ABSORPTION LOSS OF PEPTIDES TO THE PLASTIC TUBE IN RADIORECEPTOR ASSAY OF CALCITONIN AND β -ENDORPHIN: PROTECTION BY DETERGENTS

Masao KOIDA, Hiromichi NAKAMUTA, Junko MATSUO
and Yoshinobu YAMAMOTO

Department of Pharmacology, Faculty of Pharmaceutical Sciences,
Nagasaki University, Nagasaki 852, Japan

Ronald C. ORLOWSKI

Armour Pharmaceutical Company, Kankakee, Illinois 60901, U.S.A.

Accepted January 7, 1982

Abstract—During development of the calcitonin binding assay using rat brain particulate fraction, it was noticed that ^{125}I -salmon calcitonin-I (^{125}I -SCT) was partly absorbed by the polypropylene tube surface in an irreversible manner. Absorption was partially prevented by cold SCT and complete prevention was achieved by bacitracin, Triton X-100, Brij 36T, and some Zwittergents in such concentration ranges which appeared not to disrupt the ^{125}I -SCT binding ability of brain tissue. The brain fraction itself exhibited a similar preventive effect. The anti-absorbing effect of Brij 36T was also observed in an opiate receptor binding assay for β -endorphin. These results led us to recommend that the binding assay for these peptides should be done only in the presence of an appropriate anti-absorbant.

Radioreceptor assay usually employs a radioligand in highly diluted concentrations, and it is not rare that nonspecific absorption by the surface with which the ligand comes in contact presents a serious problem. Recently we characterized the calcitonin-specific binding site in the rat brain as exhibiting a high regional variation and low K_{DS} (1, 2). Our observations were later confirmed by other investigators (3). In our studies, it was noticed that calcitonin was easily lost

by absorption to the polypropylene tube (see the footnote) used for the binding assay. The use of bacitracin, employed expecting inhibition of absorption and enzymatic degradation of ^{125}I -SCT, has enabled us to characterize the calcitonin-specific binding site, but the necessity of a better agent as a substitute for bacitracin had been keenly felt.

In this paper, we report that bacitracin can be replaced by some detergents such as Triton X-100 and Brij 36T, and these detergents would be also useful in radioreceptor assays of other physiologically important peptides.

MATERIALS AND METHODS

Human β -endorphin was a generous gift

The material of the tube was described to be polyethylene in our previous report (1) based on wrong information from the manufacturer, but the actual material of the tube was polypropylene. It should be noted that the polyethylene tube from the same maker (Sample Cup 1A) also exhibited a similar binding property for ^{125}I -SCT.

of Dr. H. Yajima of Kyoto University. Salmon calcitonin-I was synthesized by the Armour Pharmaceutical Co. Ltd. (Illinois, U.S.A.). Carrier-free Na ^{125}I and (N-allyl-2,3- ^3H) naloxone (50 Ci/mmol) were purchased from The Radiochemical Centre (Amersham, England); Triton X-100, Brij 36T (polyoxyethylene 10 lauryl ether), bovine serum albumin (BSA), sodium dodecylsulfate (SDS) and bacitracin from Sigma Chemicals (Missouri, U.S.A.); Zwittergents 3-06, 3-08, 3-10, 3-12, 3-14 and 3-16 from the Calbiochem-Boehringer Corp. (California, U.S.A.); polypropylene tube (Sample Cup 4A) from Ikenkizai (Osaka, Japan); Protosol from the New England Nuclear Corp. (Boston, U.S.A.). Commercially available Triton X-100 and Brij 36T contain considerable amounts of dithiothreitol-oxidizing activity (4). This activity was able to be removed by incubating a 1% (w/v) solution of the detergent in Tris-HCl buffer (pH 7.4) containing 10 mM methionine and 1 mM EDTA at 4°C overnight.

