Regulation of calcitonin release from the 6.23 rat C-cell line by cyclic nucleotide analogues and pharmacological mediators

J.-L. Gilgenkrantz, T. J. Hall[a] and T. J. Chambers[a]

MDRI, 16 Rue D'Ankara, F-67009 Strasbourg Cedex (France), and [a] Department of Histopathology, St. George's Hospital Medical School, London SW17 ORE (England)
Received 21 February 1991; accepted 8 May 1991

Abstract. Calcitonin release from 6.23 rat medullary thyroid carcinoma C-cells was stimulated by dibutyryl cyclic AMP and inhibited by dibutyryl cyclic GMP in concentration dependent fashion. Histamine, isoproterenol, prostaglandin E_2 and Bay K 8644 stimulated calcitonin release, while acetylcholine and serotonin had no significant effect on CT release.
Key words. Calcitonin release; 6.23 rat C-cell line; cyclic nucleotides; pharmacological mediators.

Calcitonin (CT) is a 32 amino acid hormone involved in the regulation of plasma calcium levels[1,2]. CT is synthesized by thyroid C-cells and medullary thyroid carcinoma (MTC) C-cell lines, and is stored in cytoplasmic secretory granules[3,4]. The CT gene, by tissue specific alternative RNA processing[5], can also result in the production of the CT gene-related peptide (CGRP) primarily in neural tissues, but also by MTC C-cell lines[6,7]. The release of CT from C-cells in vivo[1,8] and C-cell lines in vitro[9,10] has been shown to be primarily controlled by extracellular calcium levels. In addition, there is evidence to suggest that cyclic nucleotides can also modulate the release of CT from C-cells, inasmuch as dibutyryl cyclic AMP (DiBcAMP) has been shown to stimulate CT release in vivo[11] and in vitro[12]. We have used the 6.23 rat MTC C-cell line[4,9] to examine the effects of DiBcAMP and DiBcGMP and pharmacologic mediators including histamine, serotonin, prostaglandin E_2 (PGE_2) and acetylcholine (ACh) on the release of CT measured by a sensitive and specific ELISA.

Materials and methods

Chemicals: Histamine, serotonin, PGE_2, ACh, isoproterenol were obtained from Sigma. The calcium channel activator Bay K 8644, was kindly provided by Dr. R. Miller, MDRI, Strasbourg. Rat CT, rat CGRP and rat neurotensin were obtained from Peninsula Labs., U.K.

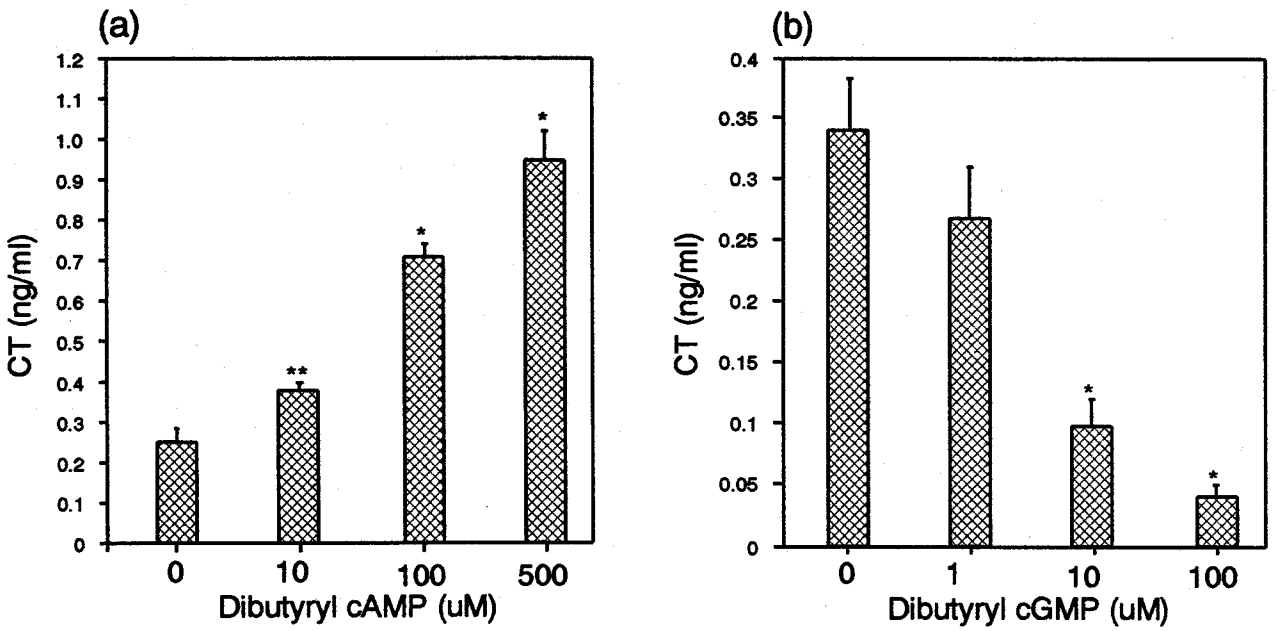
Rat C-cell line: The rat MTC C-cell line, 6.23[4,9] was obtained from the ATCC. The cells were routinely cultured in complete medium: RPMI 1640 containing 20% horse serum, 2 mM 1-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin (Gibco, Paisley, U.K.). For CT release experiments, the cells were trypsinized and 3×10^5 cells were added to the wells of 24 well plates (Costar) in 2 ml of complete medium, which was changed after 1 day. After 2 days, the cells were washed twice in medium without serum and then incubated at 37 °C with test substances freshly made up in medium, or vehicle (medium alone). After 2-h incubation, cell free supernatants (centrifuged at 700 × g for 5 min to remove cells and debris) were collected and frozen at −20 °C. 6.23 cell viability was assessed after each experiment by trypan blue staining and was always > 90%.

