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Established theory of radiation-induced decay is not generalizable to Bolton–Hunter labeled peptides

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Abstract

Peptide hormones radiolabeled with ^{125}I are widely used for the pharmacological characterization of cognate receptors. As a prerequisite for calculating ligand affinities from competition binding assays, and for estimating receptor densities from such studies, it is necessary to know the concentration of bioactive radioligand that is used in respective experiments. It has been demonstrated previously that radioiodinated peptides undergo decay catastrophe, i.e. disintegration of the radioactive label leads to the concomitant destruction of the carrier peptide. Here, we demonstrate that decay catastrophe does not apply to two peptide hormones that are iodinated by Bolton–Hunter conjugation: cholecystokinin octapeptide and glucagon-like peptide 2. The function of aged samples of these radioligands at corresponding recombinantly expressed receptors was assessed by measuring ligand-induced inositol phosphate production or generation of cyclic AMP, respectively. Both of the tested compounds, although predicted by decay catastrophe to contain little or subthreshold remaining bioactivity, stimulated an unexpectedly high level of receptor-mediated second messenger signaling. Quantitative comparison of observed functions with those of corresponding unlabeled peptides suggested that the bioactivity of each radioligand had been largely conserved despite the radioactive decay of the iodine label. Consistent with an apparent absence of decay catastrophe, we noted that the specific radioactivity, when determined immediately following peptide iodination, was close to the theoretical maximum but exponentially decreased over time. These findings raise the possibility that attachment of a Bolton–Hunter conjugate may shield labeled peptides from radiation-induced damage, a scenario that should be considered when performing radioligand binding experiments.

Keywords: Radioligand; Glucagon-like peptide; Cholecystokinin; Receptor; Radioactive decay; Peptide hormone

1. Introduction

Peptide hormones radiolabeled with ^{125}I are widely used for the pharmacological characterization of cognate receptors, e.g. for estimating receptor densities and for assessing ligand affinities by competition binding experiments [1]. Typically, these assays require that the peptides be labeled to a high specific radioactivity while conserving the chemical structures that are critical for biological function. High specific activity can be achieved by incorporation of ^{125}I into the peptides through the chloramine-T and Iodogen methods; however, these direct iodination techniques carry a considerable risk of oxidative damage to the peptide to be labeled [2,3]. Furthermore, each of these widely applied approaches also relies on the presence of either a tyrosine or histidine residue within the target peptide where the ^{125}I moiety can be incorporated, thereby limiting the applicability of these methods [4,5]. Bolton–Hunter conjugation, which involves the addition of an ^{125}I-labeled 3-(4-hydroxyphenyl) propionyl moiety to an amino group within the target peptide, provides an alternative strategy for the labeling of peptides where direct iodination techniques are not applicable or would result in a loss of biological activity.

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Abbreviations: GLP-2, glucagon-like peptide 2; BH-GLP-2, Bolton–Hunter labeled glucagon-like peptide 2; CCK-8, cholecystokinin octapeptide; BH-CCK-8, Bolton–Hunter labeled cholecystokinin octapeptide; cAMP, cyclic AMP; CCK_2 receptor, human cholecystokinin type 2 receptor.

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Evidence in the literature suggests that radioiodinated molecules disintegrate over time through a process termed decay catastrophe. This concept is based on the observation that when ^{125}I decays, the process leads to the concurrent fragmentation of the structure which carries the radioactive atom [6,7]. Decay catastrophe is well defined in small molecules such as iodotyrosine and iododeoxyuridine, where the decay of iodine leads to total fragmentation of the compound [6,8,9]. Radiation-induced disintegration has also been demonstrated in peptides such as insulin and α-bungarotoxin, which had been directly iodinated by either the chloramine-T or lactoperoxidase methods [7,10]. It has therefore been concluded that radiolabeled peptides will undergo decay catastrophe by a mechanism similar to that described for small molecules. Through this process, peptide radioligands are predicted to lose biological function in parallel with the decay of iodine [5], whereas the specific activity which relates to the remaining bioactive compound is predicted to remain essentially unchanged. Here, we present evidence that, in contrast to the established connection between radioactive and biological decay in directly radiolabeled molecules, certain Bolton–Hunter labeled peptides remain bioactive despite the ongoing decay of ^{125}I. The apparent absence of decay catastrophe leads to an exponential decrease in specific activity over time, which has important implications for interpreting the results of binding experiments performed with such radioligands.

