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Calcitonin and calcium ionophores: Cyclic AMP responses in cells of a human lymphoid line

(calcitonin analogues/lymphocytes/receptors)

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ABSTRACT **Receptors for calcitonin, as assayed by the specific binding of ^{125}I-labeled salmon calcitonin and stimulation of cyclic AMP formation, were found in 8866 cells derived from a human lymphoid line. The affinity of calcitonin from different species and of various analogues of human calcitonin for the binding sites and their ability to stimulate cyclic AMP formation were closely related to their hypocalcemic activity and presumably reflected biological properties of the hormones. Besides calcitonin, prostaglandin E_1 and β-adrenergic catecholamines stimulated cyclic AMP formation in these cells. The calcium ionophores, A23187 and Br-X-537A, did not influence the specific binding of ^{125}I-labeled salmon calcitonin. A23187, however, suppressed basal and calcitonin-stimulated formation of cyclic AMP in the presence of at least 0.6 mM calcium in the incubation medium. Br-X-537A did not require extracellular calcium to suppress basal and calcitonin-stimulated formation of cyclic AMP, suggesting that the release of calcium from internal stores may regulate adenylyate cyclase activity in 8866 cells.**

Calcitonin is a polypeptide hormone that causes hypocalcemia by means of inhibition of the release of calcium from bone and stimulation of urinary calcium excretion (1, 2). The initial step of the action of calcitonin is binding to receptors on plasma membranes of renal and bone cells (3). Subsequently and prior to the characteristic biological response, the formation of cyclic AMP (cAMP) is stimulated (3, 4). Additional receptors for calcitonin have been found on bronchial epidermoid carcinoma cells (5) and on cultured human lymphocytes (6). Marx *et al.* (6) have demonstrated calcitonin-specific binding sites, and we have found a stimulation of cAMP formation in 8866 cells derived from a patient with chronic myelogenous leukemia (7).

In the present report we have confirmed the binding characteristics of ^{125}I-labeled salmon calcitonin (^{125}I-calcitonin) to 8866 cells and present evidence that binding correlated with the stimulation of cAMP formation and the hypocalcemic activity of various calcitonins. In view of the close relationship between changes in calcium and cAMP concentrations (8), we have studied the influence of extracellular calcium and calcium ionophores on calcitonin binding and on basal and calcitonin-stimulated cAMP formation.

MATERIALS AND METHODS

Synthetic human calcitonin-(1–32) and analogues thereof, porcine calcitonin-(1–32), human parathyroid hormone -(1–34) corresponding to the structure obtained by Brewer *et al.* (9), adrenocorticotropin (ACTH)-(1–24), somatostatin, and purified porcine insulin were donated by W. Rittel, Ciba-Geigy AG, Basel, Switzerland; synthetic salmon calcitonin-(1–32) by W. Doepfner, Sandoz AG, Basel, Switzerland; 1,25-dihydroxycholecalciferol by U. Gloor, Hoffmann-La Roche Company, Basel, Switzerland; prostaglandin E_1 by J. W. Hinman, The Upjohn Company, Kalamazoo, MI; propanolol by Imperial Chemical Industries, Macclesfield, England; and the calcium ionophores A23187 by R. L. Hamill, Lilly Research Laboratories, Indianapolis, IN, and Br-X-537A by L. U. Solms, Hoffmann-La Roche Co., Basel, Switzerland. Purified bovine parathyroid hormone-(1–84) was purchased from Wilson Laboratories, Chicago, IL; human growth hormone (somatotropin) from Kabi Diagnostica, Stockholm, Sweden; synthetic bovine parathyroid hormone-(1–34) from Beckman Instruments Inc., Palo Alto, CA; epinephrine and norepinephrine from Fluka AG, Buchs, Switzerland; isoproterenol from Winthrop Products Company, Surbiton-on-Thames, England; and 3,3',5-triiodothyronine from Sigma Company, St. Louis, MO.

