Homologous desensitization of calcitonin receptors and calcitonin-dependent adenylate cyclase in T47D cells

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Prolonged use of calcitonin (CT) in vivo leads to a loss of biological response (escape). To understand the molecular basis of this phenomenon, we examined desensitization of calcitonin receptors and down-regulation of adenylate cyclase response to CT in T47CD cells after a preincubation with CT. Preincubation with salmon or human CT (sCT, hCT) for 3 h led to a concentration-dependent loss of $^{125}$I-sCT binding and a similar loss of adenylate cyclase response to a maximal stimulatory dose of sCT. At the same time there was an increased basal activity of the adenylate cyclase. After 24 h preincubation with sCT, basal cAMP levels fell considerably but not to basal levels. Time course experiments showed a delayed decline of maximally sCT-stimulated cAMP levels, which started after 1 h, while binding values declined over the first 60 min to one-third of the original values. Upon removal of CT from the medium, recovery of hormone binding occurred in parallel with the recovery of the adenylate cyclase response to sCT. T47D cells incubated with 25 μmol/l monensin, a lysosomal inhibitor, showed a persistent $^{125}$I-sCT binding after removal of hormone, consistent with diminished intracellular receptor degradation. However, despite persistent binding to the cells, basal and stimulated cAMP levels dropped in the same manner as seen in controls. Our experiments support the view that tight binding of CT to its receptor stimulates adenylate cyclase in T47D cells, until receptors are removed by internalization. The physiological role of internalization of the CT receptor might be to end continuous stimulation of the adenylate cyclase, which occurs after binding.

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The hormone calcitonin (CT) is a potent inhibitor of bone resorption and is, therefore, used therapeutically in humans in conditions of rapid bone turnover such as Paget’s disease of the bone. Continuous pharmacological administration of CT, however, leads to a loss of its bioactivity, a phenomenon which has been termed “escape” (1). Loss of CT binding sites has also been observed after continuous exposure to salmon CT in rat calvarial cultures in vitro (2) and in rat kidneys in vivo (3) and occurs together with the loss of bioactivity. The loss of binding sites could be due to the tight and quasi-reversible binding between CT and its receptor, which has been described in cancer cell lines (4) and in osteoclasts (5). Furthermore, CT receptors are internalized and degraded after binding of CT and this might contribute to the observed loss of subsequent binding.

The human breast cancer cell line T47D is a useful model system for studying the CT receptor (6). It possesses functional CT receptors coupled to adenylate cyclase (AC) via a G protein and the CT receptor complex is internalized after binding of ligand (4, 7). CT produces a persistent activation of AC in these cells (8), as it does in osteoclasts (9) and in kidney cells (10).

Recently, we showed that the ionophore monensin blocks degradation of internalized $^{125}$I-sCT in T47D cells (7). We have extended these studies to examine the relationship between down-regulation of CT binding sites and the loss of AC response to CT after binding of the hormone to its receptors in these cells. Furthermore, we have assessed recovery of hormone binding and the AC response to CT after hormone withdrawal.

Materials and methods

Synthetic salmon calcitonin (sCT) was provided by Dr Stähelin, (Sandoz AG, Basel, Switzerland), human calcitonin was donated by Dr Kabay (Ciba Geigy, Basel, Switzerland), monensin was purchased from Serva (Heidelberg, Germany), Na$^{125$I] was from Amersham (Braunschweig, Germany).

sCT was iodinated according to Hunter and Greenwood (11) as described (12). T47D cells were generously provided by T] Martin, Melbourne, Australia, and were grown in RPMI 1640 (Gibco, Eggenstein, Germany) with 10% fetal calf serum (FCS, Biochrom, Berlin, Germany), 1 mmol/l Hepes (Sigma, Deisenhofen, Germany) and added antibiotics (1% penicillin and streptomycin). Cells were seeded in 24-well plates (Nunc,
creased incubation human trations pmol/1. However, basal (Fig. 1). Pretreatment of cells with SCT led to increased basal cAMP concentrations, the magnitude of which depended on the preincubation concentration of SCT. However, as with binding, concentrations as low as 100 pmol/l were sufficient to block subsequent SCT-dependent AC stimulation. The same effect was also seen with human CT though at higher concentrations (Fig. 2).

