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SALMON CALCITONIN BINDING AND STIMULATION OF CYCLIC AMP GENERATION IN RAT SKELETAL MUSCLE

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Salmon calcitonin potently competes for amylin binding sites in rat brain and has amylin-like actions upon glucose metabolism in rat muscle. We report here that [^{125}I]-salmon calcitonin binds to rat hindlimb muscle membranes with high affinity (Kd = 0.47 pM). Binding was inhibited by rat amylin (Ki = 2 nM), rat αCGRP (Ki = 8 nM), rat ßCGRP (Ki = 11 nM), and rat calcitonin (Ki = 64 nM). Binding was maximal when measured in a hypotonic NaHepes buffer, and was significantly reduced in affinity when salts of Mg^{++}, Ca^{++}, Na^{+} or K^{+} were present. Incubation of rat hindlimb muscle membranes with salmon calcitonin at concentrations of 10 pM and above stimulated cyclic AMP generation. These results describe a skeletal muscle binding site which may mediate some of the actions of exogenous salmon calcitonin and of endogenous amylin and related peptides upon skeletal muscle fuel metabolism.

Amylin is a 37 amino acid peptide hormone that is released from the pancreatic beta cell along with insulin (1, 2). Both amylin and the structurally-related neuropeptide CGRP inhibit the ability of insulin to stimulate glucose incorporation into glycogen in skeletal muscle (3-5). Recently, binding sites with high affinity for amylin (Kd = 27 pM) were identified in rat brain (6). These sites had high affinity for rat amylin, lower affinity for rat CGRPs, and very low affinity for rat calcitonin. A notable feature of these binding sites was their high affinity for sCT, which shares only a 34% amino acid sequence identity with rat amylin. Like amylin, sCT strongly inhibited insulin-stimulated glucose incorporation into glycogen in rat soleus muscle (6). Thus, in addition to being a potent calcitonin agonist in bone, sCT is a potent mimetic of amylin's metabolic actions in rat skeletal muscle.

In an effort to characterize the receptors in skeletal muscle through which sCT alters glucose metabolism, we measured the binding of radioiodinated sCT to rat skeletal muscle membranes. In addition, we determined whether sCT was able to stimulate adenylyl cyclase activity in rat muscle membranes.

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Abbreviations: sCT, salmon calcitonin; CGRP, calcitonin gene-related peptide; VIP, vasoactive intestinal peptide.

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MATERIALS AND METHODS

Membrane Preparation. Male rats (Harlan-Sprague Dawley, 200-250 g) were fasted for 4 - 20 hours prior to decapitation and collection of lower hind limb muscles or kidneys. Muscles were frozen in liquid nitrogen for adenylyl cyclase experiments or were processed immediately for binding studies. Muscles or kidney cortices were homogenized with a Polytron homogenizer in ice-cold 20 mM Hepes buffer (pH adjusted to 7.4 with NaOH). Muscle homogenates were centrifuged at 120 x g for 10 min at 4°C and the supernatant was passed through a 500 µm nylon filter to remove connective tissue. Membranes were collected by centrifugation for 15 min at 48,000 x g and were washed twice by resuspending in fresh buffer and centrifuging. Membrane pellets were resuspended in Hepes buffer and stored at -70°C until use.

Receptor Binding. Membranes from 7 mg original wet weight of tissue were incubated in triplicate polypropylene tubes for 90 min at 23°C with [^{125}I]sCT (iodotyrosyl, 2000 Ci/mmol; Amersham Corp., Arlington Heights, IL) in 20 mM Hepes buffer containing 1 mg/ml bacitracin, 1 mg/ml bovine serum albumin, and 0.2 mM phenylmethylsulfonyl fluoride. Preliminary experiments established that specific binding reached a steady-state following this incubation period and increased linearly in proportion to the amount of muscle membrane present up to at least 0.25 mg membrane protein/ml. Incubations were started with the addition of membrane and were stopped by filtration through glass fiber filters presoaked in 0.3% polyethyleneimine and prewashed with 5 ml cold phophate-buffered saline. After harvesting, filters were washed with 15 ml cold phosphate-buffered saline. There was no measurable specific binding of [^{125}I]sCT to filters. Non-specific binding was measured in the presence of 10^{-7} M unlabeled sCT. Competition curves were generated by measuring binding of 0.4 pM [^{125}I]sCT in the presence of 10^{-13} to 10^{-6} M unlabeled peptide. At a concentration of 0.4 pM [^{125}I]sCT, total binding = 801 ± 33 cpm (n = 10), nonspecific binding = 92 ± 14 cpm (n = 10). Protein was measured by the method of Bradford (7) with bovine serum albumin as standard.

