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Calcitonin receptor gene expression in K562 chronic myelogenous leukemic cells

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Abstract

Background: The peptide hormone calcitonin (CT) can significantly effect the proliferation rate of CT receptor (CTR) positive human cancer cells. We wish to identify additional human cancers expressing CTRs and assay the effects of CT on their growth rates and signal transduction pathways.

Results: The expression of the human calcitonin receptor (hCTR) gene in the chronic myelogenous leukemia cell line K562 was examined. RT-PCR on total RNA extracted from K562 cells detected the presence of hCTR mRNA. Further analysis demonstrated that multiple hCTR isoforms were present. Incubation of K562 cells with salmon calcitonin (sCT), but not amylin, caused an increase in intracellular levels of cAMP similar to that induced by forskolin treatment. We further demonstrated that butyrate induced erythroid differentiation of K562 cells caused a significant decrease in hCTR mRNA levels. However, phorbol myristate acetate (PMA) induced megakaryocytic differentiation of these cells had no significant effect on hCTR mRNA levels. We demonstrated that exposure to various concentrations of sCT had no effect on the cellular proliferation of K562 cells *in vitro*.

Conclusion: Chronic myelogenous k562 cells express multiple CTR isoforms. However, CT does not effect K562 proliferation rates. It is likely that the small increase in intracellular levels of cAMP following CT treatment is not sufficient to interfere with cellular growth.

Background

The hypocalcemic hormone calcitonin (CT), first identified by Copp et al. [1], is produced by the parafollicular cells of the thyroid gland in response to increases in extracellular levels of calcium. CT regulates serum calcium levels by inhibiting osteoclast mediated bone resorption and enhancing calcium excretion by the kidney [2–4]. However, the presence of non-thyroidal forms of CT in tissues such as the central nervous system and prostate suggests it may play a role in other physiological processes [5,6]. The hypocalcemic effects of CT are mediated by high affinity

CT receptors (CTR). The CTR is a member of a subfamily of the seven trans-membrane domain G protein-coupled receptor superfamily that includes glucagon-like peptide 1 [7], glucagon [8], pituitary adenylate cyclase-activating polypeptide [9], corticotropin-releasing factor [10], gastric inhibitory polypeptide receptors [11] and PTH/PTH-related peptide [12]. CTRs have been identified in a variety of tissues such as skeletal muscle [13], kidney [14,15], osteoclasts [16,17] and spermatozoa [18]. Through the use transgenic mouse technology we recently identified a variety of novel, developmentally regulated sites of CTR gene

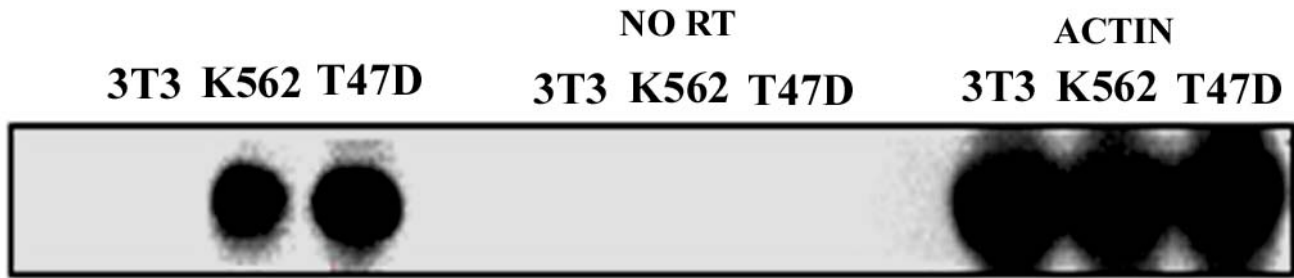


Figure 1

Southern blot analysis of RT-PCR products. RT-PCR and no RT controls were carried out on total RNA from K562 cells, T47D (hCTR positive) and 3T3 (hCTR negative) cells employing hCTR and actin specific oligos. PCR products were size fractionated on a 1 % agarose gel and Southern blotted. hCTR and actin specific ^{32}P end labelled probes were hybridized to blot. These results are representative of 3 independent experiments all giving similar results.

expression [19]. Studies have also demonstrated that breast cancer cell lines and primary breast tumors also express high levels of CTRs [20–22]. CT induces a marked growth inhibition of these cells *in vitro* [23]. It would be of clinical interest to identify other human cancers that express CTRs as they may play a role in the differentiation and growth of these cells *in vivo*.

