptor.

Effect of stepwise changes of TSH concentration on R. Readdition of TSH to prestimulated cultures after washout caused again a rapid decrease of R to almost the same level as that induced when trypsin was omitted; thus the TSH receptor was still capable of binding TSH and transmitting the signal into the cell. On the other hand, the bioactivity of TSH was lost when TSH-containing medium was trypsinized before being added to the cells (Fig. 9C). Together these observations indicate that trypsin affected mainly the ligand rather than the receptor.

Fig. 5. Effects of amiloride and furosemide on transepithelial R (A) and transepithelial PD (B) in cultures prestimulated with TSH for 48 h. Amiloride (0.1 mM, ■) and furosemide (0.1 mM, ▲) were added to apical and basal medium, respectively (arrows). Gradual changes in R and PD in TSH-stimulated cultures not exposed to inhibitors (○) are generally observed during repeated transfer of wells in and out of CO₂ incubator. Data shown are means ± SD, n = 3.

Fig. 6. Effect of IBMX on transepithelial R with or without TSH prestimulation (0.1 mU/ml) for 48 h. IBMX (0.5 mM) was added to basal medium (arrow); prestimulated cultures were simultaneously exchanged for TSH-free medium. ○ Untreated cultures, ■ IBMX, ▲ washout of TSH, △ washout of TSH in presence of IBMX. Data shown are means ± SD, n = 3.

Fig. 7. Effect of cell surface trypsinization on transepithelial R in cultures prestimulated with TSH (0.1 mU/ml) for 48 h. Basal medium was switched to TSH-free solution without (○) or with trypsin at different concentrations: 0.001% (■), 0.01% (■), and 0.1% (▲). Corresponding end-point levels of PD were 48 mV (washout of TSH), 23 mV (0.001% trypsin), 14 mV (0.01% trypsin), 0 mV (0.1% trypsin). Note decrease of R after treatment with 0.1% trypsin to level below that recorded in cultures continuously exposed to TSH in absence of trypsin (○). Data shown are means ± SD, n = 3. See text for further comments.

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Fig. 8. Estimation of R and PD in TSH-stimulated cultures not exposed to inhibitors. Data shown are means ± SD, n = 3.

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Fig. 9. C effects of TSH concentration on R and PD. Data shown are means ± SD, n = 3.
Deactivation of TSH-regulated vectorial electrolyte transport. A major theme of the present study was to examine the effect of TSH withdrawal on ion transport evaluated by measurements of transepithelial R and PD in filter-cultured pig thyrocytes. The ultimate purpose was to elucidate the mechanism by which TSH-regulated thyroid functions may be turned off in response to decreasing levels of ligand and a corresponding silencing of TSH receptor signaling. Deactivation was investigated in two situations, 1) after acute stimulation (for 30 min) with a moderate TSH concentration (0.1 mU/ml) in previously TSH-deprived cells, and 2) after prolonged exposure (for 48 h) to the same dose of TSH (referred to as prestimulation). As previously described (2, 29, 32) and confirmed in the present study, TSH stimulates Na\(^+\) and Cl\(^-\) conductance as reflected by a rapid (within minutes) but long-lasting (for days) decrease in R and an increase in PD. We found that withdrawal of TSH in acutely stimulated cells resulted in a very slow recovery of the bioelectrical parameters, which did not reach control levels (as in untreated cultures) until after several hours. In contrast, in prestimulated cultures, full restitution of R was observed within 30 min after the switch to the TSH-free medium. Based on the effects of amiloride, an inhibitor of the apical Na\(^+\) channel (3), and furosemide, an inhibitor of the basolateral NaKCl\(_2\) symporter (22), the prevailing electrolyte transport mechanisms were also found to differ between acutely and chronically TSH-stimulated cells. The rapid changes of R and PD occurring in response to acute TSH have been previously known to be largely due to enhanced Na\(^+\) influx through an amiloride-sensitive channel in the apical plasma membrane (15). In contrast, the present results collectively indicated that Cl\(^-\) cotransport contributed more to the R and PD levels measured in prestimulated cells. In fact, furosemide added to the basal medium mimicked a TSH washout response (increasing R and decreasing PD), even in the presence of TSH, whereas amiloride administered apically did not. Altogether, these observations indicate that deactivation of TSH-regulated electrolyte transport as a result of TSH withdrawal is accelerated in prestimulated thyrocytes and that this is predominantly an effect of turning off the basal-to-apical transport of Cl\(^-\).

