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Peripheral benzodiazepine receptors in potatoes (*Solanum tuberosum*)

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Abstract

The peripheral benzodiazepine receptor (PBR), an internal protein of the mammalian mitochondrial membrane, is involved in several metabolic functions such as steroidogenesis, oxidative phosphorylation, and regulation of cell proliferation. Here we report the presence of PBRs in parenchymal and meristematic tissues of potato (*Solanum tuberosum*). PBRs are heterogeneously distributed in potato and are highly expressed in meristematic cells. In particular the receptor protein is mainly localised in the meristematic nuclear subcellular preparation. This 30–36 kDa protein, which corresponds to PBR, is increased, indeed, in meristematic compared to the parenchymal tissue. This suggests an involvement of this receptor in the regulation of cell plant growth. In addition, the demonstration that PBRs are also present in vegetables supports the hypothesis of a highly conserved receptor system during phylogenesis.
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The peripheral-type benzodiazepine receptor (PBR) was initially described as a recognition binding site for benzodiazepines present in peripheral tissue. PBR is a heteroligomeric complex composed of at least three subunits, a 18 kDa protein that binds benzodiazepines and isoquinoline carboxamide derivatives, a 32 kDa protein identified as a voltage-dependent anion channel, and a 30 kDa protein referred to as adenine nucleotide translocator [1]. In particular the 18 kDa PBR protein in its native environment exists in various higher molecular mass complexes ranging from 30 to 200 kDa. Indeed, membrane extracts photo-labelled with various probes resulted in the identification of PBRs as 18 and 30–35 kDa proteins as well as a 70 kDa protein complex [2]. The 18 kDa protein forms polymers both in vitro and in vivo in response to reactive oxygen species (ROS) and PBR ligands exhibit higher binding activity to the polymers [3].

The PBR activation involves numerous biological functions including cell proliferation, steroid biosynthesis, mitochondrial respiration, apoptosis [4–6] and mRNA expression of PBRs was found increased in human neoplastic tissues [7–9]. Furthermore, PBR ligands, like benzodiazepines, affect the proliferation rate of C_6 glioma cells in a dose-dependent manner, suggesting a role of the PBRs in the mitotic processes [10].

Benzodiazepine-like molecules are relatively common constituent of plants and foods [11–13]. Extracts of potato exhibit after HPLC separation a series of compounds identified by MS analysis as *N*-desmethyldiazepam and diazepam [14,15]. In addition, an increase of natural benzodiazepines in potato has been described during germination processes [16]. These evidences led us to investigate the existence of specific receptors in potato (*Solanum tuberosum*). PBRs were characterised in potato by radioreceptor binding assay using a specific radioligand ([^{3}H]RO 5-4864) for PBRs. The PBR expression was also studied in potato by immunohistochemistry and confirmed by SDS–PAGE using a specific antibody recognising PBR.

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Materials and methods

Binding assay. All experiments were performed using tuber of potato (*S. tuberosum*, Spunta variety) grown in experimental fields in Italy.

Potatoes were homogenised in 0.32 mM sucrose and centrifuged at 20,000*g* for 40 min. The pellet was resuspended in 50 mM Tris–HCl buffer, pH 7.0, and centrifuged at 20,000*g* for 40 min. The pellet was resuspended in 50 mM Tris–HCl buffer, pH 7.0, to have a final concentration of 500–1000 μg/ml.

Assays were performed incubating, 100 μl buffer (with or without 10 μM RO 5-4864) 100 μl [^{3}H]RO 5-4864 (specific activity 3.15 TBq/mmol, NEN), and 800 μl membranes (500–1000 μg/ml) at 0 °C for 45 min. The reaction was terminated by vacuum filtration through Whatman GF/B filters and washed twice with buffer. The filters were transferred to scintillation vials with scintillation cocktail (Beckman) and the radioactivity was determined by conventional liquid scintillation spectrometry. Binding assay was performed using [^{3}H]RO 5-4864 (0.37–11 nM). [^{3}H]RO 5-4864 specific binding was defined as that binding which was displaceable by 10 μM RO 5-4864. In the characterisation experiments performed to assess the effect of different temperatures or buffer pH on the [^{3}H]RO 5-4864 specific binding, the standard incubation conditions were appropriately modified. Different incubation temperatures (0–37 °C) and pH buffer (6.5–8) were tested. Association of [^{3}H]RO 5-4864 (0.37 nM) was studied by incubating the membrane with 10 μM RO 5-4864 at various times before filtration. When dissociation experiments were performed the membranes were incubated to equilibrium (30 min) followed by addition of 10 μM RO 5-4864 to all samples. The samples were then filtered at various intervals of time.