Measurement of ^{125}I -SCT binding: ^{125}I -SCT was prepared as described (1). For estimation of the binding to the polypropylene tube, the tube contained in 1.0 ml of 50 mM Tris-HCl buffer (pH 7.4), 1.0 pmole of ^{125}I -SCT (0.9×10^5 cpm), and 0 or 1 μM of SCT. After incubation at 4°C for 30 min, the supernatant was removed by decantation. The tube was washed once with buffer, 2% SDS solution added, and incubated at 60°C overnight. The radioactivities in the supernatant, in the washing buffer, and in the SDS solution were estimated. The rat brain particulate fraction was prepared from a homogenate of lower brain regions (hypothalamus, medulla oblongata, midbrain and pons) of Wistar male rats (150–170 g) as described (1). Binding assay conditions were the same as used above except that the tube additionally contained 1.6 mg protein of the particulate

fraction and before decantation, the ^{125}I -SCT-bound fraction was pelleted by centrifuging at $15,000 \times g$ for 30 min. Tissue protein was quantified by the method of Lowry et al. (5) using BSA as the standard.

Measurement of opiate receptor binding of β -endorphin: A P_2 fraction was prepared from rat brain (void of cerebellum) according to the method of Gray and Whittaker (6). The fraction was lysed in 50 mM Tris-HCl buffer (pH 7.4) by incubating at 37°C for 30 min. The lysate was centrifuged at $15,000 \times g$ for 10 min, and the pellet suspended in the buffer was used as the P_2 membrane fraction. The binding assay was carried out in a polypropylene tube as described by Terenius (7). The reaction mixture contained in 1.0 ml of Tris-HCl buffer (pH 7.4), 2 pmole of [^3H]-naloxone, membrane fraction (0.80 mg protein), and β -endorphin (0–1.0 μM), in the presence or absence of 0.0003% Brij 36T. The presence also means here that dilution and pipetting of β -endorphin were carried out in the buffer containing 0.003% detergent. The reaction mixture was incubated at 37°C for 30 min and centrifuged at $15,000 \times g$ for 10 min. The pellet was solubilized by Protosol for counting. The specific binding was designated as the binding inhibited by 1 μM morphine.

RESULTS

^{125}I -SCT absorption to assay tube: protection by SCT and brain particulate fraction: Since ^{125}I -SCT was prepared and diluted in the presence of Triton X-100, the reaction mixture invariably contained less than 0.00005% of the detergent as the final concentration. In this concentration, the detergent did not inhibit ^{125}I -SCT absorption by the tube. Table 1 shows a low recovery rate of ^{125}I -SCT when the assay tube first received ^{125}I -SCT and then buffer or SCT-containing buffer. In the absence of SCT,

Table 1. Absorption of ^{125}I -SCT to the polypropylene tube and preventive effects of SCT and rat brain particulate fraction

	SCT	Recovered in sup. and washing	Recovered in SDS- solubilized sup.	Total recovery rate	Apparent SCT- replacable binding
		$\times 10^3$ cpm			
a)	0	$18.8 \pm 0.49^*$	$13.1 \pm 1.8^*$	38%	5.8%**
	$1.0 \mu\text{M}$	45.5 ± 2.0	8.0 ± 1.9	60	
b)	0	44.2 ± 0.64	27.5 ± 0.75	81	13
	$1.0 \mu\text{M}$	65.7 ± 5.6	16.2 ± 3.2	93	
c)	0	75.8 ± 1.4	10.2 ± 0.51	97	4.0
	$1.0 \mu\text{M}$	81.1 ± 2.1	6.6 ± 0.26	99	

*: Each value is the mean $\pm$ S.E. of 5 separate experiments. **: Calculated from the count in the SDS-solubilized supernatant. Throughout this experiment, SCT was the first addition to the tube which significantly increased the recovery of ^{125}I -SCT in the supernatant plus washing and decreased the recovery in the SDS solution. The adding order of ^{125}I -SCT (I, 8.8×10^4 cpm), buffer (II), and rat brain particulate fraction (III) which followed the SCT addition in the control, was in a) I and II, in b) I, II, and III, and in c) III, I, and II.

