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Serotonin induces selective cleavage of the PKA RI subunit but not RII subunit in *Aplysia* neurons

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Abstract

PKA type I and type II are activated in *Aplysia* neurons by stimulation with serotonin (5-HT), which causes long-term facilitation (LTF). The proteolysis of the regulatory subunit (R) is thought important for the persistent activation of PKA, which is necessary to produce LTF. In this study, we report that the type I regulatory subunit (RI) and type II regulatory subunit (RII) are differentially regulated by proteolytic cleavage. RI, but not RII, was selectively cleaved after 5-HT treatment for 2 h in *Aplysia* neurons. Interestingly, the proteasome inhibitor MG132 inhibited the cleavage of RI caused by 5-HT treatment in *Aplysia* neuron. Besides extracts from *Aplysia* ganglia treated with 5-HT cleaved ^{35}S-labeled RI synthesized *in vitro*, but not ^{35}S-labeled RII. This suggests that 5-HT induces the activation state of RI-specific proteolytic cleavage.

Key

PKA; cAMP; long-term facilitation; long-term memory; proteolysis; cleavage; *Aplysia*

Introduction

The cAMP second-messenger pathway is critical for learning and synaptic plasticity. [1]. The cAMP-dependent protein kinase (PKA) plays a major role in this pathway. Animals have two isotypes of PKA, which differ in R subunits [2]. Both types of R subunits from yeast to humans are conservatively built. Although the two types of PKA share a similar structure, they differ in their intracellular distribution: type I is mainly cytosolic, whereas most of type II is bound to membranes through the association of RII with A kinase-anchored proteins (AKAPs) [3].

During the formation of long-term facilitation (LTF) by application of the neurotransmitter serotonin (5-HT), PKA is persistently active for at least 24 hr [4],[5],[6]. This persistent activation leads to persistent phosphorylation of the transcription factor cAMP response element-binding protein (CREB), which regulates the expression of genes to produce LTF [7]. The biochemical properties of PKA R subunits in *Aplysia* nervous tissue were first characterized by Eppler [8]. Bergold et al. [9] cloned and characterized *Aplysia* RI, and Hegde, et al. [10] demonstrated that *Aplysia* R subunits and mammalian R subunits are degraded through the ubiquitin proteasome pathway. The degradation of R subunits produces the persistent activity of R subunit to activate transcriptional factors, which are necessary for LTF.

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Chely, et al. On the other hand, [11] showed that *Aplysia* RII is autophosphorylated and bind AKAPs. Furthermore, 5-HT increases RII protein levels at *Aplysia* nerve endings to at the late stages of LTF [12]. Presumably, it is to strengthen synaptic connections. Thus, PKA type I and type II have distinct roles in the synaptic facilitation produced by 5-HT. PKA type I most likely functions early in the formation of LTF by providing persistent kinase activity. PKA type II may support LTF at a late stage by acting with AKAP to strengthen synaptic connections by phosphorylating proteins at sensory neuron synapses to enhance the release of neurotransmitters [12]. However, it remains unclear how neurons differentially regulate each type of PKAs. In this study, we found that treatment of *Aplysia* ganglia with 5-HT induced cleavage of RI, but not RII. Besides extracts from *Aplysia* ganglia treated with 5-HT induced cleavage of ^{35}S-labeled RI synthesized *in vitro*, but not ^{35}S-labeled RII. We propose that selective cleavage of RI is one of the mechanisms to produce the persistent activity of PKA type I and to protect RII from proteolysis.

Materials and methods

Cerebral, buccal, pleural, pedal and abdominal ganglia were obtained from *Aplysia* as described previously [13]. Ganglia were treated in the presence/absence of 500 μM of 5-HT (Sigma, St. Louis, MO) for 2 h at 18°C. For time course experiments, ganglia were cultured in L15 medium at 18°C until recovery. Then, ganglia were homogenized in 1% NP-40, 10mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, and 0.1 % protease inhibitor cocktail (Sigma, St Louis, MO) on ice. To remove cell debris, the homogenate was centrifuged for 2 min at 3,000 × g. The recovered supernatant was used as extract of *Aplysia* ganglia. For the MG132 treatment, ganglia were treated with 10 μM of MG132 (Boston Biochem, Cambridge, MA)) 10 min before treatment with 5-HT and continuously treated with MG132 during the 5-HT treatment.

