A NOVEL EFFECT OF SALMON CALCITONIN ON IN VITRO Ca-UPTAKE BY RAT BRAIN HYPOTHALAMUS: THE REGIONAL AND HORMONAL SPECIFICITIES

Masao KOIDA, Yoshinobu YAMAMOTO, Hiromichi NAKAMUTA, Junko MATSUO, Masaharu OKAMOTO and Toshihiko MORIMOTO
Department of Pharmacology, Faculty of Pharmaceutical Sciences, Nagasaki University, Nagasaki 852, Japan

Jay K. SEYLER and Ronald C. ORLOWSKI
Armour Pharmaceutical Company, Kankakee, Illinois 60901, U.S.A.

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Abstract—It was found that salmon calcitonin-1 (sCT) inhibited in vitro 45Ca2+-uptake by rat brain hypothalamus blocks in a dose-dependent manner. The minimum effective concentration was estimated to be 10 nM or less. The effect appeared to be specific to the hypothalamus and was not observed with the pons plus medulla oblongata or the cerebral cortex. Two C-terminal fragments of the fish hormone, sCT (10-32) and sCT (22-32), and porcine calcitonin failed to inhibit the ion-uptake though tested in concentrations abolishing 125I-sCT binding to these brain tissues, indicating that the whole structure of sCT is essential for the inhibitory effect but not for the binding. Another finding to be noted was a possible dependency of this effect on the integrity of the cell membrane structure. A crude synaptosomal fraction subsequently prepared from sCT-exposed hypothalamus blocks exhibited a decreased uptake of 45Ca2+, while a corresponding fraction from unexposed tissue did not respond to the hormone. These characteristics of this novel in vitro effect of sCT suggest its possible relevancy to the anorectic effect which also appears to be specific to the fish hormone.

Recently, the calcitonin-specific binding sites were characterized in the rat brain (1-4) and based on the regional and hormonal specificities of the sites, a functional relevancy to some in vivo effects of the hormone (e.g. anorectic) (5, 6) has been suggested. Thus far, an elegant study by Miyahara and Oomura (7) seems to have succeeded in pinpointing the hypothalamic glucoreceptor as one of the sites. Peripherally, calcitonin regulates the blood Ca2+-level by changing the Ca-metabolism of bone and kidney tissues (8). An assumption was made that also in the central nervous system, the hormone would exert such behavioral effects by directly affecting the ion-metabolism, and using tissue blocks obtained from some brain regions, its effect on 45Ca2+-uptake was examined in vitro. We report herein that sCT specifically modified the ion-uptake of the rat hypothalamus, and

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a possible relevancy of this in vitro effect to the anorectic effect was suggested.

**MATERIALS AND METHODS**

Salmon calcitonin-I (sCT), sCT (10–32), sCT (22–32) and porcine calcitonin (pCT) were synthesized and/or isolated by the Armour Pharmaceutical Company (Kankakee, Ill., U.S.A.). 45CaCl2 (30 Ci/g) and carrier-free Na125I were purchased from the Radiochemical Centre (Amersham, England). Protosol was purchased from the New England Nuclear (Boston, Ma., U.S.A.). All other reagents were of analytical grade.

**Tissue preparation and measurement of 45Ca2+-uptake:** Wistar male rats (200–250 g) were killed by decapitation, and three regions (hypothalamus, pons plus medulla oblongata and cerebral cortex) were dissected out as described by Glowinski and Iversen (9). The tissue was sliced and cut finely to blocks. Blocks corresponding to less than one third of each region were transferred into a polypropylene tube with 5.0 ml of a modified Krebs Ringer bicarbonate buffer (KRB: 118 mM NaCl, 3.7 mM KCl, 1.8 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 20 mM NaHCO3, 11 mM glucose) and, unless otherwise described, the tube was incubated at 37°C for 70 min under gassing with an O2-CO2 mixture (95%; 5%). The pH of the buffer was 7.4±0.2 during incubation. At the 5th min of incubation, the tube received 50 µl of KRB containing a peptide to give the final concentration indicated for each experiment, and at the 10th min, 1 µCi of 45CaCl2 in 0.10 ml KRB was added. Incubation was terminated by filtration on a moist Millipore filter, tissue blocks collected on the filter, washed 5 times with 2 ml of ice-cold Ca2+-free KRB containing 20 mM Tris-HCl (pH 7.4) instead of 20 mM NaHCO3, pelleted, and resuspended in the buffer. The suspension (0.2 to 0.4 mg protein in 0.10 ml per tube) was incubated at 37°C for 5 min and mixed with 0.20 ml of buffer containing 0.40 µCi 45CaCl2. Incubation was continued for 2 min and terminated by adding 1.0 ml of ice-cold Ca2+-free buffer containing 5 mM EGTA. The tissue fraction was collected by centrifugation at 12,000×g for 5 min and solubilized with Protosol for counting radioactivity. When a P2 fraction prepared from fresh hypothalamus was used, the fraction was first incubated with 0 or 1.0 µM sCT for 10 or 60 min and then uptake of 45Ca2+ estimated as described above.