In this report, we identified the expression of CTRs in the human chronic myelogenous leukemia cell line K562. RT-PCR analysis revealed the presence of multiple hCTR isoforms. Butyrate induced erythroid differentiation of these cells caused a significant reduction in the expression of CTR mRNA. In contrast, PMA induced megakaryocytic differentiation of K562 cells had no effect on hCTR mRNA levels. We further demonstrated that CT, but not amylin, induced an increase in intracellular levels of cAMP of these cells. Finally, we demonstrated that CT did not significantly suppress cellular proliferation of K562 cells *in vitro*.

Results

CTR mRNA in K562 cells

Ongoing research in our laboratory detected the presence of a tissue specific factor associated with hCTR gene expression in K562 cells (G. Partington, unpublished results). We therefore wished to determine if the hCTR gene was transcriptionally active in K562 cells. RT-PCR was performed on K562 total RNA employing oligonucleotides that would enable us to detect the three most common hCTR isoforms, hCTR_{1a'}, hCTR_{1b} and hCTR_{1a} truncated [26–28]. Ethidium bromide staining of the RT-PCR products revealed two poorly resolved bands differing in size by approximately 50 bp (data not shown). This suggested

the presence of two hCTR isoforms. It was likely that these products corresponded to hCTR_{1a} and hCTR_{1b}, previously shown to differ in size by a 16 amino acid insert present in hCTR's first intracellular loop [27,28]. While a ^{32}P end labelled hCTR specific probe hybridized to the RT-PCR products, it was unable to resolve both isoforms (Fig. 1). We therefore carried out RT-PCR again, separated and purified the bands from a 2% agarose gel followed by additional electrophoresis and Southern blotting. The blot was hybridized with the same hCTR probe. Autoradiography demonstrated the presence of two CTR positive bands differing in size by approximately 50 bp (Fig. 2A). When the blot was stripped and re-hybridized with a probe corresponding to the 16 amino acid insert, only hybridization to the RT-PCR product in lane 1 was evident (Fig. 2B). These data suggest that K562's express two of the three known hCTR mRNA isoforms hCTR_{1a} and hCTR_{1b}.

Butyrate and PMA treatment of K562's

It is well known that K562 cells undergo erythroid differentiation when treated with butyrate [29] whereas treatment with PMA induces megakaryocyte differentiation [30]. We wished to determine the effect these treatments had on the expression of hCTR mRNA. K562 cells were incubated with either butyrate or PMA for a period of 48 hours followed by isolation of RNA and semi-quantitative RT-PCR analysis. Results indicated that treatment with 2 mM sodium butyrate caused a 5–6 fold reduction (as measured by densitometry) in hCTR mRNA. This suggests that erythroid differentiation represses hCTR gene expression in K562 cells. In contrast, megakaryocytic induced differentiation with 100 nM PMA had no significant effect on hCTR gene expression (Fig. 3).

