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Radioreceptor assay for formulations of salmon calcitonin

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Summary

Receptors on cultured human lymphocytes (IM-9) have been shown to bind salmon calcitonin (sCT) specifically. The aim of the present study was to develop an in vitro assay for sCT based on this binding. The assay should fulfil pharmacopoeial requirements for quantitation of biologicals and should be validated against an established in vivo assay of CT. The binding of ^{125}I-sCT was studied as a function of time and concentration of unlabelled sCT and other peptides. Also dissociation of bound sCT and degradation of sCT in the incubation medium were studied. From these studies, conditions for a radioreceptor assay for sCT were chosen. Concentrations from 7×10^{-11} to 2×10^{-9} M or from 1 to 30 mIU ml^{-1} caused a linear reduction of binding of ^{125}I-sCT. This concentration interval was chosen for a three-dose assay. The results obtained with the radioreceptor assay correlated with results from the established in vivo bioassay but were more precise. Since the in vitro method is also less resource demanding than the in vivo bioassay, it is suggested that the former is suitable as a screening method for potency determinations of sCT preparations.

Introduction

Pharmaceutical preparations of salmon calcitonin are expressed in international units (IU). The pharmacopoeial monograph includes a biological assay based on the lowering effect of calcitonin on plasma calcium in vivo (European Pharmacopoeia, 1986). The method requires the use of at least 30 but often considerably more rats to reach the prescribed fiducial limits of the estimated potency corresponding to 64–156% of the stated potency of the preparation (European Pharmacopoeia, 1986). One reason for the lack of precision is the narrow range for the biological effect, lowering the plasma calcium level from a basal concentration of around 10 mg/100 ml to a minimal level of approx. 8 mg/100 ml. To obtain sufficient precision of the potency estimate, combinations of several assays (European Pharmacopoeia, 1971) may be necessary, each requiring at least a day's work by two technicians.

Thus, an alternative in vitro assay for calcitonin would be attractive if it gave relevant but more precise results in a less animal- and time-consuming way. A practical technique for in vitro assays of biologicals is the radioreceptor assay (Sjödin et al., 1982; Sjödin, 1985; Sjödin and Viitanen, 1987; Nederman and Sjödin, 1987). This type of assay more readily reflects the biological activity of the assayed compound than a radioimmunoassay, which measures an immunoreactive determinant which may be located in a part of the molecule that by itself does not evoke biological activity

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(Freychet et al., 1971; De Meyts, 1976; Reichert and Abou-Issa, 1976).

Binding of radioiodinated sCT to receptors has been characterized in a number of tissues (Teitelbaum et al., 1985), such as skeleton (Nicholson et al., 1986), kidney (Fitzpatrick et al., 1969) and brain (Rizzo and Goltzman, 1981). Specific binding of sCT to various continuous cell lines including skeletal cells (Michelangeli et al., 1986), kidney cells (Wohlwend et al., 1985), human lymphoid cells (Marx et al., 1974) and human lung cancer cells (Findlay et al., 1980) has been described. In the present study, the established human IM-9 lymphoblastoid cell line was used to develop a radioreceptor assay for sCT designed to fulfil pharmacopeial requirements for assay validity (European Pharmacopeia, 1971). The method is specific and sensitive, yielding more precise results than the in vivo assay with less consumption of animals and time.

Materials and methods

Hormones

International Reference Preparations of calcitonin, salmon and human for bioassay were from the WHO International Laboratory for Biological Standards, London, U.K. The IRP of sCT has an assigned activity of 4 IU μg^{-1}. The commercial preparation of sCT was Miacalcic from Sandoz, and the preparation of human calcitonin (hCT) was Cibacalcin from Ciba-Geigy, Basle, Switzerland. Regular insulin was Actrapid from Novo Industri AS, Bagsvaerd, Denmark, and human growth hormone (hGH) was Genotropin from Kabi AB, Stockholm, Sweden. Glucagon was obtained from Novo and substance P (SP) from Peninsula Labs, Belmont, CA.