Cell Culture, Isolation, and Incubation. The 8866 cells were derived from a patient with chronic myelogeneous leukemia (6) and were generously provided by S. J. Marx (NIAMDD). They were cultured in RPMI 1640 medium with 2 mM L-glutamine, supplemented with 10% fetal calf serum (vol/vol; Gibco, Glasgow, Scotland) and 0.5 μg of neomycin sulfate per ml, under a humid atmosphere of 95% air/5% CO_2 at 37°. Cells were fed two to three times weekly by 1:3 dilution in fresh growth medium. The cells were harvested at the late phase of exponential growth by sedimentation for 10 min at 300 × *g* at 4°, washed three times in the incubation medium, and resuspended to a density of 10^7 cells per ml.

The incubation medium contained 25 mM Tris·HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 15 mM Na acetate, 10 mM dextrose, 0.1% bovine serum albumin, 10 mM theophylline, and, unless otherwise stated, 4 mM Na_2 [ethylenebis(oxyethylenenitrilo)]tetraacetate (EGTA) and 200 μg of ACTH-(1–24) per ml. The A23187 and Br-X-537A ionophores were added in dimethyl sulfoxide and the controls received dimethyl sulfoxide alone; the final concentration of dimethyl sulfoxide was 0.08% vol/vol, and did not affect our measurements.

Binding Studies. Salmon calcitonin and the virtually biologically inactive free acid of human calcitonin (calcitonin-OH) (10) were iodinated with chloramine-T as described (11) to specific activities of 350–500 Ci/mmol. Cells were incubated with salmon ^{125}I-calcitonin in the absence and presence of unlabeled salmon, porcine, and human calcitonins and analogues thereof, and with human ^{125}I-calcitonin-OH in the ab-

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Abbreviations: cAMP, cyclic AMP; ^{125}I-calcitonin, ^{125}I-labeled calcitonin; ACTH, adrenocorticotropin; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

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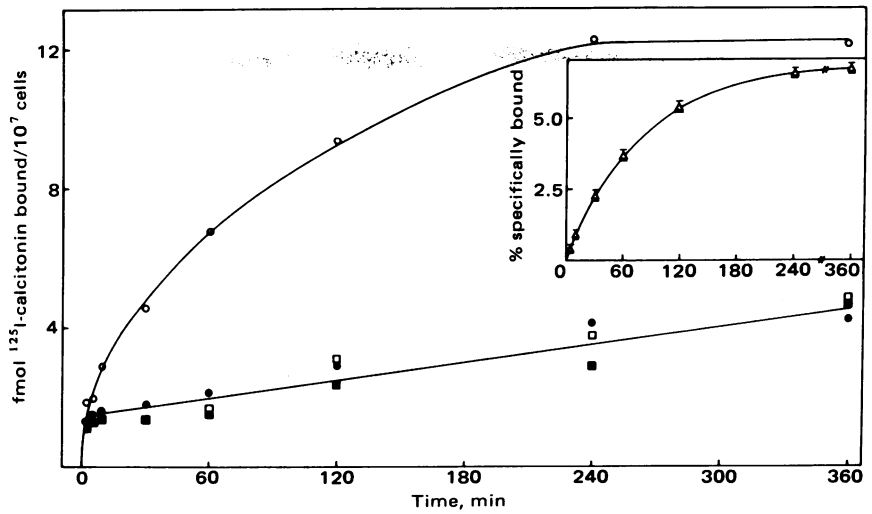

FIG. 1. Time course of binding of salmon ^{125}I-calcitonin to 8866 cells in the absence (○) and presence (●) of 50 nM salmon calcitonin and of human ^{125}I-calcitonin-OH in the absence (□) and presence (■) of 50 nM human calcitonin-OH. Cells (5 × 10^6) were incubated at 37°. (*Inset*) Specific binding of salmon ^{125}I-calcitonin (n = 4 experiments).

sence and presence of unlabeled human calcitonin-OH, at 37° in 0.5 ml. The viability, determined by trypan blue exclusion, was above 90% after the incubations. Bound radioactivity was measured in triplicate 200-μl aliquots by a microfuge phase separation technique (6).