Immunoblotting

Proteins extracted from ganglia were dissolved in Laemmli and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with anti-RI, anti-RII, or anti-α-tubulin antibodies. The anti-α-tublin antibody (T5168) was from Sigma and the polyclonal antibodies to *Aplysia* RI and RII were described previously [12], [14].

Plasmid construction

Total RNA was isolated using Trizol reagent (Life Technologies, Rockville, MD). Reverse transcription was performed with random primers and the resulting cDNA was used as a template for PCR. Gene-specific primer sets were used to amplify specific genes. These primer sets were: (1) RI, 5′-GGCGAATTCATGGCGGCCAACACCGACGAGG -3′ and 5′-GGCGATATCCTACACCGACAGGGATACAAAGC -3′. (2) RII, 5′-GGCGAATTCATGAATTTCGAGATCCCACCGG -3′ and 5′-GGCGATATCTCACCGCAGGTCCGAGATGTTGG -3′. The amplified sequences were inserted into the *Eco*RI/*Xho*I sites of pcDNA3.1(+) (Invitrogen, Carlsbad, CA).

Site-directed mutagenesis

Single amino acid substitutions in RI subunits were introduced by PCR. Complementary sense and antisense oligonucleotide primers were synthesized for PCR amplification of recombinant RI or RII subunits encoded in pcDNA3ApRI or pcDNA3ApRII. The primer set used to generate pcDNA3ApRI(S98A) and pcDNA3ApRI(S98E) was 5′-CATGAGGAGAGGGGCGGTGGCGGCGGAGGTATACAGGG -3′ and 5′-CCCTGTATACCTCCGCCGCCACCGCCCCTCTCCTCATG -3′ or 5′-CAT GAG GAG AGG GGC GGT GGA GGC GGA GGT ATA CAG GG -3′ and 5′-CCC TGT ATA CCT CCG CCT CCA CCG CCC CTC TCC TCA TG-3′, respectively.

Kinase assay

The recombinant gluthathion S-transferase (GST) Ap-CREB1a fusion protein was expressed in E. coli BL21 and purified with Gluthatione Sepharose 4B (GE Healthcare, Piscataway, NJ). *Aplysia* ganglia treated with or without 5-HT were immediately frozen on dry ice and homogenized in potassium phosphate buffer (10 mM potassium phosphate, 2 mM EDTA, and 150 mM NaCl). The extracts were incubated with GST-fused proteins in kinase buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 10 μCi γ-^{32}ATP, 50 μM ATP, and 1 mM Na_3VO_4) at 30°C for 30 min. Proteins from the reaction mixture were separated by SDS-PAGE, and analyzed by autoradiography.

Preparation of ^{35}S-labeled R subunits

cDNAs were transcribed and translated from pcDNA3ApRI, pcDNA3ApRII, pcDNA3ApRI (S98A), and pcDNA3ApRI(S98E) *in vitro* using a TnT T7 coupled reticulocyte lysate system (Promega, Madison, Wis) as instructed by the manufacturer in the presence of ^{35}S-labeled methionine (MP Biomedical, Solon, OH).

In vitro protein cleavage assay

^{35}S-labeled R subunits were incubated with extracts of *Aplysia* ganglia in 10 mM Tris-HCl [pH 7.4], 150 mM NaCl in the presence/absence of 10 μM of cAMP for 30 min at 30 °C. The reactions were stopped by addition of 3 × Laemmli buffer and heated for 10 min at 98 °C. The proteins were subjected to SDS-PAGE and analyzed by autoradiography.