the rate was only 38%. The low recovery rate is not due to absorption to the pipet tip which is also made of polypropylene because the radioactivity of ^{125}I -SCT in Table 1, 0.88×10^4 cpm, was the actual measured value by directly pipeting the ligand solution into a counting tube. SCT ($1 \mu\text{M}$) improved the recovery rate and about 6 % of the ligand was counted as the apparent SCT-inhibited binding by the tube itself. However, 40 % of the ligand was still missing possibly due to irreversible absorption by the tube. Such loss was also observed in the presence of the rat brain particulate fraction if ^{125}I -SCT was the first addition to the tube. The SCT-inhibited binding was doubled, but the total recovery rate of the ligand was again low. In contrast, when the tube received the tissue fraction and then ^{125}I -SCT, the rate increased to approximately 100%, and the SCT-inhibited binding was depressed to about 4%. These observations indicate that the tissue fraction protected the ^{125}I -SCT from unwanted absorption by the tube, and the SCT-inhibited binding by the brain fraction became measurable. Nevertheless, a possibility existed that the specific binding detected may still include in part a SCT-

inhibited binding to the assay tube.

Detergent effects: In order to test such a possibility, we first attempted determine different types of anti-absorbants which can selectively abolish the SCT-inhibited absorption by the tube. In addition to bacitracin, Triton X-100, Brij 36T, and the two Zwittergents 3-14 and 3-16 were found

Fig. 1. Detergent effects on absorption of ^{125}I -SCT to the polypropylene assay tube. $\square$, Zwittergent 3-16; $\circ$, Brij 36T; $\bullet$, Triton X-100; $\triangle$, bacitracin; $\blacktriangle$, BSA. Each point represents the mean $\pm$ S.E. of 2 triplicate determinations. The solid line was obtained in the absence of SCT and the broken line in the presence of SCT.

to have such an abolishing effect in relatively dilute concentrations (Fig. 1) which would not affect the ligand binding ability of brain binding sites. On the other hand, BSA and four other Zwittergents were not complete inhibitors of tube absorption in the concentrations tested. Secondly, we compared the effects of several anti-absorbant candidates on ^{125}I -SCT binding to the brain fraction.

Fig. 2. Effects of Triton X-100 and BSA on ^{125}I -SCT binding to the rat brain particulate fraction. ●, Triton X-100 and ▲, BSA. Each point represents the mean $\pm$ S.E. of 2 triplicate determinations. The solid line was obtained in the absence of SCT and the broken in the presence.

Fig. 3. Effects of Brij 36T and bacitracin on ^{125}I -SCT binding to the rat brain particulate fraction. ○, Brij 36T and △, bacitracin. The other conditions were the same as those in Fig. 2.

The order of adding to the assay tube was a candidate-containing buffer, ^{125}I -SCT, and then the tissue fraction. In the control, SCT was added after the buffer. In the concentration ranges which nullified the SCT-inhibited absorption to the tube (refer to Fig. 1), Triton X-100, Brij 36T, and bacitracin were able to depress the SCT-inhibited binding to a certain level (Figs. 2 and 3) which corresponded to the binding estimated above in the absence of any anti-absorbant by first adding the tissue fraction to the tube (Table 1, c). A Zwittergent 3-16 also exhibited a similar effect but with more variance. Though the data are not shown here, the minimum concentrations of Triton X-100 and Brij 36T estimated as significantly disruptive on the ^{125}I -SCT binding ability of the brain fraction were more than 0.1%. Judging from the results, Brij 36T would be the reagent of choice as the anti-absorbant because a stable SCT-inhibited binding was observed in a wider concentration range (3×10^{-4} to 10^{-2} w/v%) of this detergent.