cAMP Determination. cAMP was determined in cells and supernatants in 200-μl aliquots obtained by microfuge phase separation (6). Total cAMP was measured in unseparated cells and medium after immediate immersion of the incubation mixture into liquid N_2. cAMP was estimated radioimmunologically in duplicate samples by a modification of the method of Smith *et al.* (12); modifications included a different extraction procedure, with distruption of the cells by freezing and thawing three times in the presence of a total volume of 0.8 ml of 5% trichloroacetic acid and 0.1 M HCl. Cell debris was removed by centrifugation; 0.7-ml aliquots of the supernatant were extracted five times with 4 ml of ethyl ether saturated with water, the ether was evaporated at 56°, and the sample was lyophilized. The samples were dissolved in 0.05 M Na acetate (pH 6.2) and assayed for cAMP with radioimmunoassay kits (Schwarz/Mann, Orangenburg, NY). If required, cAMP samples were either succinylated or acetylated according to Frandsen and Krishna (13). The material extracted and assayed as cAMP could be destroyed by cAMP phosphodiesterase (Boehringer, Mannheim, Germany). Recoveries of [^{3}H]cAMP added at the end of the incubations ranged from 70 to 85%. The values are either means ±SEM from four independent experiments or representative values from at least four experiments.

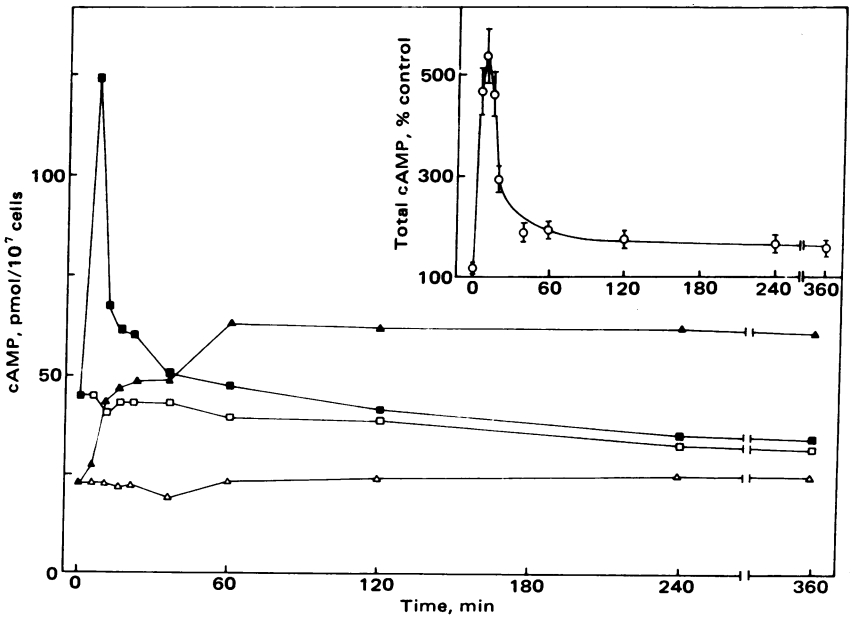

FIG. 2. Time course of stimulation of cAMP in 8866 cells in the absence (□, △) and presence (■, ▲) of 50 nM salmon calcitonin. Cells (5 × 10^6) were incubated at 37°, and cAMP was estimated in the cells (□, ■) and supernatant (△, ▲). (*Inset*) Percentage stimulation of total cAMP (in cells and supernatants; n = 4 experiments).

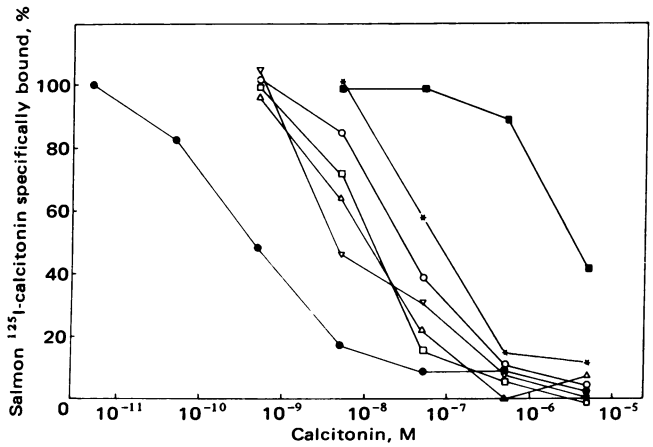

FIG. 3. Competitive inhibition by various calcitonins of the specific binding of salmon ^{125}I-calcitonin to 8866 cells. Cells (5×10^6) were incubated for 240 min at 37° with salmon ^{125}I-calcitonin and the following inhibitors: unlabeled salmon calcitonin (●), human [Leu^{12,16,19}Tyr^{22}]calcitonin (▽), human [Arg^{24}]calcitonin (△), human [Tyr^{22}]calcitonin (□), human calcitonin (*), human calcitonin-OH (■), and porcine calcitonin (○). Ordinate denotes percent initial binding (without inhibitor).