Fig. 8. Effect of trypsin on restitution of transepithelial R after short- and long-term TSH stimulation. Cultures were exposed to TSH (0.1 mU/ml) for 30 min (circles; first arrow, TSH addition) or 48 h (squares) and then transferred to TSH-free medium (second arrow) with or without addition of 0.001% trypsin. Open symbols, control; filled symbols, trypsin treatment. Data shown are means ± SD, n = 3.

Fig. 9. Effect of trypsin on action of IBMX (A), forskolin (B), and TSH (C) on transepithelial R. A: prestimulated cultures (0.1 mU/ml TSH for 48 h) were switched to TSH-free medium (bars 1 and 2) or incubated with 0.001% trypsin along with prestimulatory dose of TSH (bars 3 and 4) in absence (bars 1 and 3) or presence (bars 2 and 4) of 0.5 mM IBMX. B: TSH-deprived cultures were exposed to 0.1 mU/ml TSH (bars 2 and 3) or 50 µM forskolin (bars 4 and 5) in absence (bars 2 and 4) or presence (bars 3 and 5) of 0.1% trypsin. C: untreated cultures were exposed to 0.1 mU/ml TSH (bar 2) or to TSH-containing medium that had been preincubated with 0.01% trypsin 15 min before use (bar 3); trypsin action on cells was prevented by simultaneously adding trypsin inhibitor (0.1 mg/ml). Open bars (1), R recorded in control cultures for each experiment; hatched and solid bars, treatments without and with trypsin, respectively, in all graphs. Data shown are means ± SD, n = 3, of end-point values of R obtained after 30 (A and B) or 4 (C) min of incubation, respectively. See text for further comments.
Rate-limiting step of deactivation after long-term TSH stimulation. Deactivation of TSH-regulated thyroid functions certainly involves many steps along the TSH receptor signaling pathway, e.g., interrupted transduction of the G protein-coupled signal to adenylate cyclase, diminished adenylate cyclase activity, degradation of intracellular cAMP, and dephosphorylation of downstream signaling intermediate and effector molecules. To elucidate the rate-limiting step, we investigated whether deactivation was influenced by a mild cell surface trypsinization that did not challenge the barrier function of the epithelium. In TSH-prestimulated cultures, such treatment caused an almost instan-
As discussed above, the reversal of R after deactivation of TSH-regulated electrolyte transport. TSH concentration, dissociation of the ligand receptor in pig thyroid epithelium sensing a sudden decrease in pig thyrocytes (36). Thus, in the chronically stimulated phosphodiesterase activity is upregulated by TSH in the other hand, the receptor level seems rather resistant to TSH receptor expression in FRTL-5 cells (33). On the face. TSH has been found to regulate negatively the duration of cAMP formation is the number of TSH receptors available for ligand binding at the cell surface. TSH has been found to regulate negatively the downstream intracellular signaling cascade above the G proteins and endocytosis of the receptor (14, 21).

Possible mechanisms of TSH receptor signal deactivation. The precise mechanism by which the TSH receptor signal is rapidly deactivated when TSH is withdrawn from prestimulated cultures cannot be determined with certainty. However, it is known that the association and dissociation constants for TSH binding to the receptor are of the same order (8, 40), suggesting that TSH bound to the receptor might be in dynamic equilibrium with soluble TSH in the extracellular fluid. Thus one possibility is that a release of receptor-bound ligand directly alters the properties of the receptor, presumably by a conformational change, which then ends signal transduction. This is also supported by the data obtained in the trypsin experiments. Another possibility is that the ligand-receptor complex, similarly to that of other polypeptide hormones and their receptors, is cleared from the cell surface by endocytosis and afterward by dissociation of the complex in an endosomal compartment and that internalization is important for deactivation. In response to agonist, many G protein-coupled receptors are rapidly desensitized by phosphorylation-dependent binding to arrestins, which mediate uncoupling from the G proteins and endocytosis of the receptor (14, 21).