Quantitation of CT by ELISA: The anti-CT monoclonals Ab2 and Ab8[13], both of which bind to rat CT[3], were kindly provided by Dr A. Gnocchi, RECORDATI SpA, Milan, Italy. Ab8 was biotinylated using a kit from Amersham U.K., as per the manufacturer's instructions. The CT ELISA has been described in detail previously[13]; briefly, A/2 EIA plates (Costar) were coated with 100 µg/ml (50 µl/well) of Ab2 in pH 9.6 carbonate buffer overnight at room temperature. After washing with PBS containing 0.5% Tween 20, 25 µl of dilutions of 6.23 cell supernatants and 25 µl of biotinylated Ab8 (1:800 final dilution) were added to the wells and incubated for 4 h at room temperature. After washing, a 1:500 dilution of streptavidin-alkaline phosphatase (Amersham, U.K.) was added for 30 min. After washing, the substrate p-nitrophenyl phosphate was added in diethanolamine buffer (pH 9.8). The absorbance was read on a Dynatech MR 700 microplate reader at 405 nM, after 30 min incubation with substrate. CT levels in supernatants were quantitated from standard curves of synthetic rat CT which were included on each ELISA plate.

Results

The ELISA used to quantitate CT in 6.23 cell supernatants was highly sensitive, detecting as little as 30 pg/ml of rat CT. Since 6.23 cells also contain CGRP and neurotensin (NT) that are released concomitantly with CT[7,14], we tested the specificity of the CT ELISA. The assay was highly specific for CT, requiring 1 µg/ml of rat CGRP or NT to produce an optical density reading equivalent to that of ~ 70 pg/ml of rat CT. It has been shown previously that CT, CGRP and NT are released in approximately equimolar amounts from 6.23 cells[7,14], therefore it is unlikely that CGRP or NT in 6.23 cell supernatants would significantly affect the quantitative CT ELISA in the present experiments.

The effects of DiBcAMP and DiBcGMP on CT release from 6.23 cells are shown in the figure. DiBcAMP stimulated CT release in concentration-dependent fashion



The effects of DiBcAMP and DiBcGMP on CT release from 6.23 rat MTC C-cells. 6.23 cells were incubated for 2 h in the presence of (*a*) DiBcAMP or (*b*) DiBcGMP or with medium alone as control. CT secretion into supernatants was measured by ELISA. The results shown are the mean ± SD for duplicate 6.23 cell supernatants, assayed in triplicate in the quantitative CT ELISA. ** $p < 0.05$, * $p < 0.01$ compared to control values (Student's paired t-test).

and at the highest concentration used (500 μM), a four-fold stimulation of CT release was observed (fig., a). In contrast to the stimulatory effect of DiBcAMP, DiBcGMP was shown to be a potent, concentration dependent inhibitor of CT release, reducing CT release by ~ 70% at 10 μM and by ~ 90% at a concentration of 100 μM (fig., b).