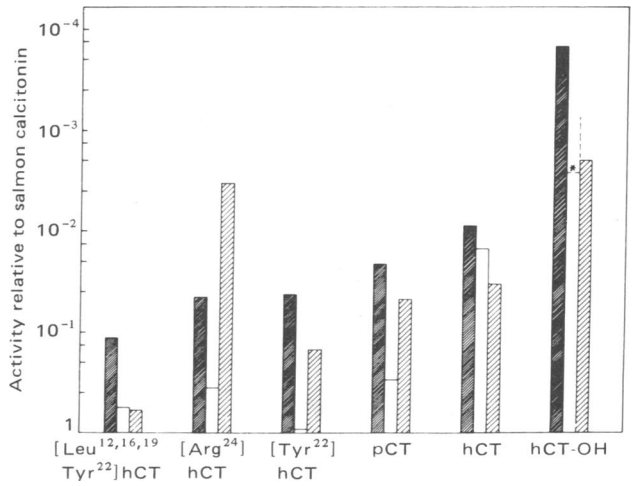

FIG. 5. Potency of various calcitonins (CT) (h, human; p, porcine) relative to salmon calcitonin in 8866 cells and in the hypocalcemic rat assay. ■, Half-maximal specific binding of salmon ^{125}I-calcitonin; □, half-maximal stimulation of total cAMP formation (asterisk indicates no stimulation of cAMP formation at 0.5 μM human calcitonin-OH; for details see Figs. 3 and 4); ▨, hypocalcemic activity (10, 15–18).

RESULTS

Binding of Salmon ^{125}I-Calcitonin. The labeled calcitonin bound specifically to 8866 cells as reported by Marx *et al.* (6). The binding increased gradually with time and reached an apparent steady state by 4–6 hr (Fig. 1). Seventy-five percent of total binding of salmon ^{125}I-calcitonin could be abolished by incubation with 50 nM unlabeled salmon calcitonin, and is referred to as specific binding. The amount of salmon ^{125}I-calcitonin remaining "bound" in the presence of 50 nM unlabeled salmon calcitonin is defined as nonspecific binding; it is similar to the binding of the radioiodinated, virtually biologically inactive, free acid of human calcitonin (calcitonin-OH) (10) both in the absence and presence of 50 nM unlabeled human calcitonin-OH. As shown by Marx *et al.* (6), the binding of salmon ^{125}I-calcitonin was linearly related to a cell density of up to 1.6×10^7 cells/ml. After 360 min of incubation, salmon ^{125}I-calcitonin was converted into smaller molecular weight fragments. Salmon ^{125}I-calcitonin in the incubation medium was degraded more than 90% in the absence and between 20 and 30% in the presence of 200 μg of ACTH per ml, as assayed by gel filtration on Bio-Gel P-6. The radioactivity eluted from the cells with 0.1 M acetic acid consisted predominantly of fragments of salmon calcitonin both in the absence and presence of ACTH; binding of fragments of radioactive salmon calcitonin to 8866 cells varied between 30 and 70%, but the binding could not be inhibited by 50 nM unlabeled salmon calcitonin (not shown).

cAMP Formation. As shown in Fig. 2, cAMP levels were already maximal in the cells after 5 min of incubation in the presence of salmon calcitonin. The time of appearance of the peak of cAMP concentrations in the cells was somewhat unpredictable, and total cAMP in the cells and incubation medium was therefore determined in most of the experiments; maximal total cAMP levels were observed between 5 and 15 min. Preincubation of the cells for various time intervals of up to 120 min in the absence of calcitonin did not affect the subsequent increase of cAMP levels by calcitonin. However, a second addition of calcitonin 30 min after the first addition did not significantly raise cAMP levels (7). The calcitonin-stimulated cAMP formation was linearly related to a cell density of up to 1.6×10^7 cells/ml. Fragments of salmon calcitonin generated during incubation for 360 min did not stimulate cAMP formation (not shown).