Potentiality by Brij 36T of opiate receptor binding activity of β -endorphin: Figure 4

Fig. 4. Dose-replacement curve by β -endorphin of $[\text{H}^3]$ -naloxone binding to the rat brain opiate receptor and the effect of Brij 36T. In the presence of Brij 36T (○), the curve (●) shifted to the left with a significant decrease of the IC_{50} from 62 nM (the 95% confidence limit: 40–100 nM) to 4.3 nM (2.4–7.4 nM). Each point represents the mean $\pm$ S.E. of 6 determinations.

compares the inhibitory effect of β -endorphin on [^3H]-naloxone binding to the rat brain opiate receptor estimated in the presence or absence of Brij 36T. Handling of the peptide in the presence of the detergent shifted the dose-inhibition curve to the left and increased its opiate receptor binding activity by more than tenfold. Since the detergent did not significantly change the [^3H]-naloxone binding in less than 0.001% concentration, it is recommended that this type of peptide be handled in the presence of a suitable anti-absorbant.

DISCUSSION

Calculation by the method of Chow and Fasman (8) predicts that SCT behaves as a highly lipophylic molecule. We can assume a similar property for β -endorphin. The results obtained here show that this type of peptide should be handled in the presence of an appropriate anti-absorbant, otherwise the binding assay would give erroneous results, especially when the labeled peptide is the first ingredient to be added to assay tube, whatever the material of the tube is. A glass surface is known to irreversibly absorb ^{125}I -SCT and absorption was never abolished by presoaking the glass fiber filter in BSA, bacitracin, Brij 36T, etc. This property of the peptide has forced us to abandon the filtration method to separate bound from free peptide (1).

One unexpected finding was that BSA is not an ideal anti-absorbant, though this protein has been widely used in radioreceptor assays of various peptide hormones including SCT. Rizzo and Goltzman (3) carried out a binding assay of calcitonin in the presence of 2% BSA. As stated previously (1) and also shown here, 1% BSA failed to abolish non-specific absorption of ^{125}I -SCT to the polypropylene tube. In contrast, the presence of the brain fraction itself was able to minimize such unwanted absorption. Though

the nature of the protecting effect is not known, it is possible that the brain fraction contains myelin basic protein which allowed Ferrara et al. (9) to apply the filtration method for the binding assay of β -endorphin.

In conclusion, it is suggested that for developing a radioreceptor assay for a basic and lipophylic peptide, special care must be taken to avoid non-specific absorption by experimental tools, and a recommended method for the calcitonin binding assay is carrying out the whole assay procedure only in the presence of Brij 36T or its substitute.

Acknowledgement: This work was supported in part by a Grant-in-Aid for Scientific Research (No. 557514) from the Ministry of Education, Science and Culture, Japan.

REFERENCES

- 1) Nakamuta, H., Furukawa, S., Koida, M., Yajima, H., Orlowski, R.C. and Schlueter, R.: Specific binding of ^{125}I -salmon calcitonin to rat brain: regional variation and calcitonin specificity. *Japan. J. Pharmacol.* **31**, 53-60 (1981)
- 2) Koida, M., Nakamuta, H., Furukawa, S. and Orlowski, R.C.: Abundance and location of ^{125}I -salmon calcitonin binding site in rat brain. *Japan. J. Pharmacol.* **30**, 575-577 (1980)
- 3) Rizzo, A.J. and Goltzman, D.: Calcitonin receptors in the central nervous system of the rat. *Endocrinology* **108**, 1672-1677 (1981)
- 4) Ashani, Y. and Catravas, G.N.: Highly reactive impurities in Triton X-100 and Brij 35: partial characterization and removal. *Analyt. Biochem.* **109**, 55-62 (1980)
- 5) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. *J. Biochem.* **193**, 265-275 (1951)
- 6) Gray, E.G. and Whittaker, V.P.: The isolation of nerve endings from brain: an electron microscopic study of cell fragments derived by homogenization and centrifugation. *J. Anat.* **96**, 79-96 (1962)
- 7) Terenius, L.: Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. *Acta pharmacol. toxicol.* **32**, 317-320 (1973)
- 8) Chow, P.Y. and Fasman, G.D.: Prediction of protein conformation. *Biochemistry* **13**, 222-

- 245 (1974)
- 9) Ferrara, P., Houghton, R. and Li, C.H.: β -Endorphin: characterization of binding sites in the rat brain. *Biochem. biophys. Res. Commun.* 89, 786–792 (1979)