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Revisión de Literatura

Calpain System in meat tenderization: A molecular approach

El sistema proteolítico calpaina en la tenderización de la carne: Un enfoque molecular

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Abstract:

Tenderness is considered the most important meat quality trait regarding its eating quality. Post mortem meat tenderization is primarily the result of calpain mediated degradation of key proteins within muscles fibers. The calpain system originally comprised three molecules: two Ca2+-dependent proteases and a specific inhibitor. Numerous studies have shown that the calpain system plays a central role in postmortem proteolysis and meat tenderization. The objective of this review is to describe the last biochemical and molecular findings in connection with this proteolytic system and their relation with meat tenderness in bovine. Findings of DNA polymorphisms and mRNA and protein expression are described as tools to predict meat tenderness. Understanding the molecular basis of meat tenderization may be useful particularly to the meat industry and may allow amendment of pre-slaughter handling practices and postmortem treatments that improves meat quality.

Keywords: Bovine, Molecular Markers, Tenderness.

Resumen:

La terneza de la carne es considerada como el atributo de mayor importancia en el concepto de calidad de carne. El proceso de tenderización de la carne post mortem es principalmente el resultado de la degradación de proteínas clave de las fibras musculares, mediado por las proteasas del sistema calpaína. Este sistema proteico está compuesto por tres moléculas: dos proteasas calcio-dependientes y su inhibidor específico. Numerosos estudios han demostrado que el sistema calpaína desempeña un papel central en la proteólisis postmortem y en la tenderización de la carne. El objetivo de esta revisión es describir los últimos descubrimientos bioquímicos y moleculares de este sistema proteolítico y su relación con la terneza de la carne bovina. Se describen los hallazgos de polimorfismos de ADN y de expresión de ARNm y proteínas, como herramientas para predecir la terneza de la carne. La comprensión de las bases moleculares de la tenderización de la carne puede ser de utilidad para la industria cárnica, permitiendo la modificación de las prácticas de manipulación antes del sacrificio y los tratamientos post mortem, mejorando la calidad de la carne bovina.

Palabras clave: Bovinos, marcadores moleculares, terneza.

INTRODUCTION

Meat quality is a main concern for livestock industries and consumers' needs. Some of the most important sensory attributes of meats are appearance, juiciness, flavour and tenderness (1). Previous studies have shown that the meat tenderization process is complex and could be affected by several different pathways including pre- and post-slaughter factors and their interaction (2).

The implementation of high-throughput analytical tools over the last two decades was an important step toward a better understanding of the complex biological systems that define muscle to meat conversion. It is well recognized that the biochemical postmorten processes are key steps in meat tenderization (3). At present, tenderization is unanimously regarded as an enzymatic process of proteolytic systems. Numerous authors suggest that calpains are the only proteases responsible for meat tenderization (4). Thus, numerous studies have focused on the factors influencing meat tenderness and their relation with calpain system. The present revision highlight the importance of the use of molecular approaches to unravel the mechanisms behind the variations in meat tenderization. The exploitation of these methodologies could deepen our understanding of the biological processes driving meat production. The thorough knowledge of the molecular mechanisms of the muscle-to-meat conversion would have a strong economic impact thanks to the improvements of the quality of the final products that would increase consumers' purchase.

CALPAIN SYSTEM

The calpain system in skeletal muscle comprises two Ca2+ dependent proteases, calpain 1 and 2, and a third polypeptide, calpastatin, whose only known function is to inhibit the two calpains. This system has a number of different roles in cells, including but not limited to the "remodeling" of cytoskeletal attachments to the plasma membrane during cell fusion and cell motility, the proteolytic modification of molecules in signal transduction pathways, the degradation of enzymes controlling progression through the cell cycle, the regulation of gene expression, substrate degradation in some apoptotic pathways, and involvement in long-term potentiation (5).

Calpains. Calpains are Ca2+-dependent, cysteine proteases. So far, 15 calpains have been described in mammalian (6) The two most often described proteases are CAPN1 and CAPN2, termed μ- and m-calpain, alluding to their micromolar and millimolar Ca2+ requirement (5). Both proteases are heterodimeric, each composed of an 80 kDa catalytic subunit and a regulatory subunit of 28kDa (7).

The 80 kDa subunits are different gene products (genes on chromosomes 29 and 16 in bovine, respectively). These subunits can be divided into four domains on the basis of their amino acid: the N-terminal anchor helix region; the protease core domains: PC1, an NH2-terminal domain, and PC2, the catalytic domain with a triad residue (Cys-His-Asn) characteristic of cysteine proteases; the C2-like domain (C2L); and the penta-EF-hand domain of the large subunit PEF(L) (8) (Figure 1). The 28 kDa subunit, common to both calpains, has two domains: the GR (glycine-rich) and PEF of the small (S) subunit (8) (Figure 1).