All the reagents were purchased from Sigma (Italy).

Immunohistochemistry. Sections of potatoes (14 μm) were cut at −4 °C and taken on poly-L-lysine slides. The slides were fixed by immersion in 20 ml LANA-fixative (formalin 20%, 14 ml picric acid, in phosphate buffered saline 0.1 M, pH 7.3). To reduce non-specific immunostaining, the sections were preincubated in PBS (pH 7.4) containing 10% BSA for 60 min. For specific immunostaining the samples were incubated for 24 h at 4 °C with specific polyclonal PBR antibody (1:500) (Trevigen); the antibody was then diluted in PBS containing 1% BSA. Furthermore, the slides were washed with PBS two times and then incubated with biotinylated goat anti-rabbit antibody (1:400; Vector Laboratories) for 30 min, washed with PBS and incubated for 30 min with ABC complex (Vectastain Elite Kit, Vector Labs). The immunoreactions were visualised by incubating the sections with a solution of 0.025% diaminobenzidine-0.01% hydrogen peroxide for 5 min. After staining, the sections were embedded in Aquamount mountaint.

Western blotting experiments. Protein extraction from meristematic and parenchymal tissues of *S. Tuberosum* was performed using Trizol reagent following the instructions of the company (Life Technologies, Italy).

Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Italy). Protein samples were solubilised in Nu-Page sample buffer (Tris base 141 mM, Tris–HCl, pH 7.4, 106 mM, LDS 2%, EDTA 0.51 mM, serva blu 0.22 mM, phenol red 175 mM, and DTT 0.05 M) (Invitrogen, Italy), boiled for 5 min, and loaded at different concentrations onto a pre-cast 12% SDS–PAGE. Separated proteins were electrophoretically transferred to nitrocellulose membrane (Bio-Rad). Membrane was blocked in TBST (20 mM Tris–HCl, 0.5 M NaCl, and 0.05% Tween 20) buffer containing 5% non-fat dried milk overnight at 4 °C and probed with specific primary anti-PBR polyclonal antibody (1:1000) for 3 h. Membrane was then washed 3 times in TBST for 10 min each time, incubated for 1 h with HRP-conjugated anti-rabbit antibody (Santa Cruz, USA), and visualised using chemiluminescence method (Amersham, UK). The specificity of the bands recognised by the antibody was demonstrated using preabsorbed antibody prepared by incubating the antibody with recombinant PBR fusion protein used also as control (Trevigen, USA).

The isolation of intact chloroplast was based on previous extraction protocol [17]. Briefly meristematic tissues of *S. tuberosum* were homogenised in frozen buffer A (10 mM $Na_2P_2O_6$, pH 6.5, containing 300 mM sorbitol and 4 mM ascorbic acid), filtered through three layers of gauze, and centrifuged at 2500*g* for 5 s. The pellet was then resuspended in buffer B (50 mM Hepes–KOH, pH 7.9, containing 330 mM sorbitol) and centrifuged twice at 4000*g* for 10 s. The pellet was resuspended in buffer B and centrifuged in a linear percoll gradient (50%) at 26,000*g* for 50 min. Chloroplasts were removed from the gradient/overlay interface and buffer B (1:2) was added and centrifuged at 2500*g* for 30 s. The pellet contained intact chloroplasts.

Mitochondria were obtained as already described [17]. Briefly meristematic tissues were homogenised in prechilled buffer A (20 mM MOPS, pH 7.4, containing 300 mM sucrose, 5 mM glycine, 4 mM cysteine, 2 mM EDTA, 0.5% (w/v) PUP-40, and 0.2% (w/v) FA-BSA) and filtered through three layers of gauze. The homogenate was centrifuged at 3000*g* for 5 min and the pellet was discarded. The supernatant was then centrifuged at 12,000*g* for 20 min and the pellet obtained was resuspended in buffer B (10 mM KH_2PO_4/K_2HPO_4, pH 7.2, containing 300 mM sucrose, 5 mM glycine, 1 mM EDTA, 0.5% (w/v) PUP-40, and 0.1% (w/v) FA-BSA). Mitochondria were in pellet.