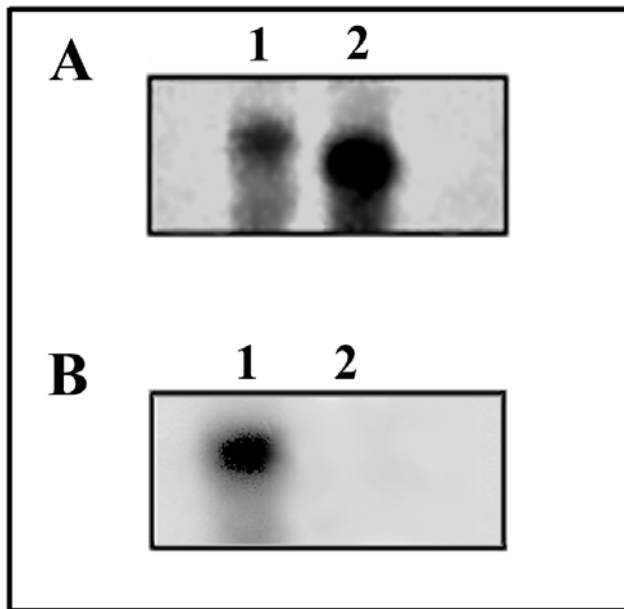


Figure 2
Southern blot analysis on gel purified K562 RT-PCR products. **(A)** RT-PCR products on total RNA from K562 cells employing hCTR specific primers were size fractionated on a 2% agarose gel. Two bands, corresponding in size to hCTR_{1a} and hCTR_{1b}, were individually purified and re-run on a 2% agarose gel followed by Southern blot analysis. The blot was hybridized with a ³²P end-labelled probe common to hCTR_{1a} and hCTR_{1b}. **(B)** Blot in Fig. 2A was stripped of probe and re-hybridized with a ³²P end-labelled probe corresponding to 16 amino acid insert in hCTR_{1b} isoform. These results are representative of 2 independent experiments giving similar results.

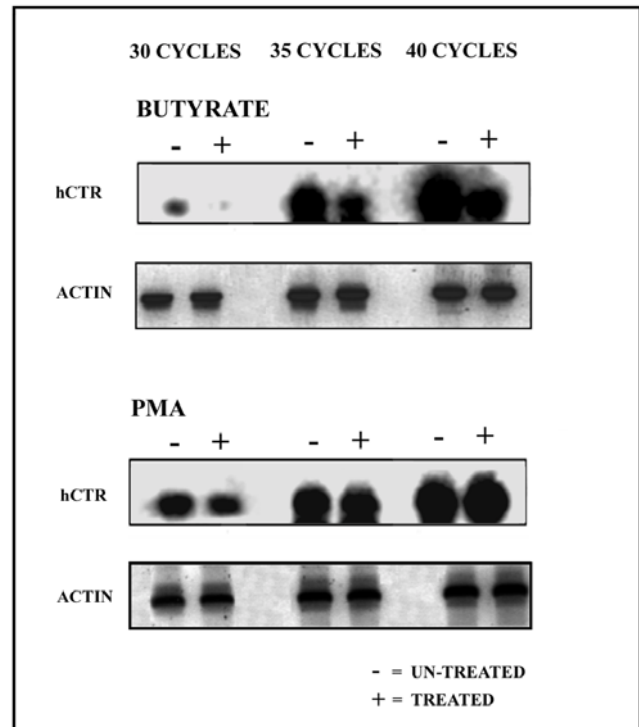


Figure 3
Semi-quantitative RT-PCR analysis on butyrate and PMA treated K562 cells. Total RNA from butyrate, PMA and untreated K562 cells was reverse transcribed and subjected to 30, 35 and 40 cycles of PCR employing actin and hCTR specific oligos. Reaction products were size fractionated on an agarose gel and Southern blotted employing ³²P end labelled hCTR and actin specific probes. These results are representative of 3 independent experiments giving similar results.

Effect of sCT and amylin on intracellular levels of cAMP in K562 cells

It has been demonstrated that the binding of sCT to the hCTR causes a significant increase in intracellular levels of cAMP [27,28]. We therefore assayed the effect of various concentrations of sCT on intracellular levels of cAMP in K562 cells. Treatment of K562 cells with 1.0–10 nM sCT for 20 minutes caused at least a two-fold rise in intracellular concentrations of cAMP. The effect of sCT on intracellular levels of cAMP was similar to that induced by treatment with 10 μM forskolin, a known activator of intracellular cAMP (Fig. 4).

Receptor activity modifying protein (RAMP) 1 and 3 can convert the hCTR into an amylin receptor which when bound to amylin, another CT related peptide hormone, increases intracellular levels of cAMP [31]. We therefore determined the effect of varying concentrations of amylin

on intracellular levels of cAMP in K562 cells. In contrast to sCT, we were unable to detect any increase in intracellular levels of cAMP following amylin treatment.