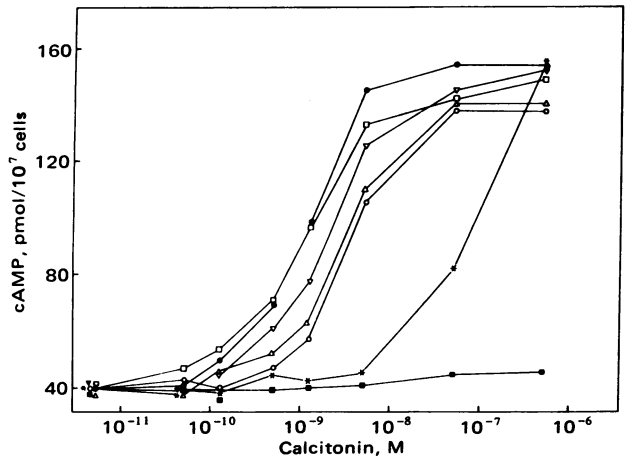

FIG. 4. Stimulation of total cAMP in 8866 cells by various calcitonins. Cells (5×10^6) were incubated for 5 min at 37° with the calcitonins given in the legend of Fig. 3.

Table 1. Stimulation of cAMP formation in 8866 cells

Hormone	Half-maximal stimulation, M
Salmon calcitonin	3.6×10^{-10}
Salmon calcitonin + propranolol (10 μM)	2.4×10^{-10}
Prostaglandin E_1	7.9×10^{-8}
Isoproterenol	1.0×10^{-6}
Isoproterenol + propranolol (10 μM)	0
Norepinephrine	1.3×10^{-6}
Epinephrine	1.6×10^{-6}

Cells (5×10^6) were incubated for 5 min at 37°. cAMP formation was stimulated 2.1- to 2.5-fold by salmon calcitonin and the catecholamines, and 9.8-fold by prostaglandin E_1 (7). ACTH-(1–24), human growth hormone, porcine insulin, bovine parathyroid hormone-(1–34) and -(1–84), human parathyroid hormone-(1–34) (9), somatostatin, 1,25-dihydroxycholecalciferol, 3,3′,5-triiodothyronine (1 and 10 μg/ml), and ACTH (1, 10, and 200 μg/ml) did not change cAMP levels.