Results

5-HT induces the cleavage of PKA RI, but not RII

A protocol producing long-term sensitization and the stimulation with 5-HT producing LTF change the level of R subunits [4],[12],[13]. We examined the protein levels of RI and RII in *Aplysia* ganglia after 5-HT treatment. Ganglia were treated with 5-HT for 2 h, then desheathed and homogenized. The protein levels of RI and RII were analyzed by Western blotting with specific anti-RI or anti-RII antibodies [12]. After the incubation with 5-HT for 2 h, the protein level of RI (52 kDa band) was decreased (Fig. 1. lane 2) compared with that in *Aplysia* ganglia cultured without 5-HT (Fig. 1. upper left panel, lane 1). On the other hand, the protein level of RII was not changed after 5-HT treatment for 2 h (Fig. 1. lane 5, compared with lane 4). Interestingly, a band showing mobility at 37 kDa was stronger after 5-HT treatment for 2 h (Fig. 1. lane 2). We suspected the 37-kDa fragment to be a cleaved product of RI as previously reported [15],[16]. It has been reported that 5-HT induces polyubiquitylation of R subunits [10] and produce the long-lasting activity of PKA [10]. Besides proteasome inhibitor MG132 blocks LTF [14]. We suspected that there might be a relation between MG132 treatment and cleavage of RI. When ganglia were treated with MG132 and 5-HT together, cleavage of RI was inhibited (Fig. 1. lane 3). We concluded that the degradation of RI is regulated by cleavage as well as by the ubiquitin-proteasome pathway. A question is why the proteasome inhibitor blocked cleavage of RI since it inhibits the degradation of polyubiquitylated proteins. Lee et al,.[17] reported that MG132 inhibits the activity of calpains as well as the 20S protease. However, neither calpain inhibitor I and II, nor EGTA inhibited the cleavage (data not shown). Presumably, the ubiquitin-proteasome pathway is indirectly involved in cleavage of RI. The treatment of ganglia with 5-HT and MG132 also increase the level of RII (Fig. 1. lane 6). Interestingly, we observed the ladder bands between 58 kDa and 75 kDa. They may be polyubiquitylated RII. The protein level of α-tubulin was not affected by 5-HT and/or MG132.

Extracts from Aplysia ganglia treated with 5-HT persistently phosphorylate Aplysia CREB1a

Persistently activated PKA targets ApCREB1a, which is the *Aplysia* homologue of CREB, for the formation of LTF [7]. To examine the persistent activity of PKA during the formation of LTF, we performed an *in vitro* kinase assay using GST-CREB1a produced in bacteria. *Aplysia* ganglia were treated with 5-HT, immediately frozen on dry ice, and homogenized. Extracts from *Aplysia* ganglia were incubated with GST-CREB1a in the presence of γ^{32}-ATP and phosphorylated GST-CREB1a was analyzed by autoradiography. Extracts from ganglia treated with 5-HT for 2 h efficiently phosphorylated CREB1a (Fig. 2B. lane 2 compare with lane 1) and a high level of phosphorylation lasted for at least 8 h (Fig. 2B. lane 4). Extracts from ganglia prepared 24 h after 5-HT incubation phosphorylated CREB1a although the level was decreased compared with that prepared 8 h after 5-HT incubation (Fig. 2 lane 6). We confirmed that 5-HT treatment induces persistent kinase activity and ability of *Aplysia* ganglia to phosphorylate CREB1a. This observation corresponds to previous reports [6], [18].