Chemicals

Carrier-free ^{125}I (15 mCi/μg) was from Amersham International, Amersham, U.K., whereas bovine serum albumin (BSA) was from ICN Life Sciences, Cleveland, OH. Pepstatin A and phosphoramidon were obtained from Sigma, St. Louis, MO, while captopril was from Squibb, Princeton, NJ. Sephadex G-25 was purchased from Pharmacia AB, Uppsala, Sweden. All other chemicals were of the highest grade commercially available.

Cells

The continuous human lymphocyte cell line IM-9 (American Type Culture Collection certified cell line 159) was bought from Flow Labs Svenska AB, Stockholm, Sweden. The cells were handled according to local safety guidelines for work with continuous human cell lines. The cells were cultured suspended in an RPMI 1640 medium complemented with fetal calf serum (10%), L-glutamine (2 mM), streptomycin (100 μg ml^{-1}) and penicillin (100 IU ml^{-1}) (Flow Labs Svenska AB). Cells were examined for viability and proliferation by trypan blue exclusion and cell counting in a Coulter Counter, model D Ind (Coulter Electronics, Luton, U.K.), respectively.

Iodination

Salmon calcitonin was labelled by the chloramine-T method (Hunter and Greenwood, 1962). To 1.5 μg of sCT in 30 μl of 0.3 M phosphate buffer (pH 7.5) were added 1 mCi of ^{125}I and 10 μl of chloramine-T (8.8 mM). After mixing for 30 s, the reaction was stopped with 25 μl of sodium metabisulfite (11 mM). The mixture was placed on a Sephadex G-25 column (PD-10) after diluting with 100 μl of 1 M acetic acid with 1% BSA. The column was eluted with acetic acid (1 M) containing 1% BSA. Fractions were collected and counted for radioactivity in a Packard 5780 or 800 C gamma counter (Packard Instruments, Downer's Grove, IL). The fractions eluted with the void volume having highest radioactivity were pooled and rerun on a 60×0.9 cm Sephadex G-25 superfine column eluted in 1 ml fractions with 10 mM acetic acid and 1% BSA before individual experiments. The fractions were counted and screened for binding activity (see binding assay). Two to three fractions displaying the highest binding were pooled and used for further binding experiments. The specific activity was calculated to be approx. 1000 Ci/mmol.

Binding assay

Cells in late log or early plateau phase were centrifuged (150 *g* for 5 min) and washed in a

PBS buffer (NaCl, 8 g l^{-1}; KCl, 0.2 g l^{-1}; Na_2HPO_4, 1.15 g l^{-1}; KH_2PO_4, 0.2 g l^{-1}; with 1% BSA at pH 7.4). Cells were resuspended in this assay buffer at a density of $1-3 \times 10^7$ cells ml^{-1} and preincubated in a shaking water-bath at 30 °C for at least 30 min before incubations with hormones were started. Cell suspension was added to tubes containing ^{125}I-sCT ($2-8 \times 10^{-11}$ M) and buffer with or without various concentrations of unlabelled peptides. In some experiments, the incubation buffer contained a cocktail of enzyme inhibitors (captopril, 2×10^{-5} M; pepstatin A, 10^{-5} M; and phosphoramidon, 10^{-6} M) to minimize degradation of sCT. Incubations were normally carried out at 30 °C in shaking water-baths. At specified times, incubations were stopped by withdrawal of duplicate 100-μl samples which were layered on top of 300 μl of ice-cold PBS with 1% BSA in polypropylene microfuge tubes which were centrifuged at $9000 \times g$ in a Beckman type 12 microcentrifuge (Beckman Instruments, Palo Alto, CA). The resulting pellets were washed three times with iced assay buffer. The radioactivity of the washed pellets and corresponding samples of incubation mixture was counted for 10 min. Total binding was calculated as the percentage of added radioactivity. Non-specific binding was determined in the presence of 7×10^{-7} M of unlabelled sCT, and subtracted from total binding in order to obtain specific binding.