The time course of the loss of binding of 

3 h desensitization with 100 pmol/l SCT a second challenge with SCT did not stimulate cAMP production.

After 24 h preincubation with SCT, AC could not be stimulated with a second challenge of SCT, if initial hormone concentrations were 1 nmol/l or higher. Cellular cAMP levels dropped considerably in comparison to the levels after 3 h stimulation. At preincubation concentrations higher than 1 nmol/l SCT, binding fell to non-specific levels after 24 h (Fig. 4).

The following experiments were conducted in order to investigate the process of recovery from homologous desensitization: after 3 h preincubation with unlabelled SCT, the medium was removed; the cells were washed and then incubated for up to 24 h with fresh medium containing no hormone. Again, preincubation with SCT resulted in a parallel loss of specific binding of [123I]-SCT and loss of AC responsiveness (Fig. 5) concurrent with
increased basal levels of cAMP. After withdrawal of hormone, recovery of specific $^{125}$I-sCT binding and AC responsiveness to sCT also occurred in parallel. Over the same period basal cAMP levels in preincubated cells fell to control levels.

In order to determine the cAMP levels during processing of bound CT, $^{125}$I-sCT was incubated with the cells for 30 min and the media were then changed. After 30 min binding to T47D cells $^{125}$I-sCT is mostly bound to the cell surface and not yet processed (7). Cellular cAMP levels were elevated by the exposure to $^{125}$I-sCT and remained elevated for another 30 min. After washing away the tracer, before beginning to fall again (Fig. 6). Inclusion of monensin, which blocks CT receptor metabolism (7), did not change the relative cellular cAMP concentrations while absolute values were higher than in controls. Maximally stimulated cAMP levels in response to the second challenge with sCT followed the same time course with or without monensin.

Discussion
The loss of CT activity which is seen after continuous administration of the hormone in vivo is well known and severely limits its therapeutic use. In model systems the loss of biological response is accompanied by a loss in CT receptor binding (2, 3). It is thought that this is initially due to the tight and quasi-irreversible nature of hormone-receptor binding, which had been described in tumor cells like T47D (4) and in normal tissues and cells such as placenta (15) or osteoclasts (5). Evidence suggests that this tight binding leads to persistent stimulation of AC, which has been observed after stimulation with sCT in CT responsive cells (8, 9). After binding, CT receptors are internalized and degraded in tumor cell lines (4, 7) and in normal kidney cells (16).

The present experiments were designed to understand the regulation of AC in T47D cells after short- and long-term exposure to CT and to determine the influence of internalization of the hormone-receptor complex on CT-activated AC. Cellular cAMP levels were measured following preincubation and then subsequent challenge with CT. We also examined $^{125}$I-sCT binding in order to correlate receptor binding to AC activity.

After preincubation with sCT, T47D cells showed a time and dose-dependent loss of specific binding of $^{125}$I-sCT...
sCT. Concomitant with this, two effects on cellular AC were observed. First, there was an initial increase in cellular cAMP concentration. These results confirm earlier observations using broken cell preparations to measure AC activity (8, 17) and show that our system, using intact cells, was capable of detecting the described "persistent activation" of the AC. Second, there was a decreased ability to stimulate AC upon the second challenge with sCT. If the human analogue of CT was substituted for the more potent salmon hormone, the same loss of binding and AC responsiveness was seen, although higher hormone concentrations were required. After preincubation for 3 h with concentrations above 10 nmol/l hCT, a loss of the AC response to sCT was seen, while one-third of the specific binding was retained. This finding might be explained by a lower receptor affinity of hCT than sCT or by the spare receptor concept; accordingly, only a subgroup of receptors needs to be activated to allow the full biological response (18).