The extract from Aplysia ganglia treated with 5-HT cleaves ^{35}S-labeled RI synthesized in vitro, but not ^{35}S-labeled RII

The difference in proteolysis of RI vs. RII may be explained by the different cellular distribution of two subunits forms [12]. If cleavage occurs only in the cytoplasm and not at the synapses where RII is located, then extract from *Aplysia* ganglia treated with 5-HTshould cleave both exogenous RI and RII. To test this hypothesis, ^{35}S-labeled R subunits synthesized *in vitro* were incubated with extracts from *Aplysia* ganglia treated with/without 5-HT in the presence of 10 μM of cAMP. The extracts from ganglia cultured without 5-HT did not proteolyze RI (Fig. 3A. lane 1). On the other hand, the extracts from *Aplysia* ganglia treated with 5-HT for 2 h cleaved ^{35}S-labeled RI (Fig. 3A. lane 2) and produced a 37kDa-fragment (Fig. 3A. closed star) but did not cleave^{35}S-labeled RII (Fig. 3A. lane 4, compare with lane 3). This result suggests that the difference in proteolysis between RI and RII is due to the difference in their molecular characteristics, not to the difference in their localizations.

We observed at 37-kDa fragment but not the other fragments (Fig. 1). We performed SDS-PAGE using 15 % gel to obtain a higher resolution of the cleaved products. ^{35}S-labeled RI incubated with extracts from ganglia treated with 5-HT produced a 37kDa fragment (Fig. 3B. star) and a 15-kDa fragment (Fig. 3B. closed circle). This suggests that cleavage of RI after 5-HT treatment occurs at a protease-sensitive region as previously shown [19],[20]. Both RI and RII subunits, have a protease-sensitive region in the hinge region. The hinge region contains a catalytic subunit-binding domain. Therefore, once cleaved by proteases, R subunits loose the ability to hold C subunits for inhibition of kinase activity, and PKA is activated [20]. The hinge region of both RI and RII are sensitive to proteolysis. Why RI is selectively cleaved? We further studied to answer this question.

When the concentration of cAMP increases, cleavage of R subunits efficiently occurs [19]. When the concentration of cAMP increases, C subunits are released and the hinge region is exposed to the outside of the molecule. Then, R subunits become more sensitive to the protease [20]. We performed the above experiments in the presence of 10 μM of cAMP and used 20 μg of extracts. We questioned if cleavage of RI is cAMP-dependent. 10 μg of extracts from ganglia treated with 5-HT completely cleaved ^{35}S-labeled RI (Fig. 3C. lane 3) but cleaved about 50% of ^{35}S-labeled RI in the absence of cAMP (Fig. 3C lane 2). This suggests that cAMP enhances cleavage of RI.

Next, we suspected that the modification of R subunits by phosphorylation might paly a role in cleavage of R subunits. Functionally, RI and RII can be distinguished on the basis of their potential for autophosphorylation [19]. RII, but not RI, contains an autophosphorylation site at the 96th amino acid residue where is located in the hinge region. We suspected that

autophosphorylation may prevent cleavage of RII. However, ^{35}S-labeled RII S96A mutant was still resistant to cleavage (data not shown). Then, we examined the phosphorylation site of RI located around the hinge region. Hashimoto, et al. [21] reported that a serine residue at position 98, where is located in the hinge region, is phosphorylated by a cGMP-dependent protein kinase. ^{35}S-labeled RI S98A mutant was incubated with 1 μg of extract from *Aplysia* ganglia treated with 5-HT. The extract did not cleave wild-type RI (Fig. 3D. compare lanes 1 and 2) but partially cleaved the RI S98A mutant (Fig. 3D. compare lanes 3 and 4). Next, we examined RI S98E mutant, which mimics phosphorylation. The wild-type RI was cleaved after incubation with 2 μg of extract from *Aplysia* ganglia treated with 5-HT (Fig. 3E compare lane 2 and 3) but the S98E mutant was relatively resistant (Fig. 3E compare lane 3 and 4). We concluded that the phosphorylation of 98S in RI partially protected RI from cleavage.