2. Materials and methods

2.1. Radiolabeling of peptides

The Bolton–Hunter conjugated peptides assessed in this study were prepared at the PerkinElmer Life Sciences radiolabeling facility. Briefly, Bolton–Hunter reagent [*N*-succinimidyl-3-(4-hydroxy 5-[^{125}I]iodophenyl) propionate] was first labeled with ^{125}I by the chloramine-T method [5]. The products of this reaction were purified by normal-phase HPLC, thereby separating monolabeled Bolton–Hunter reagent from all other products. Monolabeled Bolton–Hunter reagent was collected and conjugated to either CCK-8 (human cholecystokinin 26–33, sulfated) or GLP-2 (human preproglucagon 126–158) by minor modification of the Bolton–Hunter method [3]. Individual components within the reaction mixture were separated by reverse-phase HPLC using a C-18 column and a gradient system containing 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile. This preparation yielded distinct peaks containing product and precursors, respectively, thereby enabling the isolation of essentially carrier-free, radiochemically pure, monolabeled product. Based on the clear separation of native peptide from monoiodinated peptide, a specific activity close to the theoretical maximum of 2176 Ci/mmol[1] was predicted [11]. This assumption was supported by bioassays of both the ^{125}I-Bolton–Hunter conjugated CCK-8 (BH-CCK-8) and the ^{125}I-Bolton–Hunter conjugated GLP-2 (BH-GLP-2) preparations (see Section 3).

2.2. Receptor expression

COS-7 cells (1×10^6/10 cm plate) were transfected with 5 μg of cDNA encoding either the CCK_2 or GLP-2 receptor [12,13], each subcloned into the expression vector pcDNA 1.1 (Invitrogen). Transfections were performed using the DEAE/dextran method, as previously described [14]. The following day, cells were split into either 12-well plates for measuring peptide-induced generation of inositol phosphates (200,000 cells/well) or 24-well plates for assessment of cAMP production (100,000 cells/well). Signaling assays were performed 48 hr after transfection.

2.3. Measurement of second messenger signaling

Prior to assessment of inositol phosphate production, CCK_2 receptor expressing cells were labeled overnight with 3 μCi/mL of *myo*-[^{3}H]inositol (45–80 Ci/mmol; PerkinElmer Life Sciences). The cells were then stimulated for 1 hr with the indicated amounts of either BH-CCK-8 or unlabeled CCK-8, in the presence of 10 mM LiCl. The [^{3}H]inositol phosphate products resulting from receptor stimulation were recovered from cell homogenates by chloroform extraction and then separated from *myo*-[^{3}H]inositol precursors by strong anion chromatography [15]. Together, these biochemical purification steps reduced any residual ^{125}I radiation below the detection limit of γ-counting (data not shown). [^{3}H]inositol phosphates from the column eluates were quantified by β liquid scintillation counting; results were normalized to the total cellular ^{3}H incorporation. To enable correction for residual γ-radiation when determining ^{3}H activity in cell extracts that had not undergone anion chromatography, β counting was performed simultaneously in two distinct, low- and high-energy windows (Beckman LS1801).

For measurement of ligand-induced cAMP production, GLP-2 receptor expressing cells were stimulated for 1 hr with the indicated amounts of BH-GLP-2 or unlabeled GLP-2, in the presence of 1 mM isobutyl-methyl-xanthine [16]. After freeze–thawing in 0.1 M HCl, cell lysates were acetylated and diluted 50-fold prior to cAMP radioimmunoassay. Samples were measured by solid-phase proximity scintillation counting (PerkinElmer Life Sciences, FlashPlate®). The radiolabeled cAMP was captured by specific antibodies and brought into close proximity to adjacent scintillant molecules, thus generating a signal. In contrast, residual BH-GLP-2 in the samples did not generate any appreciable non-specific signal (data not shown).

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2.4. Estimation of added peptide concentrations/ determination of specific activities

BH-CCK-8 and BH-GLP-2 that were present in the bioassays were measured by γ-counting (Beckman Gamma 5500B). From these data, peptide concentrations were predicted under the assumption that decay catastrophe had occurred and the specific activity was 2176 Ci/mmol, as on iodination day. Alternatively, concentrations were also calculated under the assumption that the radiolabeled peptides had remained intact whereas the specific activity had exponentially decreased over time ($T_{1/2} = 60$ days). These theoretical values were compared with experimentally determined peptide concentrations, as assessed by signaling assays. Apparent specific activities were calculated as a quotient of radioactivity/measured bioactivity.