The procedure adopted for isolation of nuclei has been described by Mayer and Piechulla [17]. Briefly mersitematic tissues have been triturated in liquid nitrogen and added of buffer A (25 mM Mes, 10 mM KOH, pH 6.2, containing 5.5 M glycerine, 600 mM sorbitol, 0.05% (w/v) Triton X-100, 0.1 mg/mL FA-BSA, 0.5 mM DEDTC, 0.1 mg/mL PVP-40, 5 mM EDTA, 0.5 mM spermidine, 0.2 mM spermine, 0.5 mM AEBSF, and 10 mM mercaptoethanol) filtered, and centrifuged twice in a percoll cushion gradient at 3000*g* for 15 min. The pellet was then resuspended in buffer B (25 mM Mes, 10 mM KOH, pH 6.0, containing 2 M glycerine, 0.1 mg/mL FA-BSA, 0.5 mM DEDTC, 0.1 mg/mL PVP-40, 5 mM EDTA, 0.5 mM spermidine, 0.2 mM spermine, 0.5 mM AEBSF, and 10 mM mercaptoethanol) and centrifuged at 2000*g* for 5 min. The obtained pellet was enriched of nuclei.

Five micrograms of each subcellular fraction (chloroplasts, mitochondria, and nuclei) was loaded onto a pre-cast 12% SDS–PAGE for immunoblot analysis as previously described.

Results and discussion

Binding assay

In order to characterise the properties of PBRs, we performed radioreceptor-binding studies on homogenised potato using the specific radioligand [^{3}H]RO 5-4864. The study of the influence of the incubation temperature on [^{3}H]RO 5-4864 specific binding showed that the binding capacity was optimal at 0 °C (Fig. 1a). Specific binding, in fact, decreased at 24 °C by 15% and at 37 °C by 40%. As regards the influence of the pH buffer, it was found that [^{3}H]RO 5-4864 binding was very low at pH 6.5 but increased to a maximum at pH 7.0 beyond which it rapidly declined (Fig. 1b). The half-time of association was 3 min and equilibrium was attained at 20–30 min of incubation, which remained unchanged for at least an additional 20 min (Fig. 1c). This indicates that neither loss of receptors nor degradation of ligand occurred during the incubation. The