Studies to examine the time course of receptor binding and intracellular cAMP accumulation during incubation with sCT revealed a dissociation between these parameters. Specific binding of [125I]-sCT immediately dropped after preincubation with sCT (Fig. 3, top); following a second and maximal challenge with sCT, however, it took more than 1 h before a reduction of cAMP levels was seen (Fig. 3, bottom). Within the first 60 min of the preincubation period, cAMP levels increased to a maximum, while cAMP levels after the second sCT stimulation remained unchanged in that time. Persistent activation by occupied cell surface receptors may explain these observations. Michelangeli et al. (17) showed that loss of CT binding and AC responsiveness after preincubation with sCT could be largely prevented by an early acid washing, which removes cell surface bound CT. Later acid washing did not resolve binding capacity or AC responsiveness. Furthermore, Findlay and Martin (19) showed that the loss of binding after preincubation with sCT could be prevented for up to 4 h if the cells were kept at 4°C and acid washed before rebinding. These results taken together suggest that initial tight binding of CT to its receptor causes persistent activation of the AC via G protein. Another explanation for dissociation of binding and activation of AC may be the fact that the CT receptor alternatively couples to an additional signal pathway via...
incubation with high concentrations of sCT after 24 h preincubation with 1 nmol/l or more sCT leads to a fall of cAMP compared with the basal levels. We have no explanation for this result at the moment.

We hypothesize that the fall in maximally stimulated cAMP levels in CT-preincubated T47D cells is due to internalization of CT receptor. This is supported by the recovery studies after preincubation with submaximal doses of sCT (Fig. 5). Since we have previously shown that CT receptors are not recycled (7), recovery of CT binding to the cells should be due to the appearance of new CT receptors on the cell surface. In fact the recovery of CT binding (Fig. 5, top) is very closely mirrored by the recovery of the maximally stimulated cAMP levels (Fig. 5, bottom). Based on these data we hypothesized that internalization would separate CT receptor from the Gs protein coupled AC thus ending the state of “persistent activation”. To test this hypothesis, the effect of monensin was examined. Monensin acts by increasing lysosomal pH (24) and prevents intracellular degradation of the hormone-receptor complex. Monensin has been shown to prevent recycling of several receptors (including transferrin, LDL, insulin, EGF), while it does not interfere with internalization (25). Indeed, in previous experiments monensin blocked the release of internalized CT without interfering with internalization (7). While monensin clearly prevented a decrease in cell-associated radioactivity (Fig. 6, top), it did not influence the time course of basal and subsequently stimulated cAMP levels in these cells (Fig. 6, bottom). This indicates that internalization terminates the persistent activation of the G protein activated AC in T47D cells. The absolute cAMP levels were slightly higher in monensin-treated cells than in untreated cells. This could be due to an effect of monensin on formation or breakdown of cAMP. In preincubated cells, the time course of the decrease in basal cAMP levels correlates well with the time course of the internalization of CT seen in previous experiments (7).

These data are consistent with the view that tight binding of CT leads to persistent activation of the cellular AC in T47D cells. Internalization uncouples receptor from the G protein activated AC and ends this activated state. Loss of receptors from the cell surface makes the cells unresponsive to further stimulation by CT. This model might explain part of the “escape” phenomenon, which is seen after continuous treatment with CT.

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References


different G proteins (20-22). Interactions between the different CT receptor-generated second messengers could modulate the activity of the AC. The CT receptor shows no sequence similarity to other reported G protein-coupled receptors but is homologous to the parathyroid hormone-parathyroid hormone-related peptide receptor, indicating that the receptors for these hormones, which regulate calcium homeostasis, represent a new family of G protein-coupled receptors (23).

After 24 h preincubation with sCT, initial and maximally stimulated, i.e. following a second sCT challenge, cAMP levels were clearly reduced compared with the levels after 3 h preincubation (Fig. 4), while specific binding values were similar to those after 3 h of desensitization. The low levels of cAMP could be due to the internalization and degradation of a large part of the receptor pool. After 3 h desensitization the loss of [125I]sCT binding would be due to the tight binding of unlabelled CT to the receptor and an associated increase in basal AC activity, while after 24 h the receptor levels would be reduced to such an extent that stimulation of the AC with CT was not possible. Oddly enough,
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