Discussion

We found that the treatment of *Aplysia* ganglia with 5-HT causes cleavage of RI but not RII and extracts from *Aplysia* ganglia treated with 5-HT for 2 h have an ability to cleave ^{35}S-labeled RI selectively *in vitro*. Our observation suggests that *Aplysia* neurons have a mechanism whereby only RI is cleaved in response to 5-HT. Bergold, et al. [22] found that loss of the regulatory subunit of PKA depends on protein synthesis during exposure to 5-HT, and then Hegde, et al. [18] provided evidence that a ubiquitin carboxy-terminal hydrolase (Uch) is induced as an immediate early gene (IEG) product only in neurons in response to 5-HT. Presumably, up-regulation of Uch does not directly act on cleavage of RI since it appears to associate with the proteasome and to increase the rate of release of ubiquitin from intermediates of proteolytic degradation. However, Uch may indirectly contribute cleavage since MG132 inhibited cleavage of RI (Fig. 1). Proteolytic regulation of R subunits by cleavage has been reported in rat alveolary type II cells where it is caused by calpain [23]. We propose that cleavage of RI is one of the mechanisms to produce persistent kinase activity of PKA in neuron.

In our study, the phosphorylation of RI at 98S partially protected against cleavage. The serine residue of RI at position 98 is phosphorylated by a cGMP-dependent protein kinase (PKG) [21]. Activation of PKG and inhibition of PKA is sufficient to induce long-term depression (LTD) of synaptic transmission at Schaffer collateral-CA1 synapses in the rat [24],[25]. In contrast, the cGMP-PKG pathway produces long-term potentiation (LTP) in the rat hippocampus [26]. Furthermore, the cGMP-PKG pathway triggered by nitric oxide (NO) produces transcription-dependent, long-term hyperexcitability (LTH) of nociceptive sensory neurons in *Aplysia* [27]. For these regulation through cGMP-PKG pathway, cleavage of RI may have a role.

Persistent activation of PKA by proteolysis is thought necessary to produce long-lasting memories [4],[13],[18], [28]. However, it appeared to be difficult to explain how neurons regulate the level of two different PKAs at the same time if both R subunits are susceptible to proteolysis. Here, we proposet that selective cleavage of RI provides the persistent kinase activity of PKA type I in the nucleus to phosphorylate transcription factors for initiating and maintaining the gene expression cascade required for long-term facilitation, whereas PKA type II is protected from cleavage and is provided to synaptic endings in order to strengthen synaptic connections.

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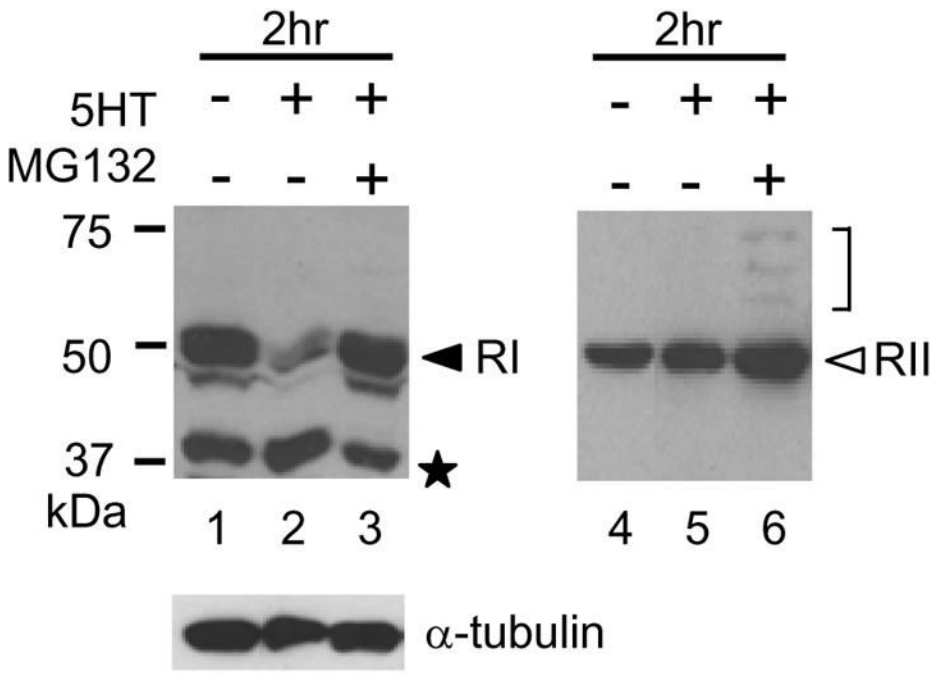

Fig 1.