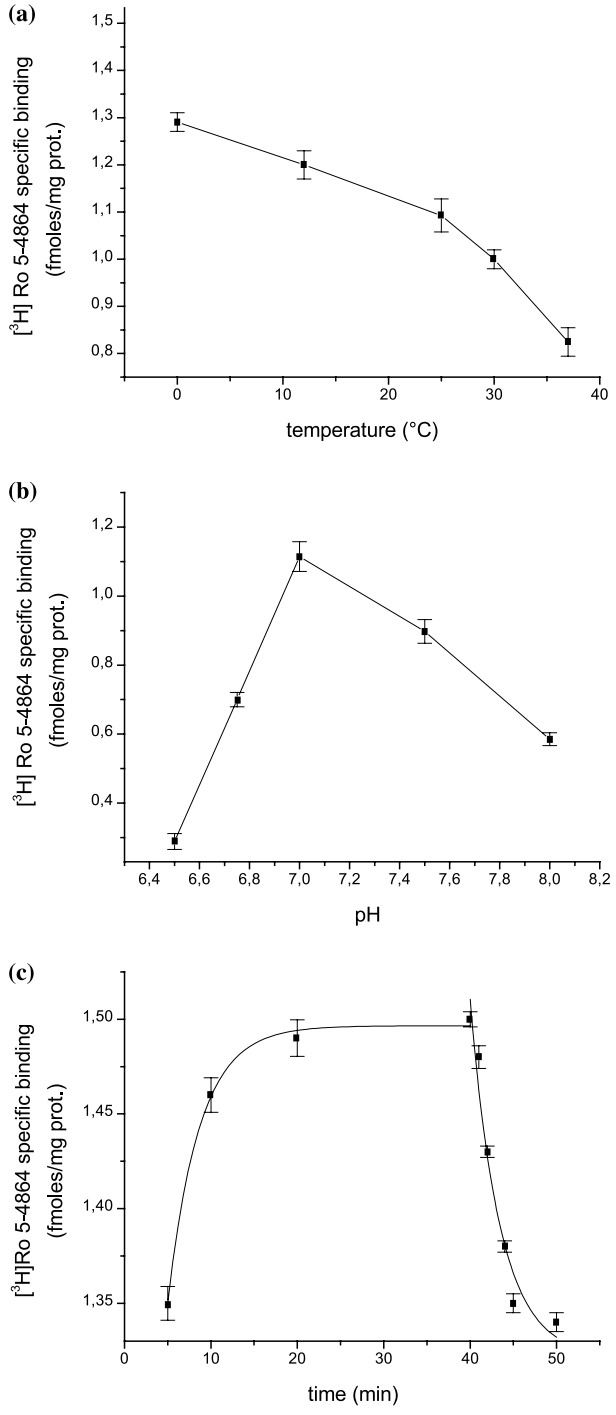

Fig. 1. [^{3}H]RO 5-4864 binding to potato (*S. tuberosum*, Spunta variety) membranes. Influence of temperature (a) and buffer pH (b) incubation on [^{3}H]RO 5-4864 specific binding. Association and dissociation kinetic characteristics of [^{3}H]RO 5-4864 binding to potato membranes (c).

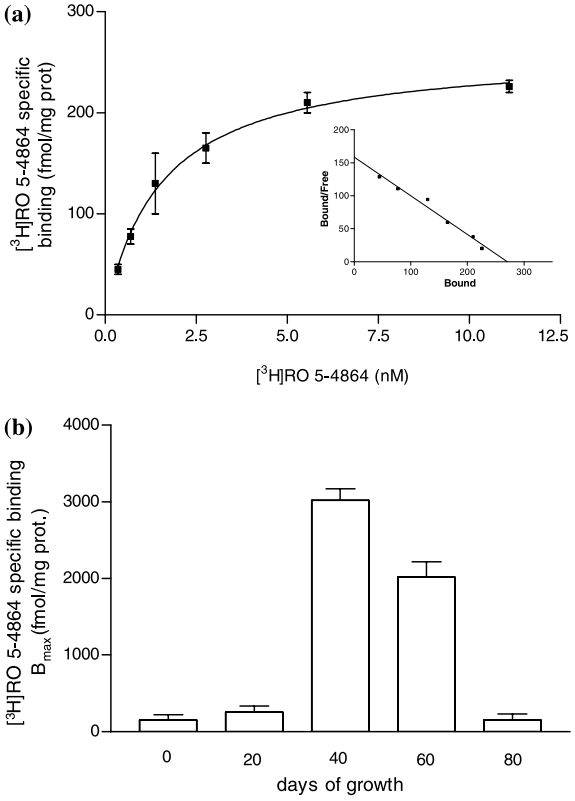

Fig. 2. Saturation curve and Scatchard analysis of [^{3}H]RO 5-4864 binding to potato membrane preparations (a). All data are plotted as average of 6 different saturation curves performed in duplicate. Variation of B_{max} (maximum number of binding sites) (b) during tuber growth. Each column represents the B_{max} obtained by Scatchard analysis of saturation curves of [^{3}H]RO 5-4864 binding to potato membranes. Data are represented as means ± SEM.

half-time of dissociation was 2 min and binding was completely reversible. A Scatchard analysis of the saturation curves performed with [^{3}H]RO 5-4864 yielded the following: $K_D = 1.54 \pm 0.283$ nM, $B_{max} = 261 \pm 15.5$ fmol/mg protein (Fig. 2a). Moreover to assess the functional implication of PBRs we tested the density of the receptor during tuber growth. As shown in Fig. 2b the amount of PBRs changed in parallel with the appearance and disappearance of meristem. The density was found to reach a maximum at 40 days when meristem is highly present and to decrease following the progressive meristem differentiation. The B_{max} value of PBRs ranged from 261 ± 16 fmol/mg at 20 days to a maximum of 3021 ± 100 fmol/mg protein at 40 days of growth. A binding study on tuber meristem preparation revealed a very high density of PBRs (3920 ± 98 fmol/mg protein) with a K_D of 4.78 ± 1.3 nM showing a similar affinity to that found in mammalian tissues.