Dissociation of bound sCT

Cells were incubated with and without an excess of sCT at 30 °C. After 2 h, samples of the incubation mixtures were withdrawn and centrifuged for 2 min at $800 \times g$, washed once with assay buffer with and without excess sCT, respectively, and resuspended in a volume of corresponding buffer giving the same cell density as in previous incubation and incubated for an additional 2.5 h at 30 °C. Aliquots were taken at specified times and treated as described above for detection of cell-bound radioactivity.

Binding specificity

These experiments were performed in a similar way to the binding assays but in addition to tubes with various dilutions of sCT, tubes with different concentrations of other peptides, known to interact with receptors on IM-9 cells, were included. Incubations were carried out at 30 °C for 2 h.

Trichloroacetic acid precipitability of ^{125}I-sCT following incubation

Incubations were carried out at 7 or 30 °C. At specified times, duplicate 100-μl aliquots of the incubation supernatants were layered on 300 μl of ice-cold 20% trichloroacetic acid in microfuge tubes and spun at $9000 \times g$ for 30 s. The resulting pellets were washed 3 times with 20% trichloroacetic acid. The radioactivities of the pellets were counted and compared with total radioactivity of the corresponding aliquots of supernatants.

Potency determinations

Three concentrations of unlabelled sCT were chosen from the linear portion of the ^{125}I-sCT competition curve. Each assay usually included three separately prepared but identical incubation series, each with tubes containing standard and test preparations of sCT in these three concentrations. In addition, tubes without unlabelled sCT for determination of maximal binding and tubes with excess sCT (7×10^{-7} M) for non-specific binding were included in each series.

Results, normally from three series of incubations carried out in a single experiment, were employed for calculation of the relative potency of the test preparations in terms of the standard preparation, with the concentration of unlabelled ligand as dose and the specific binding as response. Estimated potency was calculated by analysis of variance for a three-dose, or in a few experiments for a two-dose, assay according to pharmacopoeial principles (European Pharmacopoeia, 1971). The statistical weight is defined as the reciprocal value of the variance of the log potency estimate (European Pharmacopoeia, 1971). The index of precision is calculated by dividing the standard deviation of responses by the slope of the log dose response relationship (Loraine and Bell, 1966). The weighted log potency estimates were used for combination of results from separate assays, after being tested for homogeneity (European Pharmacopeia, 1971).

In vivo assay of sCT

Test preparations of sCT or hCT were assayed against International Reference Preparations by the method described in the European Pharmacopoeia (1986). In short, 50–80 male Sprague-Dawley rats (Alab, Sollentuna, Sweden) weighing around 100 g were divided into six to eight groups. Three or four doses of test and standard preparations (2–50 mIU of sCT/animal) were given intravenously. Exactly 1 h after injection the animals were anaesthetized with ether. Blood samples were obtained into heparinized tubes by cardiac puncture or retroorbital sampling after which the animals were killed. Plasma was analyzed for calcium concentration by atomic absorption spectrophotometry. The relationships between calcium concentrations and logarithms of the doses were calculated.

Results

Time course for binding of ^{125}I-sCT

Binding of ^{125}I-sCT at 30 °C increased during the first 2 h (Fig. 1). Specific binding amounted to 70% of total binding at 2 h and declined thereafter (not shown). 82% of total radioactivity in solutions of ^{125}I-sCT could be precipitated by 20% trichloroacetic acid. After incubation with cells at 30 °C for 2 h, 42% of added radioactivity was precipitated by trichloroacetic acid, suggesting 50% degradation of tracer during the incubation period. Addition of captopril (2×10^{-5} M), pepstatin A (10^{-5} M) and phosphoramidon (10^{-6} M) did not markedly affect binding of ^{125}I-sCT (not shown).

When cells were centrifuged and resuspended in fresh buffer without ^{125}I-sCT after an initial 2 h incubation with tracer, cell-bound radioactivity dissociated only slowly. The rate of dissociation was not accelerated by addition of an excess of unlabelled hormone (Fig. 1).