Subsequent desensitization to the preligand-exposed state is believed to involve endosomal dephosphorylation and recycling of the receptor to the cell surface. Evidence for ligand-induced internalization of the TSH receptor has been obtained in nonthyroid cells overexpressing the transfected receptor (17, 18). Moreover, desensitization of the TSH receptor might also be arrestin dependent (20, 24, 25). However, TSH-induced desensitization is generally recognized after acute stimulation, like the present experiments on TSH-deprived cultures in which deactivation was markedly delayed. Moreover, we did not observe any signs of desensitization on the long-term action of TSH on PD at the given dose. It thus remains an open question whether TSH receptor internalization influences the responsiveness of prestimulated thyrocytes to reduced ligand concentrations. Regardless of this, the almost instantaneous deactivation observed after clearance of soluble and surface-bound TSH by trypsin treatment strongly suggests that internalization is not a prerequisite for turning off TSH receptor signaling. An alternative, albeit speculative, mechanism for deactivation might be removal of ligand-bound receptor by proteolysis. Indeed, shedding of the ectodomain of the TSH receptor as a result of matrix metalloprotease and protein disulfide isomerase activities has been identified (9, 39).

On-off signaling of the TSH receptor: possible physiological implications. Cultures prestimulated with TSH are conceivably a more adequate model for studying the normal regulation of thyroid function than cultures deprived of TSH. There is reason to believe, therefore, that the dynamic deactivation of TSH receptor signaling and effector function as has been observed in prestimulated cultures represents a mechanism of physiological significance. This is further supported by the findings that the steady-state level of R was rapidly
adjusted to small variations in the TSH concentration within or close to the physiological range (12) and that such adjustments were possible to reproduce in on-line recordings of the same culture during a considerable period of time. Depending on the concentration of ligand, it thus seems that the TSH receptor cycles between on-off signaling and that this is transduced to corresponding modulation of the effector mechanisms that govern plasma membrane conductivity. Altogether, the present data indicate that TSH exerts a close surveillance on thyroid activity. The influence of TSH is tonic but can be adjusted rapidly, almost on a minute-to-minute basis, by means of an altered concentration of the ligand. This in turn supports the notion that the pulsatile and circadian release of TSH from the pituitary (7, 19) is likely transmitted to rapid changes in thyroid structure and function (19, 37, 43, 44). In terms of altered electrolyte transport, there are several indications pointing to the possibility that this may have important physiological implications. For instance, in rats with inhibited TSH secretion caused by hypophysectomy or thyroxin treatment, the viscosity of the colloid in the follicle lumen is very high, and the diffusion of newly iodinated thyroglobulin is consequently slow (31, 34). However, shortly after injection of TSH, the iodinated molecules are spread out evenly in the colloid as a sign of unrestricted diffusion (16). Such altered properties of the colloid, which are probably caused by changes in the ion content and water content, may facilitate iodination and endocytosis of thyroglobulin and may ultimately enhance the release of thyroid hormone to the bloodstream (35). Transepithelial ion transport identified in thyrocytes is bidirectional with respect to the apical-basal polarity of the epithelium (2). Na+ flux occurs preferentially in the apical-to-basal direction via apical sodium channels and the Na\(^+\)-K\(^+\)-ATPase located in the basolateral plasma membrane (32). In contrast, Cl\(^-\) is transported mainly in the opposite direction through a basolateral NaKCl\(_2\) symporter and anion channels present in the apical plasma membrane (1, 6, 11). TSH positively regulates both transport mechanisms via the cAMP-PKA pathway, but, as suggested by the present data, the relative importance may vary depending on the duration of TSH stimulation. Activation of tyrosine kinase receptors and protein kinase C has also been found to induce distinct changes in transepithelial R and PD in filter-cultured thyrocytes (13, 29), further indicating that these parameters are closely related to the functional status of the thyroid epithelium.

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