Immunohistochemistry and Western blot analysis

Furthermore, to assess the distribution of PBRs, we performed an immunohistochemical analysis using a specific polyclonal antibody for PBRs. In meristem a very intense immunostaining was found in the majority of the cells while in parenchyma a few isolated cells were immunopositive (Fig. 3).

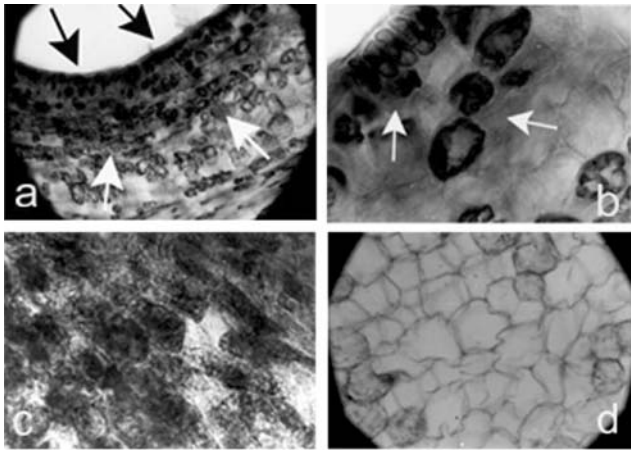

Fig. 3. PBR immunostaining in potato (*S. tuberosum*, Spunta variety). Intensive staining in meristem at low magnification (a). Higher magnification from meristem (b). Intense PBR staining in meristematic cells (c). Labelling in parenchymatic cells (d).

To confirm the presence of PBRs in our potato samples we investigated the expression of PBR protein by Western blot analysis. The antibody recognised predominately a 36 kDa protein whereas at 18 kDa molecular mass level only a faint band was seen. Neither treatment with increasing concentration of SDS, chaotropic agents, nor glycosidase enzymes were able to break this protein in smaller fragments suggesting an involvement of covalent cross-links. Interestingly an increase of 36 kDa PBR protein dimer was seen in tuber meristem compared to the parenchymal sample preparation (Fig. 4a). When the specificity of the immunoreactivity was evaluated by preabsorbing the specific PBR antibody with the recombinant PBR fusion protein, no activity was detected after the incubation (Fig. 4b). Moreover, we investigated the presence of PBR in different subcellular preparations of meristematic tissues, such as chloroplasts, mitochondria, and nuclei. As showed in Fig. 5 the presence of 36 kDa PBR protein was detected mainly in the nuclear fraction whereas in chloroplasts the protein was barely detected. No signal was detected in mitochondria preparation.

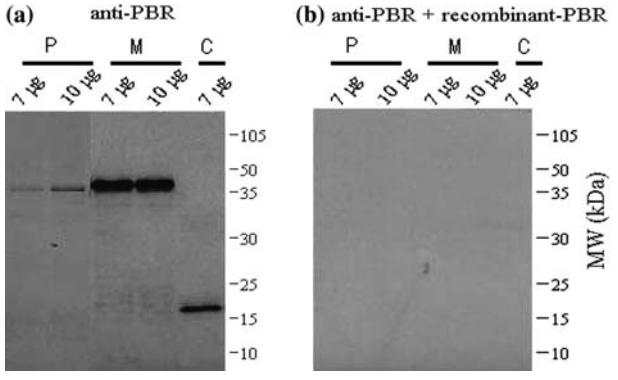

Fig. 4. Detection of PBR proteins in meristem and parenchymal membrane preparations. (a) Immunoblot analysis of meristem M and parenchyima P tissue extract loaded at different concentrations using specific polyclonal anti PBR antibody. C, immunoreactivity of recombinant PBR protein referred to as control. (b) Specificity of immunodetection seen in (a) examined by preabsorption of the ant-PBR antibody with the recombinant PBR protein.