Effect of sCT on K562 cellular proliferation

sCT has been found to inhibit the cellular proliferation of hCTR-positive T47D and MCF7 human breast cancer cells *in vitro* while increasing cellular proliferation of hCTR-positive prostate cancer cells [23,32]. We therefore wished to determine the effect of sCT, at various concentrations, on the proliferation of K562 cells *in vitro*. Results of cell counts over a 4 day period indicated no effect of sCT, at any concentration, on cellular proliferation (Fig. 5).

Discussion

CTRs have been reported in a number of human cancer cell lines including those of lung [33], prostate [32] and

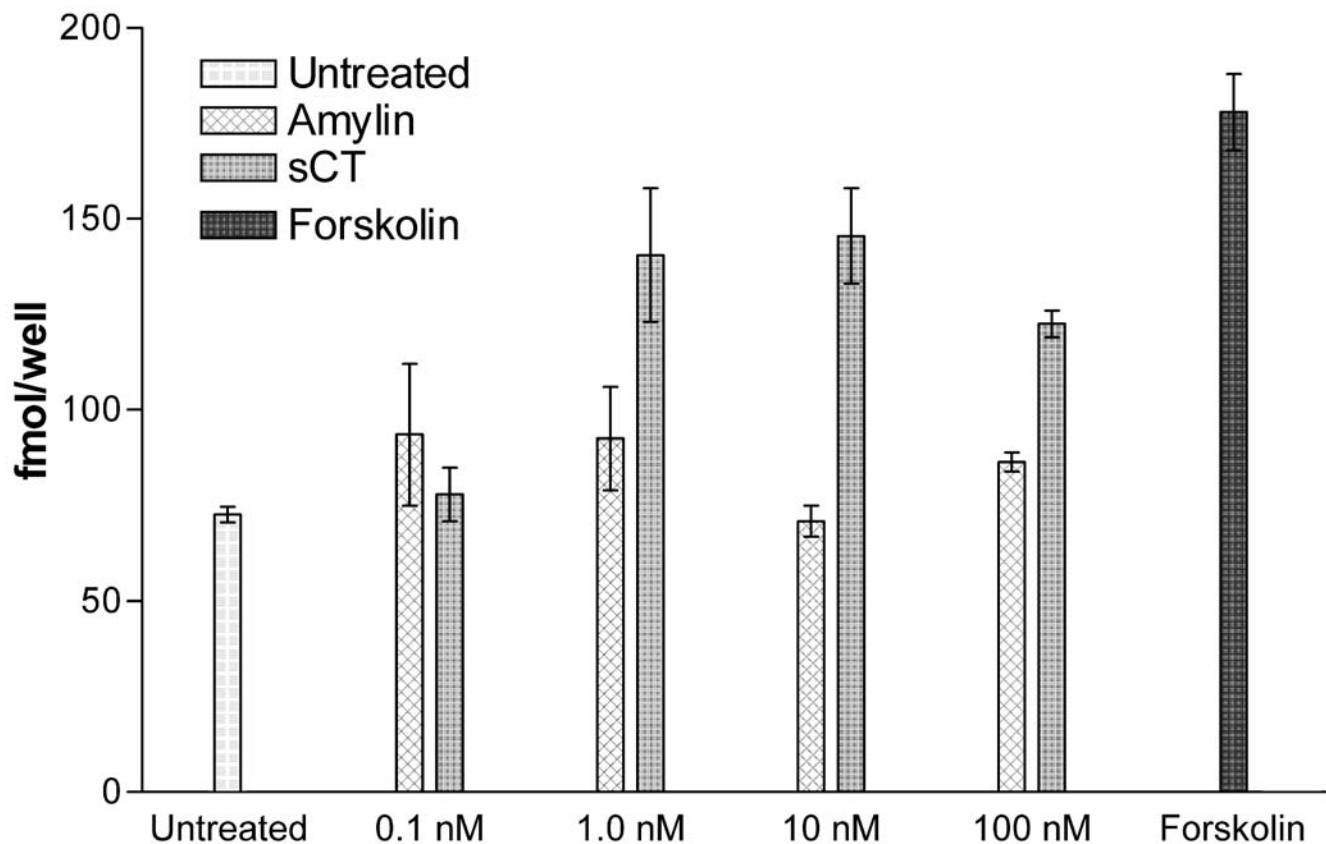


Figure 4

Intracellular levels of cAMP in amylin, sCT and forskolin treated K562 cells. K562 cells were treated with varying concentrations of amylin and sCT for 20 minutes followed by analysis of intracellular cAMP levels. Forskolin, a known activator of cAMP, was employed as a positive control. Each data point represents an average of 2 assays. These results are representative of 2 independent experiments each giving similar results.

breast cancer origin [21,22]. Since studies have demonstrated that CT can modulate the growth of these cells it is of clinical interest to identify other human cancers that express CTRs. In this study, we demonstrate that the hCTR gene is transcriptionally active in the chronic myelogenous leukemia (CML) cell line K562. The K562 line was originally established by Lozzio and Lozzio [34] from the pleural effusion of a patient with chronic myelogenous leukemia. This