3. Results

Two physiologically important peptides, CCK-8 and GLP-2, were utilized to explore the issue of decay catastrophe with Bolton–Hunter labeled peptides. Both of these ligands have attracted interest because of their dual roles as gastrointestinal hormones and neurotransmitters [17,18]. The functions of radiolabeled peptides were initially tested after relatively long storage at −80°, i.e. after periods that exceeded the half-life of ^{125}I decay by at least 2-fold and were therefore predicted to result in considerable decay catastrophe. Second messenger signaling induced by BH-CCK-8 was assessed by monitoring inositol phosphate production in COS-7 cells transiently transfected with the human CCK_2 receptor [12]. We initially analyzed two different batches of BH-CCK-8, one (sample A) that had been stored for 272 days (4.5 times the half-life of ^{125}I) and one (sample B) that had been stored for 563 days (9.4 times the half-life of ^{125}I) after iodination. Cells expressing the CCK_2 receptor were stimulated with sample A or B at final concentrations of 7.0×10^4 or 4.4×10^3 dpm/mL, respectively. If decay catastrophe had occurred (i.e. if the peptide had disintegrated in parallel with ^{125}I), the specific activity of BH-CCK-8 should not have changed and, based on the measured radioactivity, the concentration of intact peptide would be predicted at 1.5×10^{-11} M (sample A) or 9.2×10^{-13} M (sample B). Although, at these low concentrations, little if any receptor stimulation is expected [15], we noted that both sample A and sample B triggered a several-fold increase of inositol phosphate production over basal levels when incubated with CCK_2 receptor expressing cells (Fig. 1, left panel). In contrast, no effect of either sample A or sample B was detected when tested using control cells without CCK_2 receptor expression (data not shown).

Quantitative analysis of observed BH-CCK-8 effects was performed by comparison with a full concentration–response curve generated with unlabeled CCK-8 in the same experiment (Fig. 1, right panel). Projection of BH-CCK-8-induced inositol phosphate production on this standard curve suggested that close to nanomolar concentrations of bioactive radiolabeled peptide were present. If it is assumed that the specific peptide radioactivity, but not the amount of peptide *per se*, had decreased in parallel with ^{125}I, the concentration of bioactive peptide would be predicted to be 3.4×10^{-10} M for sample A and 6.6×10^{-10} M for sample B (see Fig. 1). The proximity of our experimental findings to the predicted values suggests that BH-CCK-8 retains close to full bioactivity despite the disintegration of ^{125}I.

To assess whether this conclusion might also apply to a different Bolton–Hunter labeled peptide, we studied cAMP

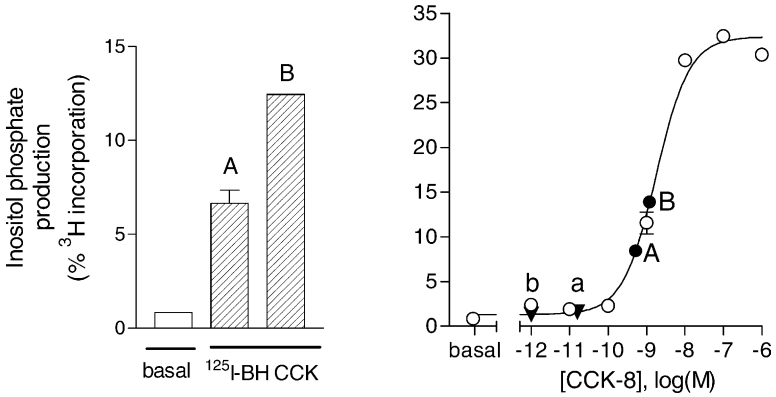

Fig. 1. Retention of full biological activity of BH-CCK-8 after prolonged radioactive decay. BH-CCK-8 stimulated receptor-mediated inositol phosphate production was measured in COS-7 cells expressing the human CCK_2 receptor. Data represent means ± range of duplicate determinations, performed in parallel. Left panel: effects of two aged samples of BH-CCK-8 (A and B; hatched bars, 272 and 563 days after iodination, respectively) on inositol phosphate production vs. corresponding signaling in the absence of ligand (basal, open bar). Right panel: signaling levels induced by BH-CCK-8 samples A and B (closed circles) were projected on a standard concentration–response curve generated with unlabeled CCK-8 (open circles). Extrapolation from the level of signaling suggests bioactive radioligand concentrations of 5.2×10^{-10} and 1.2×10^{-9} M, respectively. These values are close to prediction, assuming the labeled peptides had remained intact despite the decay of ^{125}I (see text). In contrast, if decay catastrophe had occurred, the predicted levels of ligand-induced signaling are represented by "a" and "b" (closed inverted triangles).