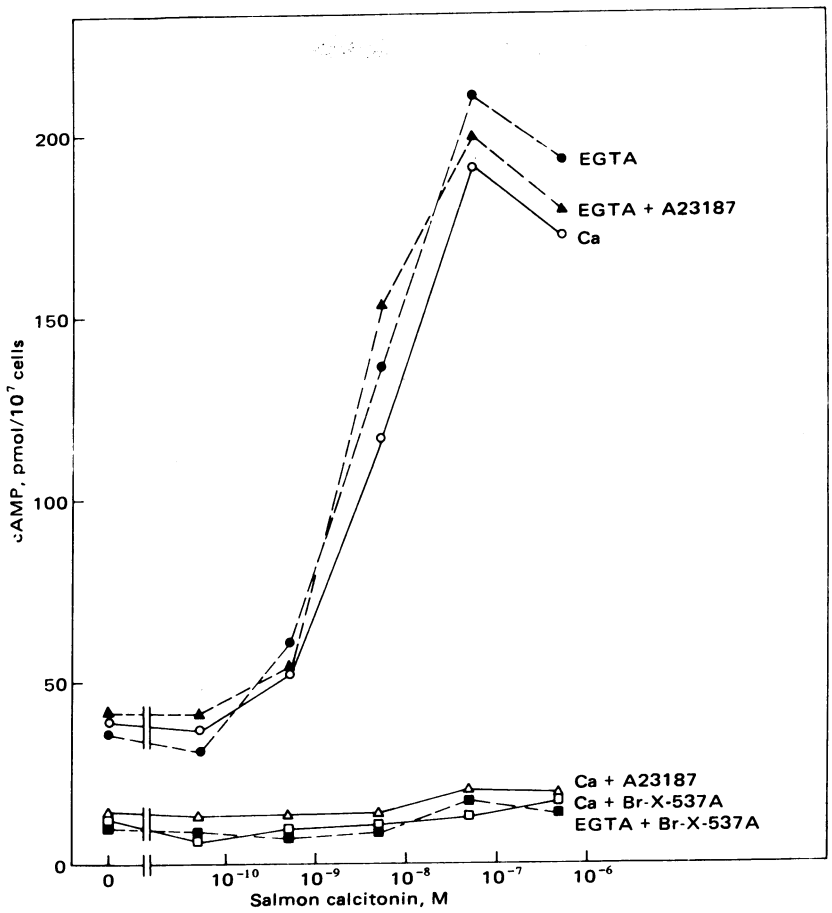

FIG. 6. Total cAMP responses to salmon calcitonin in the presence of A23187 and Br-X-537A. Cells (5×10^6) were incubated for 5 min at 37° with 4 mM EGTA (closed symbols) and with 1 mM $CaCl_2$ (open symbols) in the absence of (●, ○) and presence of (▲, △) 0.7 mM A23187 or (■, □) 0.7 mM Br-X-537A. Statistical difference between (▲, ○, ●) and (△, □, ■) in the absence of salmon calcitonin was determined by Student's t test ($P < 0.02$) and in the presence of salmon calcitonin by two way variance analysis ($P < 0.001$) ($n = 4$ experiments).

Specifity. The displacement of specifically bound salmon ^{125}I-calcitonin by unlabeled salmon, porcine, and human calcitonins and several analogues of the human hormone is shown in Fig. 3. The apparent association constant determined by Scatchard analysis (14) of salmon calcitonin was 2 nM^{-1} and the total number of salmon calcitonin binding sites was 400–800 per cell. The amounts of inhibitor required to displace salmon ^{125}I-calcitonin from its receptor increased in the order unlabeled salmon calcitonin, human [Leu^{12,16,19}Tyr^{22}]calcitonin, human [Arg^{24}]calcitonin, human [Tyr^{22}]calcitonin, porcine calcitonin, human calcitonin, and human calcitonin-OH. The same calcitonins behaved in a similar way when assayed for their capacity to stimulate cAMP formation (Fig. 4). In this case the order of ability to stimulate cAMP formation was salmon calcitonin, human [Tyr^{22}]calcinonin, human [Leu^{12,16,19}Tyr^{22}]-calcitonin, human [Arg^{24}]calcitonin, porcine calcitonin, human calcitonin, and human calcitonin-OH. In Fig. 5 the relative binding inhibition of the various calcitonins and their relative ability to stimulate cAMP accumulation is compared to their hypocalcemic activity (10, 15–18). The concentrations of calcitonin causing half-maximal inhibition of binding and half-maximal stimulation of cAMP formation were comparable and corresponded reasonably well to their hypocalcemic activity.

Besides calcitonin, prostaglandin E_1, isoprotenerol, norepinephrine, and epinephrine stimulated cAMP formation, whereas ACTH-(1–24), growth hormone, insulin, parathyroid hormone, somatostatin, 1,25-dihydroxycholecalciferol, and 3,3′,5-triiodothyronine were inactive (Table 1). The stimulation with isoproterenol and the suppression with propranolol suggest the existence of β-adrenergic receptor sites. These sites are probably not linked to the stimulation of cAMP formation by salmon calcitonin, which is not influenced by the addition of propranolol.

Extracellular Calcium and Calcium Ionophores in Binding of Salmon ^{125}I-Calcitonin and cAMP Formation. Addition of calcium, EGTA, and the calcium ionophores A23187 and Br-X-537A to the incubation medium did not affect the specific binding of salmon ^{125}I-calcitonin to 8866 cells. Nonspecific binding was raised from 2% to 10% in the presence of the A23187, but remained unchanged with Br-X-537A, calcium, and EGTA (not shown).