Western blotting of RI, RII and α-tubulin in *Aplysia* ganglia. *Aplysia* ganglia were dissected and treated with/without 500 μM 5-HT. Ganglia were homogenized and dissolved in Laemmli. The proteins were analyzed by SDS-PAGE and detected with polyclonal Ab for RI, polyclonal Ab for RII, and monoclonal Ab for α-tubulin. MG132 was applied 10 min before 5-HT and continuously applied during the 5-HT treatment. Lanes 1, RI and RII after treatment without 5-HT for 2 h. Lanes 2 and 5, RI and RII after treatment with 5-HT for 2 h. Lanes 3 and 6, RI and RII after treatment with 5-HT and MG132 for 2 h. Protein levels of α-tubulin are shown in the lower panel. Molecular mass markers are shown on the left (in KiloDaltons). The closed arrowhead and open arrowhead indicate RI and RII, respectively. An asterisk indicates the 37-kDa cleaved fragment. The bracket indicates the putative polyubiquitylated RII.

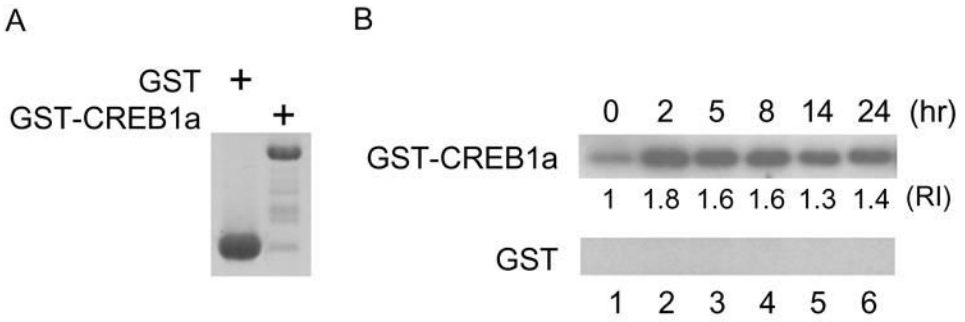

Fig 2.

Extracts from *Aplysia* ganglia treated with 5-HT phosphorylate Ap-CREB1a and this ability lasts 24 h. (A) GST alone and GST-RI were expressed in bacteria. The products were analyzed by SDS-PAGE and visualized by Coomassie Brilliant Blue staining. (B) GST or GST-CREB1a incubated with extracts from *Aplysia* ganglia and γ-^{32}ATP at 30°C for 30 min. The products were analyzed by SDS-PAGE and assessed by autoradiography. Extracts from *Aplysia* ganglia without 5-HT treatment weakly phosphorylated GST-CREB1a (upper panel, lane 1) but not GST alone (lower panel, lane 1). *Aplysia* ganglia treated with 5-HT for 2 h phosphorylated GST-CREB1a efficiently (upper panel, lane 2) and persistently phosphorylated GST-CREB1a 2 to 24 h after (upper panel, lane 2 to 6). “RI” indicates the relative intensity of phosphorylated GST-CREB1a.