We also examined the effects of a number of pharmacological mediators on CT release from 6.23 cells. As shown in the table, histamine and isoproterenol were potent stimulators of CT release (approximately 3-fold and 2-fold, respectively) at the highest, non-cytotoxic concentrations used. PGE_2 and the calcium channel agonist Bay K 8644 also increased CT release approximately 1.5 to 2-fold, while ACh and serotonin had no significant effect on CT release at the highest non-cytoxic concentrations used. In other experiments (not shown), we found no significant effect of the hormones testosterone (300 ng/ml), B-oestradiol (10 ng/ml) or hydrocortisone (1 μM) on CT release from 6.23 cells.

The effect of pharmacologic mediators on CT release from 6.23 C-cells. 6.23 cells were incubated for 2 h in the presence of compounds at the concentration shown. CT concentrations in cell free supernatants were quantitated by ELISA. The highest non-cytotoxic concentration of each compound was determined by trypan blue exclusion of 6.23 cells at the end of each experiment. The results shown are the mean ± SEM for at least 3 separate experiments. ** $p < 0.05$, * $p < 0.01$ compared to controls (Student's paired t-test)

Compound (concentration)	CT released (ng/ml)
Medium control	0.28 ± 0.04
Isoproterenol (1 μM)	0.63 ± 0.04*
Bay K 8644 (10 μM)	0.48 ± 0.04**
PGE_2 (1 μM)	0.52 ± 0.02**
ACh (100 μM)	0.30 ± 0.05
Histamine (100 μM)	0.78 ± 0.04*
Serotonin (100 μM)	0.22 ± 0.05

Discussion

In the present study we have examined the effects of dibutyryl cyclic nucleotide analogues and pharmacological mediators on the release of CT from the 6.23 rat MTC C-cell line. A highly sensitive and specific ELISA, which could detect as little as 30 pg/ml of rat CT, was used to quantitate CT levels in cell supernatants. DiBcAMP was found to increase CT release (~ 4-fold at 500 μM), confirming similar observations by others using 6.23 C-cells[15] and human MTC C-cells[12]. However, we have also shown for the first time that DiBcGMP is a potent inhibitor of CT release from 6.23 C-cells, inhibiting CT release by ~ 90% at 100 μM, the highest non-cytotoxic concentration tested.

It is noteworthy that CT release from 6.23 cells appears to be spontaneous and is modestly influenced by the extracellular calcium concentration. Thus, addition of 1 mM EGTA inhibited CT release from 6.23 cells by 25% (0 mM Ca^{++}), while 6.23 cells in Dulbecco's MEM (1.8 mM Ca^{++}) showed a 20% increase in CT release compared to that seen with RPMI 1640 medium (0.42 mM Ca^{++}). Clearly, the inhibitory effect of DiBcGMP and the stimulatory effects of histamine and DiBcAMP on CT release are much greater than those seen when the extracellular calcium concentration is altered, suggesting that cyclic nucleotide effects are oriented towards the CT secretion mechanism itself. This view

is supported by our preliminary experiments where co-addition of Bay K 8644 and DiBcAMP produces and additive increase in CT release, while co-addition of histamine and DiBcAMP shows no significant increase in CT release compared to each compound when added alone.

Of the pharmacological mediators tested, histamine, isoproterenol and PGE_2 were the most potent stimulators of CT release. These compounds are known to stimulate increases in intracellular cAMP levels, histamine acting via H2-type receptors, isoproterenol via B-adrenergic receptors and PGE_2 via receptors for PGE[16], indicating that these receptor types are expressed on 6.23 cells. In experiments using human MTC C-cells[17], it has been shown that PGE_2 (1 μM) stimulates CT release approximately 5-fold and also that PGF_{2a} (1 μM) had a lesser effect (approximately 2-fold increase), showing that such pharmacological regulation of CT release is not restricted to the rat 6.23 C-cell line. The calcium channel activator Bay K 8644 has been shown previously to increase CT release from 6.23 cells[7,18], by increasing Ca^{2+} entry into the cells. The lack of effect of ACh and serotonin on CT release suggests that functional receptors for these molecules are not present on 6.23 cells.