cell line is considered to represent an early differentiation stage of the granulocyte lineage. The results of our RT-PCR experiments suggest that K562 cells express two of the three known hCTR isoforms. The most common of these isoforms and present in K562, is hCTR_{1a} [27,28]. The insert positive hCTR_{1b}, also present in K562 cells, is expressed in fewer tissue types [27,28]. Both of these isoforms increase intracellular levels of cAMP when bound to CT. Our result demonstrating that sCT increases intracellular levels of cAMP in K562 cells is consistent with these studies. However, sCT does not in-

crease cAMP levels to the extent reported in other CTR positive cell lines (3–4 fold) such as T47D and MCF7 [20]. Furthermore, our experiments indicate that K562 cells do not appear to be responsive to amylin. Studies have shown that receptor activity modifying protein (RAMP) 1 and 3 can convert CTRs into amylin receptors [31]. Amylin receptors like CTRs, activate intracellular levels of cAMP when bound to amylin. The absence of increased levels of cAMP in K562 cells following amylin treatment suggests RAMP 1 and/or 3 are not active/expressed in these cells. Alternatively, amylin receptors may be present in K562 cells but our cAMP assay system is not sufficiently sensitive to detect their activity.

K562 cells are well known to differentiate along an erythroid or megakaryocytic pathway in the presence of butyrate or PMA, respectively [29,30]. Since nothing is known about CTR gene expression during erythropoiesis or megakaryocytic differentiation, we were interested in