Cyclic AMP. Frozen muscles were homogenized with a Polytron homogenizer in 50 mM Hepes buffer (4°C, pH 7.4) containing 250 mM sucrose. Membranes were collected by centrifugation for 15 min at 25,000 x g and were resuspended in cold buffer. sCT in 50 mM Hepes buffer (25 µl) was combined with reaction solution (25 µl) containing 2 mM phosphocreatine, 50U creatine kinase/ml, 10 mM $MgCl_2$, 3 mM ATP, 40 µM GTP, 2 mM isobutylmethylxanthine, and 0.3 TIU aprotinin/ml in 50 mM Hepes. Muscle homogenate (50 µg protein/50 µl buffer) was added and the mixture was incubated for 25 min at 37°C then heated to 80°C in a water bath. Supernatants were assayed for cyclic AMP using a scintillation proximity assay (Amersham Corp., Arlington Heights, IL).

Data Analysis. Rectangular hyperbolae were fitted to saturation binding and cyclic AMP dose-response data using a nonlinear least-squares curve-fitting program (Inplot, GraphPAD Software, San Diego). Sigmoid curves (4 -parameter logistic equation) were fitted to competition binding data, and resulting IC_{50} values were converted to apparent inhibition constants (K_is) using the Cheng-Prusoff equation. Results are reported as means ± SEs.

RESULTS

Saturable binding of [^{125}I]sCT. Binding of [^{125}I]sCT to skeletal muscle membranes (0.1 mg membrane protein/ml) was measured in a hypotonic buffer (20 mM NaHepes, pH 7.4) which yielded maximal levels of specific binding. Saturable binding of [^{125}I]sCT was observed at concentrations varying from 0.2 to 20 pM, with nonspecific binding measured in the presence of 10^{-7} M sCT (Fig.1). Scatchard analysis of specific binding yielded a dissociation constant (Kd) = 0.47 ± 0.02 pM and binding site density (Bmax) = 4.3 ± 0.4 fmol/mg protein (n = 4 experiments). To establish that high affinity binding constants obtained at low radioligand concentrations were unaffected by sequestration of ligand by membranes, saturation isotherms were generated with the assay volume increased to 4.5 mls and the membrane concentration further reduced to 0.02 mg protein/ml. Binding constants measured under these assay conditions (Kd = 0.48 ± 0.11 pM; Bmax = 4.3 ± 1.1 fmol/mg protein; n = 2 experiments) were similar to

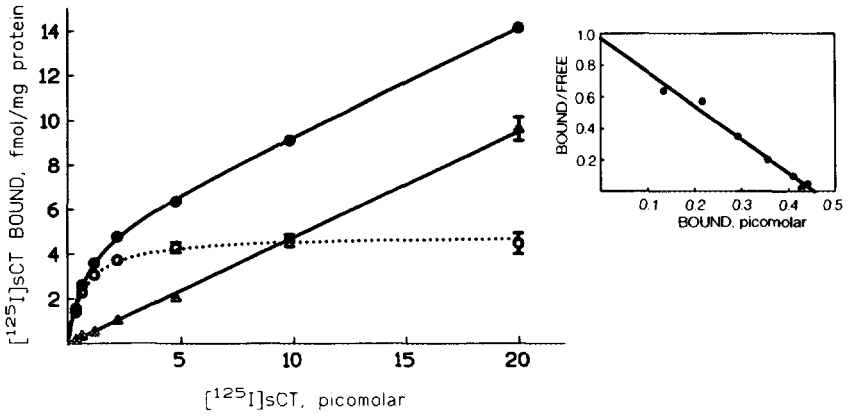

Figure 1. Saturable binding of [^{125}I]sCT to skeletal muscle membranes. Binding of [^{125}I]sCT to muscle membranes was measured in the absence (•) or presence (▲) of 10^{-7} M unlabeled sCT. (o) = specific binding. Each point represents the mean of triplicate determinations from a single experiment, which was repeated 4 times. Inset: Scatchard plot of specific [^{125}I]sCT binding.

those measured at higher membrane concentrations. In contrast to the very high affinity of [^{125}I]sCT binding to rat muscle membranes, Scatchard analysis of [^{125}I]sCT binding to rat kidney cortex membranes yielded a Kd = 60.5 pM and Bmax = 86.5 fmol/mg protein.