**125I-sCT binding and inhibition by sCT fragments:** Binding assay was carried out as described (12) using lower brain regions (hypothalamus, midbrain, pons and medulla oblongata).

**RESULTS**

Inhibitory effect of sCT on 45Ca2+-uptake: regional and hormonal specificities: The presence of sCT (1.0 µM) in the incubation medium for 60 min significantly decreased 45Ca2+-uptake by hypothalamic blocks (Fig. 1). The effect was dependent on the time of incubation with sCT and did not appear when the time was shorter. The concentration-dependency of this effect is depicted in Fig. 2. The minimum effective concentration was assessed to be 10 nM or less. Figure 3 shows the regional specificity. The response to sCT appeared to be specific to the hypothalamus and was not observed with the pons plus
medulla oblongata or the cerebral cortex.

In contrast to sCT, pCT was not inhibitory on the ion-uptake at 1.0 μM (Fig. 4), though at this concentration the mammalian hormone was able to practically saturate the $^{125}$I-sCT binding sites in the lower brain regions including the hypothalamus (2). Two fragments of sCT also failed to affect the uptake in the concentrations which potentially blocked the $^{125}$I-sCT binding. The IC50s of these fragments estimated by the binding assay are compared with that of sCT in Fig. 5.

Dependence of the sCT effect on the integrity of cell membrane structure: The sCT effect was examined at subcellular levels. Hypothalamic blocks were first incubated with or without sCT (1.0 μM) for 60 min, and

Fig. 1. sCT-induced decrease of $^{45}$Ca$^{2+}$-uptake by rat hypothalamus blocks: dependency of the effect on the incubation time. Time shows the duration of incubation after the addition of $^{45}$Ca$^{2+}$. Each point represents the mean±S.E. of five to seven duplicate determinations made in the presence (●) or absence (○) of sCT (1.0 μM). ★★ significant inhibition (P<0.01).

Fig. 2. Concentration dependency of the sCT effect. Rat hypothalamic blocks were incubated at 37°C for 70 min and at the 5th min of incubation, sCT was added to give the final concentration indicated. The extent of $^{45}$Ca-uptake, which occurred in the absence of sCT for 60 min, was taken as 100%. Each point represents the mean±S.E. of five to seven determinations. ★ and ★★ significant inhibition (P<0.05 and P<0.01, respectively).

Fig. 3. Regional specificity of the sCT effect. Tissue blocks from discrete brain regions were incubated at 37°C for 70 min and at the 5th min of incubation, sCT was added to give 1 μM as the final concentration. Each column represents the mean±S.E. of six to nine duplicate determinations made in the presence (●) or absence (○) of sCT (1.0 μM). ★★ significant inhibition (P<0.01).

Fig. 4. Hormonal specificity of the sCT effect. Each column represents the mean±S.E. of five to seven duplicate determinations. ★★ significant inhibition (P<0.01).
two particulate fractions prepared. The P$_2$ fraction from sCT-treated blocks exhibited a decreased level of $^{45}$Ca$^{2+}$-uptake as compared with the control (Fig. 6A). Such change was not induced in the P$_1$ fraction. In contrast, when sCT was directly incubated with the P$_2$ fraction of fresh hypothalamus, no inhibition ensued (Fig. 6B).