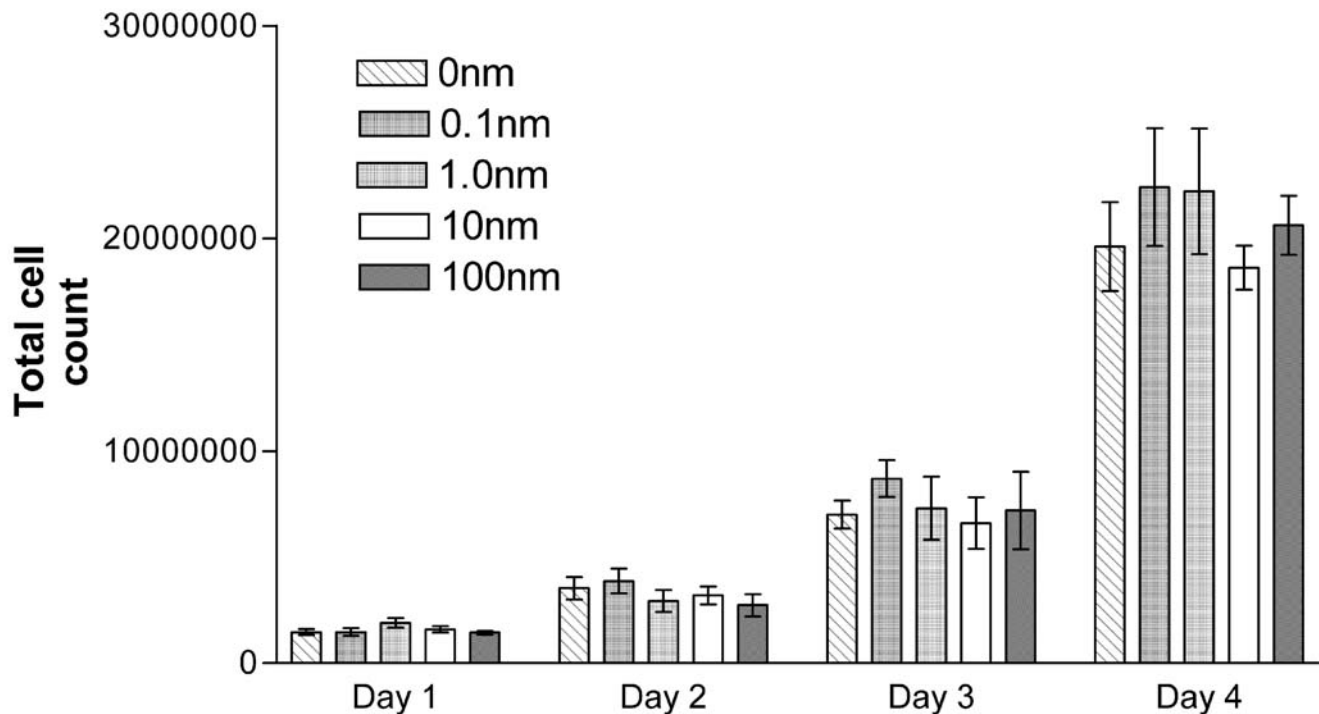


Figure 5

Cellular proliferation of sCT treated K562 cells. K562 cells were treated with varying concentrations of sCT and assayed daily over a four-day period for cell number. Each data point represents an average of four cell counts. These results are representative of 3 independent experiments each giving similar results.

determining what effect butyrate and PMA would have on CTR gene expression in K562 cells. We demonstrate that CTR gene expression during butyrate induced K562 erythroid differentiation decreases significantly. The role this down regulation plays is unknown. However, the effects of forced expression of CTRs in K562 cells combined with sCT incubation might help to determine the role, if any, CTRs play in this model system of erythropoiesis. Our result, demonstrating that the hCTR gene remains transcriptionally active in PMA treated K562 cells, suggests the possibility that CTRs may be expressed in normal megakaryocytes. Studies to examine hCTR gene expression in normal human megakaryocytes are now underway.

Studies have demonstrated that CT/CTRs can modulate cellular proliferation [23,32]. Our results clearly demonstrate that CT, at a variety of concentrations, has no significant effect on the growth of K562 cells. We hypothesize this is due to the small effect CT has on increasing intracellular concentrations of cAMP in K562 cells. Yin et al [35] demonstrated that forskolin, a known activator of cAMP, had no effect on the proliferation of K562 cells. They hy-

pothesized that this was due to the inability of forskolin to increase intracellular levels of cAMP beyond the threshold needed to effect cellular proliferation. We demonstrate that CT/CTRs have a similar effect on levels of intracellular cAMP as forskolin in K562 cells. It is likely that the lack of effect CT has on cellular growth of K562 cells is due to its inability to increase production of cAMP in these cells to a level necessary to interfere with cellular proliferation. Interestingly, Yin et al., [35] demonstrated that a K562 subclone (K/Dau600) showed significantly high levels of intracellular cAMP in response to forskolin. Cellular proliferation of this subclone was also inhibited by forskolin. It would be of interest to determine if CT has a greater ability to increase intracellular levels of cAMP in this subclone than in the parental line and subsequently modulate its proliferation rate.

Conclusions

The results of our study demonstrate that the hCTR gene is transcriptionally active in K562 CML cells. The expression hCTR's in this cell line acts to increase intracellular levels of cAMP when bound to CT. However, CT does not effect the proliferation rate of these cells. The expression

of CTR's in K562 cells will prompt future studies to examine hCTR expression in equivalent cells from patients in CML blast-crisis. If the hCTR gene is active in these cells, the effects of CT on their growth and differentiation should then be assessed.