Selectivity of binding. sCT potently inhibited [^{125}I]sCT binding to muscle membranes with a Ki = 0.7 ± 0.3 pM (Table 1). Of the structurally-related hormones endogenous to the rat, amylin was the most potent inhibitor tested, while rat αCGRP and rat ßCGRP were somewhat less potent (Fig. 2). Rat calcitonin was 10,000-fold less potent than sCT. [^{125}I]sCT binding was also inhibited by rat VIP (Table 1). Peptides that inhibited binding of [^{125}I]sCT to muscle membranes by less than 50% at 10^{-6} M included rat parathyroid hormone, human glucagon, human secretin, bovine insulin, and substance P.

Effects of ions. Specific binding was maximal in 20 mM NaHepes buffer. Increasing the concentration of NaHepes or addition of salts of Mg^{++}, Ca^{++}, Na^{+} or K^{+} significantly reduced specific [^{125}I]sCT binding (Table 2). Scatchard analysis of the specific binding of [^{125}I]sCT

Table 1. Inhibition of 0.4 pM [^{125}I]sCT binding to rat skeletal muscle membranes, measured in 20 mM NaHepes buffer. Results are means ± SEs from 3-5 separate experiments.

PEPTIDE	Ki ± SE (nM)
sCT	0.0007 ± 0.0003
rat amylin	2.0 ± 0.4
rat αCGRP	8.3 ± 1.2
rat ßCGRP	10.8 ± 3.8
rat calcitonin	64 ± 13
rat VIP	153 ± 90

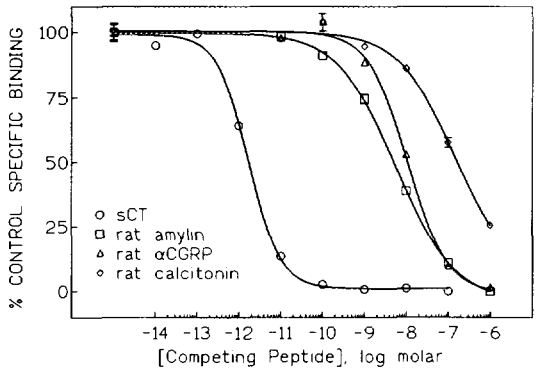

Figure 2. Competition by peptides for [^{125}I]sCT binding to rat muscle membranes. Nonspecific binding was measured in the presence of 10^{-7} M sCT. Results are means ± SEs of triplicate tubes.

measured in 20 mM NaHepes buffer in the presence of 100 mM KCl + 2 mM $MgCl_2$ yielded a Kd = 39 pM and Bmax = 6.4 fmol/mg protein. Thus, the additional ions reduced the affinity but not the binding capacity of the [^{125}I]sCT binding sites in muscle membranes.

Cyclic AMP. Incubation of rat hindlimb skeletal muscle membranes with sCT produced an increase in cyclic AMP generation of up to 250% over background levels of 243 ± 33 pmol cyclic AMP/mg protein/25 min (Fig. 3). For mean values from 4 experiments performed in triplicate, a double hyperbola (two site model) fit the data significantly better than a single hyperbola, with EC50s (95% confidence intervals in parentheses) for the two sites of 15 pM (1 - 31 pM) and 1.6 nM (0.6 - 3.8 nM).

DISCUSSION

These studies provide the first report of sCT binding sites in skeletal muscle membranes and also demonstrate that sCT potently stimulates cyclic AMP generation in muscle membranes.

Table 2. Effects of ions and buffer strength upon [^{125}I]sCT binding to rat muscle membranes. [^{125}I]sCT was present at a concentration of 0.4 pM. Results are means ± SEs of triplicate determinations from a single experiment.