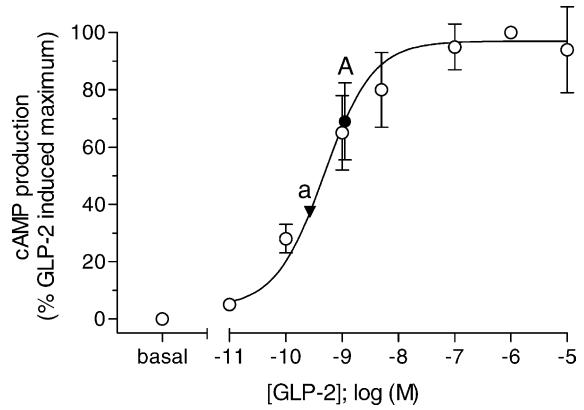

Fig. 2. Conservation of BH-GLP-2 bioactivity despite radioactive decay. The production of cAMP induced by BH-GLP-2 (158 days after iodination) was measured in COS-7 cells expressing the human GLP-2 receptor. Data represent means ± range of duplicate determinations. The standard curve illustrating signaling induced by increasing concentrations of unlabeled GLP-2 (open circles) was generated from five independent experiments (means ± SEM). All measurements were normalized to the maximum effect induced by 10^{-6} M unlabeled GLP-2 (=100%). Projection of signaling levels induced by BH-GLP-2 (A; closed circle) on the standard curve suggests a bioactive radioligand concentration of 1.1×10^{-9} M. This value is close to prediction assuming that the labeled peptide had remained intact despite the decay of ^{125}I (see text). In contrast, had decay catastrophe occurred, the predicted level of ligand-induced signaling is represented by "a" (closed inverted triangle).

production induced by BH-GLP-2 in COS-7 cells transiently transfected with the human GLP-2 receptor [13]. GLP-2 receptor expressing cells were stimulated with 1.3×10^6 dpm/mL of BH-GLP-2, 158 days after iodination (corresponding to 2.6 times the half-life of ^{125}I). Assuming that decay catastrophe had occurred, the expected concentration of intact GLP-2 peptide in the assay was 2.7×10^{-10} M. Alternatively, if the peptide had remained intact despite radioactive decay, the concentration was predicted to be 1.7×10^{-9} M. BH-GLP-2 induced a several-fold increase in cAMP production over basal levels in GLP-2 receptor expressing cells (Fig. 2) but had no effect in control cells without receptor expression (data not shown). Furthermore, comparison of BH-GLP-2-induced function with a standard curve based on unlabeled GLP-2 suggested that the actual concentration of bioactive peptide (1.1×10^{-9} M) was close to the predicted concentration if the specific radioactivity had decreased over time but the peptide had remained intact (Fig. 2).

The apparent conservation of biological activity in Bolton–Hunter conjugated radioligands may be explained in two different ways: (i) absence of decay catastrophe despite disintegration of the iodine label, or (ii) substantial contamination of the initial radioligand preparation with unlabeled precursor peptide. In the latter case, observed bioactivity in aged samples could be attributable to the *a priori* presence of unlabeled peptide that may remain intact even if adjacent iodinated peptide molecules were subject to radioactive decay. The key criterion that distinguishes between the two outlined scenarios is the level of initial specific radioactivity which should be either (i) close to the theoretical maximum if the radioligand is essentially pure, or (ii) significantly reduced if the tested preparations contained both labeled and a sizable amount of unlabeled peptide.

Experimental determination of initial specific activities suggested that respective values for both BH-CCK-8 and BH-GLP-2 approached the predicted maximum (2176 Ci/mmol) for pure radioligand preparations (Fig. 3). Consistent with this conclusion, further assessment of BH-CCK-8 and BH-GLP-2 samples over an extended period of time post-iodination indicated that specific activity values fell close to a theoretical curve based on the assumption that disintegration of the radiolabels had occurred while not triggering concomitant decay catastrophe of attached peptides. Formal analysis of the findings with BH-CCK-8 (the radioligand that was studied more extensively) revealed that the data points followed a monophasic exponential curve (dashed line in Fig. 3). A computerized fit to this function ($r^2 = 0.998$) gave an extrapolated specific activity of 2142 Ci/mmol on iodination day, in close agreement with the expected value for an essentially pure monoiodinated radioligand. While the experimental findings generally support a scenario in which decay catastrophe does not occur, it should be noted that the time-dependent decrease in the specific activity of BH-CCK-8 ($T_{1/2} = 73$ days) was slightly slower than predicted by the theoretical decay curve ($T_{1/2} = 60$ days). It is possible that this small deviation from the ideal model may reflect a limited degree of either radiation-induced or radiation-