Calcium and EGTA did not influence cAMP levels, whereas Br-X-537A inhibited basal and calcitonin-stimulated cAMP formation (Fig. 6); A23187, on the other hand, only suppressed basal and calcitonin-stimulated cAMP formation in the presence of at least 0.6 mM calcium. A23187 was inactive with EGTA or with 60 μM calcium in the incubation medium.

DISCUSSION

Calcitonin receptors on cells from the human lymphoid cell line 8866 initiate, upon binding of the hormone, cAMP responses similar to those observed in recognized target organs of calcitonin, such as kidney and bone cells (3, 4). Stimulation of cAMP formation by calcitonin was only observed initially, suggesting that desensitization of the adenylyate cyclase activity took place during prolonged incubation (7). Salmon calcitonin had not only

a higher binding affinity than human calcitonin for the 8866 cells, but it also had a greater ability to stimulate cAMP formation. This is further evidence for the evolution of human calcitonin to an intrinsically less potent form of the hormone. Substitutions of amino acids in the structure of human calcitonin towards the structure of the salmon hormone results in a potentiation of the affinity and potency of the human hormone in the 8866 cells, which reasonably well agree with their hypocalcemic activity (10, 15–18). We cannot exclude the existence of separate high-affinity binding sites for labeled human or porcine calcitonins that are not detected with salmon ^{125}I-calcitonin. The results obtained in 8866 cells, however, reflect biological properties of calcitonin.

The functional significance of calcitonin, β-adrenergic, and prostaglandin E_1 receptors coupled to the stimulation of cAMP formation remains to be elucidated. In mixed cell preparations from rat thymus, salmon calcitonin inhibited apparently cAMP-mediated stimulation by epinephrine of thymocyte proliferation (19). On the other hand, the calcitonin did not affect [^{3}H]thymidine incorporation into 8866 cells (not shown). The behavior of 8866 cells may not be comparable to rat thymocytes. Characteristic biological responses of calcitonin in leukemic cells have not been detected.

The role of cAMP as a second messenger is widely accepted, whereas the mode of action of calcium as a coupling factor is complex (8). In the present study, calcitonin stimulated and Br-X-537A suppressed cAMP formation in the absence and presence of extracellular calcium; A23187, on the other hand, only suppressed cAMP levels in the presence of calcium added to the incubation medium. Calcitonin-specific binding was unrelated to the calcium concentration and was not affected by the presence of calcium ionophores. Furthermore, as in turkey erythrocytes (20) and human leukocytes (21), cAMP levels were not influenced by changes of the calcium concentration in the medium. A23187 stimulated the calcium-dependent secretion of histamine from mast cells (22) provided calcium was present in the incubation medium, whereas Br-X-537A enhanced the release of oxytocin from the neurohypophysis in the absence of added external calcium (23). There is evidence that A23187 not only transports calcium across plasma membranes, but also stimulates calcium efflux from internal, most likely mitochondrial, stores (24–26), whereas Br-X-537A appears to release calcium from intracellular binding sites only (23). A23187, on the other hand, had no direct effect on the activity of the adenylyate cyclase in turkey erythrocytes (21). If Br-X-537A and A23187 increase, as is generally believed, the free calcium concentration in the cytosol (27, 28), they probably decrease adenylyate cyclase activity by raising cytosolic calcium levels.

The data may also imply that a fall of the calcium concentration in the cytosol is associated with calcitonin-stimulated increase of cAMP in cultured human lymphocytes. Inasmuch as the extracellular calcium concentration did not influence the cAMP responses to calcitonin, the hormone possibly stimulated the translocation of calcium from the cytosol to intracellular binding sites. This is in line with the findings of Borle (29), who presented evidence that calcitonin stimulated the rate of calcium uptake into kidney mitochondria. On the other hand, in contrast to the lack of an effect of A23187 on adenylyate cyclase activity (21), calcitonin directly stimulates the enzymatic activity in kidney and bone cells (3, 4). Nevertheless, EGTA stimulated and calcium suppressed calcitonin-sensitive adenylyate cyclase activity (30, 31). Finally, a stimulation of a calcium-sensitive cAMP phosphodiesterase cannot be ruled out, but appears unlikely, since EGTA had no effect on phosphodiesterase activators of normal and leukemic lymphocytes (32).

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