Buffer + ion	% Control
20 mM NaHepes	100
50 mM NaHepes	13.3 ± 1.7
50 mM TrisCl	10.6 ± 0.6
20 mM NaHepes	
+ 0.5 mM $MgCl_2$	39.8 ± 1.6
+ 2.0 mM $MgCl_2$	12.5 ± 0.5
+ 2.0 mM $MgSO_4$	13.7 ± 0.3
+ 2.0 mM $CaCl_2$	11.7 ± 0.5
+ 100 mM NaCl	2.9 ± 0.1
+ 100 mM KCl	3.7 ± 0.3
+ 1.0 mM NaEDTA	94.0 ± 3.2
+ 1.0 mM NaEGTA	87.9 ± 0.9
+ 300 mM sucrose	88.3 ± 3.0

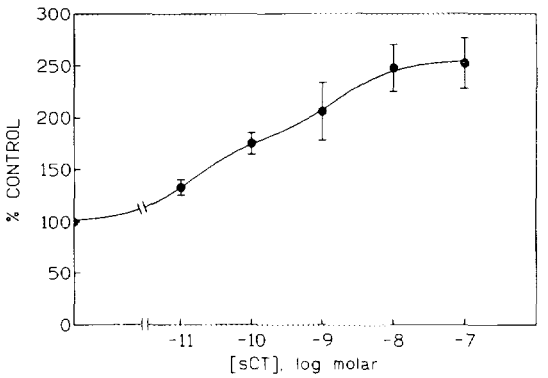

Figure 3. Stimulation of cyclic AMP generation in rat skeletal muscle membranes by sCT. Results are means ± SEs of data from 4 experiments, each performed in triplicate. Control = 243 ± 33 pmol cyclic AMP/mg protein/25 min.

Calcitonin, released from thyroid gland C-cells, is thought to act at calcitonin receptors in bone to inhibit osteoclast-mediated bone resorption. In addition to bone, [^{125}I]sCT binding has been described in several tissues including kidney (8), brain (9), lung (10), lymphocytes (11), and testes (12). Mammalian calcitonins are considerably less effective than sCT at competing for [^{125}I]sCT binding to membranes from several tissues, including rat skeletal muscle (Fig 2). In addition, calcitonin is not normally accessible to sites within the brain, suggesting that an alternative endogenous ligand for these [^{125}I]sCT binding sites may exist. Mammalian tissues have been reported to contain substances that display sCT-like immunoreactivity or biological activity (13-15), but these have not been isolated and it is not known whether these substances could have any relationship to this binding site.

Among the related peptide hormones endogenous to the rat, sCT has a 53% sequence identity (17/32 amino acids) to rat calcitonin and a 34% sequence identity (11/32 amino acids) to rat amylin. Rat calcitonin and sCT have the potential to interact with amylin receptors, depending upon the amino acid residues involved in receptor interactions. Surprisingly, such an interaction appears to hold for sCT at least one amylin binding site population, since amylin binding sites in rat brain have an affinity of approximately 30 pM for both rat amylin and sCT, although they have low affinity (>1μM) for rat calcitonin (6). Thus, some actions of sCT may be mediated by receptors that are activated physiologically by amylin. sCT, like amylin, inhibits insulin-stimulated glucose incorporation into glycogen in rat soleus muscle, while rat calcitonin is considerably less potent at producing this action (6). Recently, sCT was reported to stimulate active Na^+-K^+ transport in isolated skeletal muscle (16). Although amylin is relatively effective at inhibiting [^{125}I]sCT binding to skeletal muscle membranes, available data concerning amylin's high potency in eliciting intracellular responses (5, 17) and studies with antagonists (18) suggest that amylin's effects upon muscle glucose metabolism are mediated by receptors similar to the [^{125}I]amylin binding sites in rat brain. However, these sites have not been readily measurable in the whole muscle membranes used for the experiments reported here.

Binding sites in rat skeletal muscle membranes displayed a significantly higher affinity for [^{125}I]sCT than binding sites in rat kidney. Recently, we have cloned two receptor isoforms

from a rat nucleus accumbens cDNA library that bind [^{125}I]sCT with high affinity (19). Two similar receptors have been cloned from rat hypothalamus (20). These receptor isoforms differ by the presence of a 37 amino acid insert in the extracellular domain between the second and third transmembrane regions, which resulted in a reduction in binding affinity for sCT from 8 pM to 48 pM (19). When expressed in COS-7 cells, activation of either receptor by sCT produced stimulation of adenylyl cyclase activity. Distribution studies indicated that rat skeletal muscle contained mRNA encoding both receptors, although at differing levels (19).