Methods

Cell Culture

The human chronic myelogenous leukaemia cell line K562 was obtained from the European Collection of Cell Cultures. The cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum, 100 µg/ml penicillin and 100 U/ml streptomycin (obtained from Invitrogen). The CTR positive human breast cancer cell line T47D and the CTR negative mouse cell line 3T3 were maintained in DMEM with fetal calf serum and antibiotics as above.

Reverse Transcription and PCR

RT-PCR was carried out on total RNA isolated from the above cells by acid guanidinium thiocyanate-phenol-chloroform extraction as described by Chomczynski and Sacchi [24]. 5 µg of DNase I treated total RNA was reverse-transcribed in a 25 µl vol. containing 1 × RT Buffer, 1 mM dNTP's, 250 U M-MLV reverse transcriptase (Gibco BRL) and 150 ng random primers at 42°C. After one hour, the reaction mixture was heated to 95°C for 5 minutes. 4 µl were then employed for PCR amplification of hCTR mRNA using the following: 200 nM sense primer (TTGCT-TCTATTGAGCTGTGCC): corresponding to sequences 1–21 in hCTR complementary DNA (cDNA) and 200 nM antisense primer (ATGTTCTTGTGCAGGGTTACC): corresponding to sequences 612–632 in hCTR cDNA, 1 × Buffer, 0.2 mM dNTP's and 2 U Amplitaq DNA Polymerase (Roche) in a final volume of 50 µl. Oligonucleotides were annealed at 58°C for 45 seconds, followed by extension for 1 minute 30 seconds at 72°C and denaturing at 94°C for 45 sec. In addition, PCR employing a highly conserved mouse actin sense primer (GTTACCAACT-GGGACGACATGG): corresponding to 147–168 of the mouse actin cDNA and a conserved antisense primer (GATCTTGATCTTCATGGTGC): corresponding to 890–909 of the mouse actin cDNA was performed, as described above, on all RNA samples. RT-PCR and no RT reaction (control) products were size fractionated on a 1% agarose gel, excised, purified and run individually on two separate 2% agarose gels followed by Southern blot transfer. Two ³²P end-labelled oligonucleotides corresponding to sequences 383–405 in human CTR cDNA (present in all hCTR isoforms) and 494–542 in the "insert positive" hCTR cDNA, as described by Nakamura et al. [25], were hybridized to the blots. A highly conserved actin oligonucleotide corresponding to sequences 551–600 in the mouse actin cDNA was also employed for Southern blot analysis.

Butyrate and PMA treatment of K562 cells

Erythroid differentiation of K562 cells was induced by the addition of 2 mM butyric acid (Fluka), neutralised to pH 7.0 with NaOH. Differentiation along the megakaryocytic pathway was induced by treating cells with 100 nM phorbol myristate acetate (PMA), (Alexis). After 48 hours of treatment, cells were harvested and total RNA isolated as described above. The RNA samples were reverse transcribed as described above followed by 30, 35 and 40 cycles of PCR. Southern blot analysis of RT-PCR products was then carried out as described above.

sCT, amylin treatment and levels of K562 intracellular cAMP

96-well overnight cultures of K562 cells (seeded at a concentration of 10⁵ cells/ml) were treated with sCT or amylin (Bachem) at a concentration of 0 nM, 0.1 nM, 1.0 nM, 10 nM and 100 nM, for 20 minutes at 37°C. As a positive control for cAMP activation, two wells of cells were treated with 10 µM forskolin. A Biotrak cAMP enzyme immunoassay (EIA) system (Amersham) was used to determine effects of treatment on intracellular levels of cAMP in K562 cells.

sCT treatment and cellular proliferation

10⁶ K562 cells were treated with sCT (Bachem) at a concentration of 0 nM, 0.1 nM, 1.0 nM, 10 nM and 100 nM and maintained at 37°C over a period of four days. Cultures were grown in triplicate. Six separate aliquots from each flask were taken daily and total cell counts were obtained by hemocytometer after staining with toluidine-blue. Cell media and sCT were changed daily.

Authors' contributions

RM carried out the experiments in this paper. MP conceived and supervised the project.

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