Inhibition of ^{125}I-sCT binding by unlabelled sCT

Competition for binding of ^{125}I-sCT by unlabelled hormone was observed at concentrations of sCT from 7×10^{-11} M. Half-maximal inhibition of specific binding was achieved at around

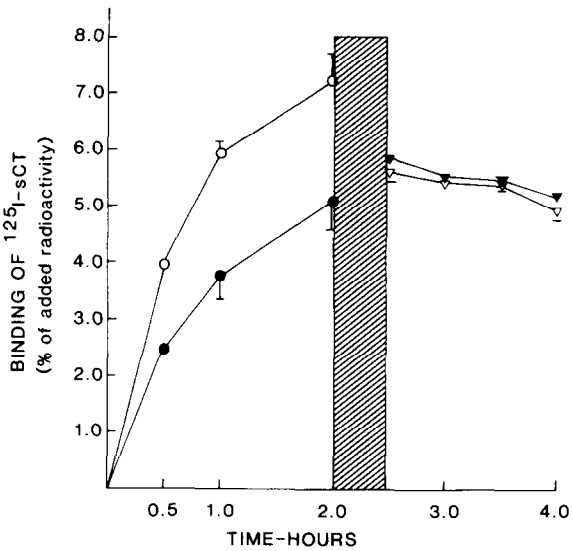

Fig. 1. Time-course of ^{125}I-sCT binding (○, total binding; ●, specific binding) and dissociation in assay buffer with (▲) or without (△) an excess of unlabelled sCT. 2.1×10^7 cells ml^{-1} were incubated at 30 °C with 5×10^{-11} M ^{125}I-sCT in the presence (non-specific binding) or absence (total binding) of 7×10^{-7} M unlabelled sCT for 2 h. The duration of centrifugation, washing and resuspension of cells was 0.5 h indicated by the hatched area. Mean values and range (when range exceeded the size of the symbols) from two separate incubations are indicated.

2×10^{-10} M and 90% inhibition was seen at an sCT concentration of 7×10^{-9} M (Fig. 2).

Specificity of binding

Glucagon, hGH, insulin and substance P did not affect binding of ^{125}I-sCT to IM-9 cells (Table 1).

Potency determinations

Using concentrations of unlabelled sCT, producing a linearly log dose related inhibition of tracer binding, results such as those in Fig. 3 were obtained when a commercial preparation of sCT was assayed against the International Reference Preparation. Using analysis of variance for a three-dose assay, the commercial preparation was found to have 102% of the activity of the IRP with limits of error of 87–115% ($p = 0.95$). The statistical weight was 1234 and the index of precision was 0.070 indicating a high precision. When the

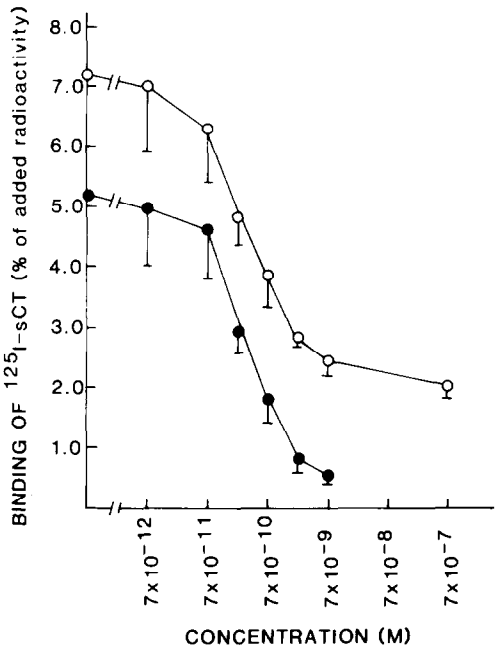

Fig. 2. Inhibition of total (○) and specific (●) binding of ^{125}I-sCT ($2-8\times10^{-11}$ M) by increasing concentrations of unlabelled sCT. Incubations with $2-3\times10^{7}$ ml^{-1} IM-9 cells at 30 °C for 2 h. Points represent means of seven separate experiments. Vertical bars denote S.E.

assay was repeated, the second potency estimate of the same preparation was 113% (Table 2).