Incubation in the presence of salts of monovalent or divalent cations markedly reduced the affinity of [^{125}I]sCT for binding sites in muscle membranes (Table 2). Cations alter the coupling of receptors to G-proteins and alter the affinity of G-protein coupled receptors (21). Since skeletal muscle contains at least two calcitonin receptor isoforms that potentially couple to multiple G-proteins (22), the effects of ions that alter this coupling are likely to be complex. Also, calcitonin receptors cloned from human breast carcinoma are reported to generate intracellular signals in response to extracellular calcium (23), indicating an additional novel interaction with divalent cations.

In summary, rat skeletal muscle contains binding sites with high affinity for sCT that appear to be coupled to stimulation of adenylyl cyclase activity. These studies are consistent with previously reported results indicating that mRNA encoding sCT receptors is present in rat skeletal muscle (19). These binding sites may mediate some of the actions of exogenously administered sCT, as well as endogenously released amylin, on skeletal muscle metabolism.

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REFERENCES

1. Cooper, G.J., Day, A.J., Willis, A.C., Roberts, A.N., Reid, K.B. and Leighton, B. (1989) Biochim. Biophys. Acta. 1014, 247-58.
2. Rink, T.J., Beaumont, K., Koda, J. and Young, A. (1993) Trends Pharmacol. Sci. 14, 113-118.
3. Cooper, G.J.S., Leighton, B., Dimitriadis, G.D., Parry, B.M., Kowalchuk, J.M., Howland, K., Rothbard, J.B., Willis, A.C. and Reid, K.B. (1988) Proc. Natl. Acad. Sci. USA. 85, 7763-6.
4. Leighton, B. and Cooper, G.J. (1988) Nature. 335, 632-5.
5. Young, A.A., Gedulin, B., Wolfe-Lopez, D., Greene, H., Rink, T.J. and Cooper, G.S. (1992) Am. J. Physiol. 263, E274-E281.
6. Beaumont, K., Kenney, M.A., Young, A.A. and Rink, T.J. (1993) Molec. Pharmacol. 44, 493-497.
7. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
8. Morel, F., Ombert-Teboul, M. and Chabardes, D. (1981) Ann. Rev. Physiol. 43, 569-581.
9 Fischer, J.A., Tobler, P.H., Kaufman, M., Born, W., Henke, H., Cooper, P.E., Sagar, S.M. and Martin, J.B. (1981) Proc. Natl. Acad. Sci., USA. 78, 7801-7805.
10. Fouchereau-Peron, M., Moukhtar, M.S., Benson, A.A. and Milhaud, G. (1981) Proc. Natl. Acad. Sci. USA. 78, 3973-3975.
11. Body, J.J., Glibert, F., Nejai, S., Fernandez, G., Van Langendonck, A. and Borkowski, A. (1990) J. Clin. Endocrinol. Metab. 71, 675-681.
12. Chausmer, A.B., Stevens, M.D. and Severn, C. (1982) Science. 216, 735-736.

13. Fischer, J.A., Tobler, P.H., Henke, H. and Tschopp, F.A. (1983) J. Clin. Endocrinol. Metab. 57, 1314-1316.
14. Sexton, P.M. and Hilton, J.M. (1992) Brain Res. 596, 279-284.
15. Shah, G.V., Deftos, L.J. and Crowley, W.R. (1993) Endocrinology. 132, 1367-1372.
16. Andersen, S.L.V. and Clausen, T. (1993) Am. J. Physiol. 264, C419-C429.
17. Weiel, J., Hull-Ryde, E., Irsula, O. and Frangakis, C. (1993) Diabetes. 42S1, 129A.
18. Beaumont, K., Kenney, M.A., Wolfe-Lopez, D., Albrecht, E.A. and Gaeta, L.S.L. (1993) Diabetes. 42S1, 215A.
19. Albrandt, K., Mull, E., Brady, E.M.G., Herich, J., Moore, C.X. and Beaumont, K. (1993) FEBS Letters. 325, 225-232.
20. Sexton, P.M., Houssami, S., Hilton, J.M., O'Keeffe, L.M., Center, R.J., Gillespie, M.T., Darcy, P. and Findlay, D.M. (1993) Mol. Endocrinol. 7, 815-821.
21. Rodbell, M. (1980) Nature. 284, 17-22.
22. Force, T., Bonventre, J.V., Flannery, M.R., Gorn, A.H., Yamin, M. and Goldring, S.R. (1992) Am. J. Physiol. 26, F1110-F1115.
23. Stroop, S.D., Moore, E.E., Thompson, D.L. and Kuestner, R.E. (1993) J. Bone Min. Res. 8S1, S147.