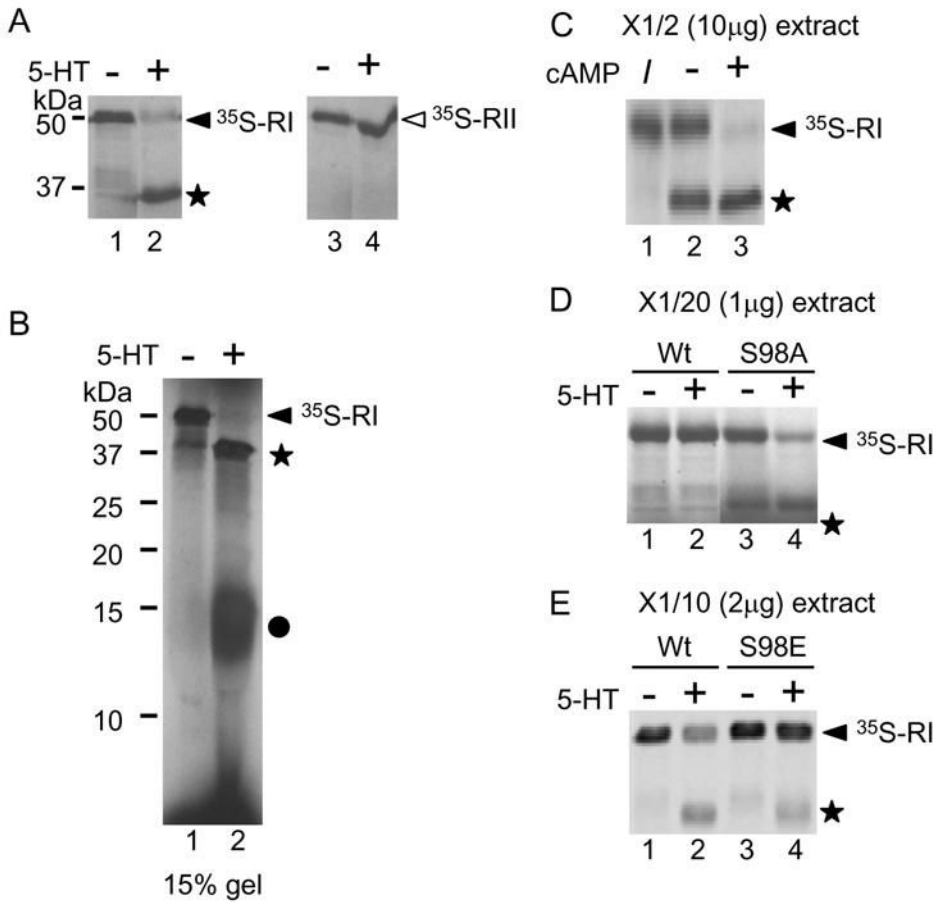

Fig 3.

Autoradiography of ^{35}S-labeled RI and RII. ^{35}S-labeled proteins were produced *in vitro* and incubated with extracts from *Aplysia* ganglia treated with or without 5-HT. (A) ^{35}S-labeled RIs were incubated with extracts from ganglia not treated with 5-HT (lane 1) and treated with 5-HT (lane 2). ^{35}S-labeled RIIs were incubated with extracts from ganglia cultured without 5-HT (lane 3) or with 5-HT (lane 4). (B) Extracts from ganglia treated with 5-HT cleave RI and produce two fragments, 15 kDa and 37 kDa. Lane 1 shows RI incubated with extract from ganglia treated without 5-HT. Lane 2 shows RI incubated with extract from ganglia treated with 5-HT. A closed circle indicates the 15-kDa fragment. (C) cAMP enhances cleavage of RI. ^{35}S-labeled RI (shown in lane 1) were incubated with ganglia treated with 5-HT in the absence of cAMP (lane 2) or in the presence of 100 mM of cAMP (lane 3). *I* indicates input ^{35}S-labeled RI before incubation with extract. (D) The S98A mutation in RI decreases its sensitivity to cleavage. Radiolabeled proteins were incubated with twenty-fold diluted extract from ganglia treated without 5-HT (lane 1 and 3) or with 5-HT (lane 2 and 4). The wild type RIs with ganglia not treated or treated with 5-HT are shown in lane 1 and 2. The S98A mutants are shown in lanes 3 and 4. (E) ^{35}S-labeled Rs were incubated with ten-fold diluted extract from ganglia treated without 5-HT (lane and 3) or with 5-HT (lane 2 and 4). The wild-type RIs are shown in lane 1 and 2. The S98E RI mutant is shown in lanes 3 and 4.