In a series of eight assays, in which the standard and test preparations were identical, an average potency estimate obtained (non-weighted) was 98% ($n = 8$). The interassay coefficient of variation was 21% (Table 3) and the mean statistical weight was 572 ± 176 (S.E.). When various dilu-

TABLE 1

Specificity of binding of ^{125}I-sCT to IM-9 cells

Peptide	Concentration (M)	Total binding (% of control)
sCT	6.9×10^{-12}	94 ± 1
	6.9×10^{-11}	90 ± 6
	6.9×10^{-10}	54 ± 6
	6.9×10^{-9}	37 ± 2
	6.9×10^{-7}	30 ± 5
Glucagon	2.8×10^{-7}	95 ± 8
	2.8×10^{-6}	101 ± 3
hGH	2.9×10^{-8}	105 ± 2
	2.9×10^{-7}	113 ± 6
Insulin	3.1×10^{-7}	105 ± 8
	3.1×10^{-6}	109 ± 5
SP	4×10^{-7}	95 ± 2
	4×10^{-6}	95 ± 2

Binding of $2-5\times10^{-11}$ M of ^{125}I-sCT to $2-3\times10^{7}$ cells ml^{-1} after incubation for 2 h at 30 °C in the presence of various peptides at specified concentrations. Control binding is binding of ^{125}I-sCT in the absence of unlabelled peptides. Means and range from two separate experiments.

TABLE 2

Potencies of sCT preparations determined against the International Reference Preparation using radioreceptor assay (R) or rat hypocalcemia assay (HC)

Preparation	Assay	Potency (% of standard)	Limits of error ($p = 0.95$) (%)	Statistical weight	Index of precision (λ)
1	R	102	87–115	1234	0.070
1	R	113	86–116	1088	0.073
Combination	R	107	91–110	2322	
1	HC	97	65–155	113	0.326
2	R	108	72–139	320	0.145
2	HC	118	50–202	44	0.486
2	HC	102	75–134	251	0.218
Combination	HC	104	77–131	295	
3	R	101	75–134	370	0.090
3	R	100	76–131	478	0.079
Combination	R	100	85–118	848	
3	HC	127	82–122	555	0.119
3	HC	105	62–162	94	0.291
Combination	HC	123	83–120	650	

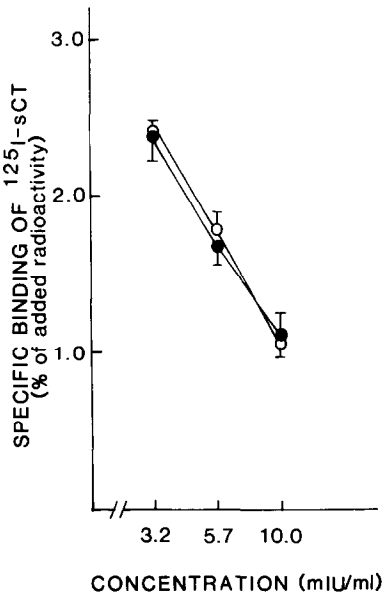

Fig. 3. Radioreceptor assay of a commercial preparation of sCT (●) against the International Reference Preparation of sCT (○). ^{125}I-sCT (5×10^{-11} M) and unlabelled sCT at specified concentrations were incubated with 2.3×10^{7} cells ml^{-1} for 2 h at 30 °C. Each point represents the mean of four separate incubations. Vertical bars denote S.E. Estimated potency of the test preparation was 102% of the standard with limits of error of 87–115% ($p = 0.95$).

tions of the preparation were assayed, the deviations of estimated potency from nominal potency were moderate (Table 3).