There is considerable evidence from in vitro and in vivo studies to show that calcium is the major physiological secretagogue for CT release from C-cells[6,8-10,12]. Although several reports have shown that DiBcAMP can stimulate CT release in vitro[9,12,15], in vivo experiments with perfused dog thyroid indicate that cyclic nucleotides have only a minimal effect on CT release alone, but augment calcium stimulated CT release[11]. Modulation of CT release by mediators such as histamine from parafollicular mast cells[19] and PGE from macrophages[16] may occur during inflammatory reactions in the thyroid due to autoimmune disease and in medullary thyroid carcinoma[20]. In addition, the thyroid gland is richly innervated by both adrenergic and cholinergic nerves which control blood flow and the activity of follicular cells at which they terminate[19]. However, there is no evidence for nervous control of C-cell activity, which is perhaps not surprising since C-cells are scattered singly or in small groups in the thyroid[19]. However, it is possible that adrenergic stimulation of CT release occurs during stress reactions when circulating levels of adrenalin and noradrenalin are increased.

In conclusion, the results of this study demonstrate that cAMP and cGMP have opposing effects on CT release and that certain pharmacologic mediators can also modulate CT release from 6.23 C-cells. This cell line is clearly a useful in vitro model system for assessing the mechanisms involved in regulating CT release.

1 Copp, D. H., Cameron, E. C., Cheney, B. A., Davidson, A. G. F., and Henze, K. G., Endocrinology *70* (1962) 638.
2 Austin, L. A., and Heath, H., N. Engl. J. Med. *304* (1981) 269.
3 Scopsi, L., Arias, J., Racchetti, G., Fossati, G. L., and Galante, Y. M., Histochemistry *88* (1988) 113.
4 Zeytinoglu, F. N., DeLellis, R. A., Gagel, R. F., Wolfe, H. J., and Tashjian, A. H., Endocrinology *107* (1980) 509.
5 Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S., and Evans, R. M., Nature *298* (1982) 240.
6 Heller-Brem, S., Muff, R., Peterman, J. B., Born, W., Roos, B. A., and Fischer, J. A., Endocrinology *121* (1987) 1272.
7 Seitz, P. K., and Cooper, C. W., J. Bone Min. Res. *4* (1989) 129.
8 Austin, L. A., Heath, H., and Go, V. L. W., J. clin. Invest. *64* (1979) 1721.
9 Gagel, R. F., Zeytinoglu, F. N., Voekel, E. F., and Tashjian, A. H., Endocrinology *107* (1980) 516.
10 Fried, R. M., and Tashjian, A. H., J. biol. Chem. *261* (1986) 7669.
11 Laurberg, P., Horm. Metab. Res. *20* (1988) 261.
12 Gautvik, K. M., and Tashjian, A. H., Horm. Metab. Res. *6* (1974) 70.
13 Racchetti, G., Fossati, G., Comitti, R., Putignano, S., and Galante, Y. M., Molec. Immun. *24* (1987) 1169.
14 Zeytinoglu, F. N., Gagel, R. F., DeLellis, R. A., Wolfe, H. J., Tashjian, A. H., Hammer, R. A., and Leeman, S. E., Lab. Invest. *49* (1983) 453.
15 Hishikawa, R., Fukase, M., Yamatani, T., Kadowaki, S., and Fujita, T., Biochem. biophys. Res. Comm. *132* (1985) 424.
16 Melmon, K. L., Rocklin, R. E., and Rosenkranz, R. P., Am. J. Med. *71* (1981) 100.
17 Roos, B. A., Bundy, L. L., Miller, E. A., and Deftos, L. J., Endocrinology *97* (1975) 39.
18 Hishikawa, R., Fukase, M., Takenaka, M., and Fujita, T., Biochem. biophys. Res. Comm. *130* (1985) 454.
19 Ahren, B., Endocr. Rev. *7* (1986) 149.
20 Deftos, L. J., and Roos, B. A., in: Bone and Mineral Research, p. 267. Ed. W. A. Peck. Elsevier 1989.

0014-4754/91/101067-03$1.50 + 0.20/0