DISCUSSION

It is known that when $^{125}$I-sCT at 1 nM was incubated in a polypropylene tube, most of the ligand would disappear from the incubation medium by irreversible absorption to the tube surface (12); and thus, all the binding assays of calcitonin reported so far have been carried out in the presence of an anti-absorbant such as bovine serum albumin, bacitracin or detergents (1-4, 12). None of them were employed in this experiment, however, because these surfactants invariably caused vigorous bubbling during incubation. Instead, we expected that the presence of tissue in the assay tube would minimize such absorption of later added sCT (12). In vitro 45Ca$^{2+}$-uptake by the hypothalamus blocks was significantly inhibited by sCT in a concentration as dilute as 10 nM. This concentration appears to be comparable to the in vivo level which would be reached by intraventricular injection of anorectic doses of sCT, rather than the IC50 of the hormone estimated by a binding assay using the lower brain regions including the hypothalamus (2). The lowest dose of sCT which Freed et al. (5) employed to decrease feeding of rats was 0.20 U (0.015 nmole) per animal. Later we confirmed that the minimum effective dose would be above 0.2 U per rat (6). Assuming the distribution volume as 2.0-0.2 ml, injection of 0.20 U would give 7.5 nM or higher as the final concentration which corresponds to the minimum effective concentration estimated herein for the novel in vitro effect. Recently, Levine and Morley (13) have also noted a similar observation in a preliminary report. A finding that sCT suppressed not only natural feeding but also CaCl$_2$-induced feeding in rats led them to a study examining the effect of the peptide on $^{45}$Ca-uptake by a rat hypothalamic "explant". 

**Fig. 5.** Competitive inhibition of $^{125}$I-sCT binding by sCT and its C-terminal fragments. Each point represents the mean±S.E. of three triplicate determinations. The IC50s of sCT (◇), sCT (10-32) (●) and sCT (22-32) (□) were calculated to be 1.08 nM (the 5% confidence limit: 0.79-1.47), 17.9 nM (11.7-27.2) and 146 nM (108-198), respectively.

**Fig. 6.** In A, the P$_1$ and P$_2$ fractions were prepared from the hypothalamus blocks preincubated with (◇) or without (□) sCT (1.0 μM). Each column represents the mean±S.E. of three to four duplicate determinations. * significant inhibition (P<0.05). In B, the P$_2$ fraction was from the fresh hypothalamus and incubated with (◇) or without (□) sCT (1.0 μM). Each column represents the mean±S.E. of five duplicate determinations.
The minimum effective concentration was estimated to be around 10 nM.

More substantial evidences to correlate the in vivo and in vitro effects would be the regional and hormonal specificities of the latter effect that the binding assay has ever failed to predict directly. While the binding assay has detected comparable $^{125}$I-sCT binding sites in the hypothalamus and the pons plus medulla oblongata (2), the effect was observed with the hypothalamus alone. This biassed regional specificity seems to be in favor of our working hypothesis that sCT might exert its anorectic effect through modifying the Ca-metabolism of the hypothalamus where the "appesat" resides. In addition, it was found that pCT at 1 $\mu$M failed to affect the ion-uptake of the hypothalamus. Though at this concentration the mammalian hormone was able to depress $^{125}$I-sCT binding to a negligible level. Two fragments of sCT were also fairly active in the binding assay, but not on the ion-uptake. This hormonal specificity tends to suggest, on the one hand, that the whole structure of sCT would be essential for the in vitro effect as have been observed with the anorectic activity (see below); but on the other hand, it clearly gives evidence that even in the hypothalamus, only a small fraction of the binding sites would function as the receptor for mediating the in vitro and possibly in vivo effects.

Recently, we have compared the anorectic potency of the three calcitonins and the two sCT fragments used in this experiment (6). It was found that sCT alone decreased feeding and the effect, though partially, could be blocked by sCT (10–32), but was never blocked by pCT or human calcitonin. This apparently demonstrates that the receptor sites responsible for the in vivo effect could recognize even a part of sCT molecule but none of the mammalian congeners. Though we have not succeeded in detecting the antagonistic property of the fragment in this in vitro study, the results we obtained so far seem to indicate that the anorectic effect of sCT is more closely related with the effect on the Ca-uptake rather than its activity to inhibit $^{125}$I-sCT binding. In the future, function-related classification of the binding sites might become feasible, however, if we carefully design the binding assay by using more a finely dissected area of a given brain region and also by comparing the effects of calcitonins or the fragments properly.

There are two more findings to be commented on. One would be the extent of inhibition inducible by sCT. At 1 $\mu$M, it was only 15%. The hypothalamus contains a number of nuclei, each being endowed with different functions. It is considered, therefore, that for clear definition of the nature of the in vitro effect, the use of a more restricted area would be advantageous; and such a study is now underway. The other is a finding implying a possible dependency of the sCT effect on the integrity of the cell membrane structure. The effect once induced at the level of tissue blocks was able to be traced down to a subcellular fraction, while on direct interaction with the fraction, sCT was no longer effective. Though the uptake and distribution of Ca$^{2+}$ into a cell is known to be a complex phenomenon, more detailed study is expected to identify the mechanism underlying the sCT effect.

It is concluded that the novel effect on the Ca-metabolism of the hypothalamus shares some characteristics with the in vivo effect and may be usable as a model in order to define the cellular events leading to the behavioral effect.

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