TABLE 3

Estimated potencies of different dilutions of an sCT preparation determined with radioreceptor assay

Nominal potency (%)	Estimated potency (%)	Limits of error ($p = 0.95$) (%)	Statistical weight	Index of precision (λ)
100	89	83–120	771	0.076
100	75	77–129	525	0.074
100	76	75–132	330	0.114
100	107	72–138	334	0.095
100	131	75–134	311	0.118
100	86	88–113	1724	0.051
100	95	75–134	410	0.085
100	121	64–157	170	0.129
80	82	85–118	972	0.067
80	89	80–125	547	0.090
50	44	55–184	86	0.121

TABLE 4

Estimated potencies of different batches of sCT and hCT determined with the in vivo rat hypocalcemia assay

Preparation	Estimated potency (%)	Limits of error ($p = 0.95$) (%)	Statistical weight	Index of precision (λ)
sCT-a	54	67–150	134	0.248
	54	58–174	73	0.306
	67	64–157	107	0.309
	89	79–126	398	0.162
	85	82–122	545	0.138
Combination	79	88–114	1257	
sCT-b	118	50–202	44	0.486
	102	75–134	251	0.218
Combination	104	77–131	295	
hCT-a	130	60–166	85	0.340
	97	49–205	42	0.580
	64	57–176	68	0.379
	162	31–326	15	
	128	57–176	70	0.408
	111	57–176	67	0.487
Combination	106	78–128	347	
hCT-b	152	51–196	48	0.352
	104	67–149	139	0.271
Combination	115	71–140	187	

In a series of in vivo assays of preparations of sCT or hCT against their respective international reference preparations (Table 4), the corresponding coefficients of variation were 24% ($n = 5$) and 29% ($n = 6$), respectively and the mean statistical weight was 139 ± 38 (S.E.). The average index of precision was 0.335, indicating a relatively low precision.

Some batches of sCT were assayed against the IRP with both the radioreceptor assay and the in vivo method (Table 2). The potency readings with both methods were similar.

Discussion

The present study confirms earlier results reported by Marx et al. (1974) demonstrating the existence of specific binding sites for CT on cultured human lymphocytes. The degree of specific binding and the binding capacity were similar to those reported by Marx et al. (1974) as was the

slow rate of dissociation of bound ^{125}I-sCT from lymphocytes. This phenomenon has also been reported from studies on kidney cells (Chao and Forte, 1983) and osteoclasts (Nicholson et al., 1986). The concentration of sCT required for 50% inhibition of specific binding of ^{125}I-sCT in the present study (2×10^{-10} M) was similar to the concentration (3×10^{-10} M) of sCT causing 50% binding inhibition in another lymphoid cell line (Marx et al., 1974), indicating sites with similar affinity for sCT.

The binding sites were specific for CT, since they did not bind glucagon, hGH, insulin or SP, of which the latter three have been reported to bind to receptors on IM-9 cells (De Meyts, 1976; Payan et al., 1986).

Relative biological potencies of various synthetic sCT analogues have been reported to be similar when tested for adenylate cyclase activating activity and receptor binding in T47D human breast cancer cells or for induction of hypocalcemia in the rat (Findlay et al., 1985), suggesting that affinity for sCT receptors on such cells reflects biological activity in vitro and in vivo.

The radioreceptor assay described in the current article is to our knowledge the first one which has been designed to fulfil pharmacopeial requirements for assay validity (European Pharmacopeia, 1971) and which has been validated in direct comparisons with the established in vivo assay. The potency readings were similar with both assays. Theoretically, derivatives of sCT may display affinity for receptors but not biological activity. The present results do not suggest that pharmaceutical formulations of sCT contain such components.

The present radioreceptor assay displays a high degree of sensitivity and specificity for CT. However, the results obtained with the present radioreceptor assay are less precise than previous data we have obtained with similar assays for glucagon (Sjödin, 1985), insulin (Sjödin and Viitanen, 1987) and hGH (Nederman and Sjödin, 1987). A major reason for this discrepancy is a considerably lower degree of specific binding obtained in the experiments with sCT compared with the studies using other ligands. Nevertheless, the present radioreceptor assay for sCT appears to yield more precise results than what has been obtained with the in vivo assay. Furthermore, the receptor assay requires no animals and is less time consuming than the in vivo assay.

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