To date PBR has been described in mammalian cells as one of the key proteins implicated in several functions including steroidogenesis, mitochondrial respiration, and cell proliferation. PBR has also been shown to be a component of the mitochondrial permeability transition pore involved in apoptosis. Recently it has been reported that excessive levels of ROS or continuously exposure to ROS could lead to the covalent polymerisation of all the newly formed PBRs, thus leading to the exclusive presence of higher polymers which could be detrimental to the mitochondrial function and cell viability [3]. In this paper we demonstrated for the first time the presence of PBR in vegetable tissue like potato tuber. In sterile cultivated tissues of *S. tuberosum* the presence of endogenous benzodiazepines has been already demonstrated [15]. The presence of such molecules in vegetables might not just be a coincidence and these molecules seem to be likely involved in the basic metabolic pathway of plant cells. Hence it was reasonable to search a receptor protein involved in several metabolic functions such as PBR.

Our results showed that [^{3}H]RO 5-4864, a specific PBR agonist, binds with high affinity ($K_D = 1.54 \pm 0.283$) to a homogeneous populations of binding sites in potato with a fast half-time of association and dissociation constant. A careful study of these recognition sites

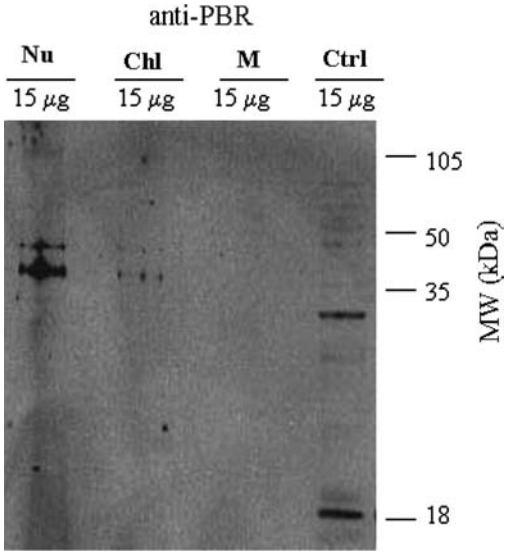

Fig. 5. Detection of PBR proteins in different subcellular preparations of meristematic tissues. Immunoblot analysis of nuclear fraction Nu, chloroplasts Chl, and mitochondria M using specific polyclonal anti PBR antibody. Ctrl immunoreactivity of rat adrenal gland protein preparation referred to as control.

showed a pH and temperature dependency with a maximal density of 3021 ± 100 fmol/mg of protein. The values of the affinity of RO 5-4864 and the kinetic characteristics of the binding sites observed in potato are quite similar to those found in rat brain. Characterisation of peripheral benzodiazepine binding sites in human brain tissue showed that [^{3}H]RO 5-4864 binds with high affinity ($K_D = 13 \pm 2$ nM) to the membranes with a density in the range of 135–340 fmol/mg protein [18].

Immunohistochemical study of PBR distribution revealed that PBR is heterogeneously distributed in potato and expressed selectively in meristematic cells. In particular the subcellular analysis showed the presence of PBR protein mainly in the nuclear matrix. When the data on the immunohistochemical distribution of PBR are compared with that on the density of RO 5-4864 binding, a striking correlation is observed: PBR immunoreactivity and RO 5-4864 binding are highly concentrated in meristem whereas the lower concentration of PBR immunoreactivity and RO 5-4864 binding are found in parenchyma.

Taken together our results suggest that high PBR expression in potato is a characteristic of nuclear meristematic cells. It was demonstrated in mammalian cells that PBRs regulate cell proliferation and/or steroid synthesis. Probably also in vegetable PBR could be involved in cell growth regulation.

Although the physiological role of the intracellular PBR remains uncertain, the patterns of their distribution in tissue suggest that these binding sites represent important control elements for the modulation of several metabolic functions. The suggestion that PBR is a target of ROS [3] together with the finding that ROS regulate plant cell expansion, a central process in plant morphogenesis [19], led us to infer that the presence of 36 kDa PBR protein in potatoes might be implicated in cell proliferation and vegetables growth. This finding is very important from the heuristic point of view, since until now, no such functional activity of receptor system was predicted in vegetables. Since PBRs have been described in mammalian tissues, the demonstration that PBRs are also present in vegetables suggests a highly conserved receptor system during phylogenesis.

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