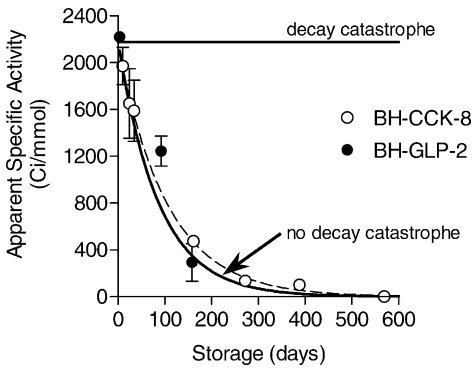

Fig. 3. Time-dependent exponential decrease in specific activities of Bolton–Hunter labeled peptides. The specific activities of BH-CCK-8 (open circles) and BH-GLP-2 (closed circles) after different lengths of storage were measured as a quotient of sample radioactivity/bioactivity. Data represent means ± range or SEM, respectively, from duplicate or quadruplicate determinations. Values obtained with BH-CCK-8 were fitted by a monophasic exponential decay curve using GraphPad Prism software version 3.0 (GraphPad). The parameters of this curve (dashed line) suggest that the initial specific activity of BH-CCK-8 was 2142 ± 97 Ci/mmol on calibration day (95% confidence interval: 2045–2239 Ci/mmol), and then decreased with a half-life of 73 days (95% confidence interval: 65–84 days). Shown as bold lines are the predicted ideal scenarios if decay catastrophe had occurred (horizontal line) or if the peptides had remained fully bioactive despite a time-dependent decrease in radioactivity (no decay catastrophe).

independent loss of CCK-8 bioactivity during storage, which would tend to increase the radioactivity/biological activity quotient. It is well established that CCK-8 is prone to losing biological activity by the oxidation of a sensitive methionine within the octapeptide [19].

4. Discussion

Prior study of radioiodinated ligand decay has relied primarily on the chemical analysis of compound integrity. In contrast, in the present study, peptide-induced receptor-mediated function served as an exquisitely sensitive experimental readout of ligand bioactivity. The applied assay designs can clearly distinguish radioactive signals attributable to second messenger molecules from those which may have originated from the radiolabeled peptides *per se*. These experiments suggest that, in contrast to the assumption that decay catastrophe occurs in all ^{125}I-labeled compounds, the structural integrity of two exemplary Bolton–Hunter labeled peptides, BH-CCK-8 and BH-GLP-2, does not seem to be affected by radiodecomposition.

Although detailed information is available regarding the radiodecomposition of small molecules, there is only a limited number of prior studies that have addressed the issue of decay catastrophe in radiolabeled peptides. To our knowledge, these experiments focused exclusively on ^{125}I disintegration in directly iodinated peptides, such as insulin and α-bungarotoxin, in which the radiolabel was directly attached to tyrosine or histidine residues within the peptide itself [7,10]. Previous physical and chemical analyses of small molecules have shown that ^{125}I decays by electron capture, leading to an Auger cascade. Together, these events generate a large amount of ionizing energy and create a large positive charge on the decay product [6,8]. The charge then delocalizes over the neighboring atoms, leading to disruption of the carrier molecule. By analogy, the observed radiation-induced decay catastrophe of directly iodinated peptides may be attributable to the structural disintegration of labeled carrier amino acids, a process that is likely triggered by a combination of coulombic repulsions during the atomic delocalization and the ionizing energy of the Auger cascade [6,7]. In contrast, functional evidence from our experiments suggests that the biologically relevant structures of BH-CCK-8 and BH-GLP-2 remain intact despite the radioactive decay of the ^{125}I moiety. Although the chemical processes that occur with aging of these radioligands remain to be established, we hypothesize that the presence of an extra aromatic group in Bolton–Hunter conjugated radioligands that spatially separates the radioactive iodine from the peptide itself may protect the latter from being damaged during the decay process. Consistent with this idea, there is evidence in the literature that increasing the distance between the radioactive moiety and the peptide backbone can mitigate peptide fragmentation due to radioactive decay [20].

Absence of decay catastrophe has significant implications for the use of radioligands in receptor binding experiments. The commonly applied protocol for these assays assumes constant specific activity and suggests calculating the amount of radioactive peptide present in a sample on this basis. However, constant specific activity is an expectation that cannot be generalized in light of our observations, which specifically focus on BH-CCK-8 and BH-GLP-2 but may be applicable to other Bolton–Hunter conjugated radioligands. Whether or not decay catastrophe occurs should be experimentally assessed for applied radioligands to enable proper interpretation of the binding data. Without adequate adjustments for possible changes in specific ligand radioactivities over time, the calculation of peptide receptor densities by established paradigms would be inaccurate [21]. Furthermore, the calculated affinities of unlabeled competitor ligands would be underestimated if the concentration of bioactive radioligand were falsely assumed to decrease during the radioactive decay process [22].

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