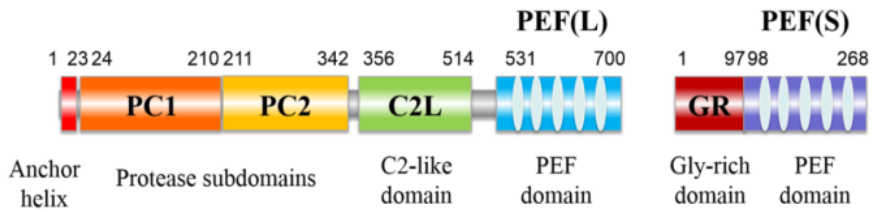

Figure 1. Schematic structure of calpain.
Conventional calpains are composed of catalytic and small regulatory subunits. Domain structures are defined according to the text. The Anchor helix, the protease core domains (PC1 and PC2); the C2-like domain (C2L); the penta-EF-hand domains of the large and small subunits (PEF(L) and PEF(S)), and the glycine-rich (GR) domain of the small subunit. Residue numbers flanking each domain indicate domain boundaries. Adapted from Sorimachi et al.(8).

FIGURE 1
Figure 1

Calpains (1 and 2) have been detected in every vertebrate cell carefully examined for their presence (5). Different tissues/cells, however, differ widely in their protein ratios. Inmunolocalization studies have shown that both, CAPN1 and CAPN2, are located intracellularly along the Z disk/I band regions in the form of

intracellular stores (9). Raynaud et al (10) found that CAPN1 is concentrated on the N1 and N2 line region of titin and suggested that this might constitute a reservoir for the cell. Many calpain substrates, including titin, nebulin, filamin, troponin-T and desmin, which attaches the sarcolemma to the Z-disc, are co-localized and proteolyzed during meat tenderization (9). Although calpains are considered to be cytoplasmic enzymes, recent researches have shown that they are also presents in several subcellular organelles such as caveolae vesicles (11), endoplasmic reticulum (ER) (12,13), mitochondria (14), Golgi apparatus and nucleus (15,16) showing the multifunction of this family.

Due to the recent increase in the number of calpain-like molecules, Goll et al (5) proposed a nomenclature system. In this system, calpain genes have been named numerically, capn1 through capn15, and the polypeptides encoded by these genes have also be named numerically, CAPN1 through CAPN15 (Table 1). At present, two small subunits, CAPNS1 and CAPNS2, have been identified as well. The CAPNS1 subunit is an absolute requirement for the stability of both conventional calpain catalytic subunits in vivo, whereas CAPNS2, similar to CAPNS1, has no clear physiological clear role to date (8).

Calpains, based on their domain composition, can be divided into two general classes: "typical" calpains, those possessing a calmodulin-like domain at their C-terminus, and "atypical" calpains, those lacking a calmodulin-like domain IV at their COOH terminus. Considering their tissue distribution, calpains can also be classified into ubiquitous or tissue-specific calpains (7). The calpain-like molecules reported in bovine to date are described in Table 1.

TABLE 1.
Table 1. Bovine genes for calpains and their regulatory subunit

Gene/ Protein	mRNA accesion code	pb	Aliases	protein accesion code	Aa	Chrom	Typical/ Atypical	Distribution	Phenotype of gene defficency in mice	Domainsa				Splice Variantsb
										PC	C2L	C2	PEF	
Catalytic subunits														
capn1	NM_174259	2948	μ-calpain μ80k	NP_776684	716	29	Typical	ubiquitously	Platelet dysfunction	+	+	-	+	-
capn2	NM_001103086	3216	m-calpain m80K	NP_001096556	700	16	Typical	ubiquitously	Embryonic lethal	+	+	-	+	-
capn3	NM_174260	2955	nCL-1 p94	NP_776685	822	10	Typical	skeletal muscle, retina and lens specific	Muscular distrophy	+	+	-	+	+
capn5	NM_001192894	4487	nCL-3 hTRA-3	NP_001179823	640	15	Atypical	ubiquitously	Sudden death	+	+	+	-	+
capn6	NM_001192231	2522	calpamodulin	NP_001179160	641	X	Atypical	placenta, uterus	Development of embryonic skeletal muscle	+	+	+	-	-
capn7	NM_001193181	3522	palBH	NP_001180110	813	1	Atypical	ubiquitously	n.r.	+	+	-	-	-
capn8	NM_001081619	1952	nCL-2	NP_001075088	381	16	Typical	exclusive to stomach mucosa and the GI track	stress-induced gastric ulcer	+	+	-	-	-
capn9	n.r.C	n.r.	nCL-4	n.r.	n.r.	26	Typical	exclusive to stomach mucosa and the GI track	stress-induced gastric ulcer	n.r.	n.r.	n.r.	n.r.	n.r.
capn10	XM_01082719	2103	calpain 10	XP_010825497	565	3	Atypical	ubiquitously	No significant phenotype	+	+	-	-	+
capn11	XM_010818270	2402	calpain 11	XP_010816572	783	23	Typical	testis	n.r.	+	+	-	+	+
capn12	NM_001192697	2166		NP_001179626	721	18	Typical	hair follicle	n.r.	+	+	-	+	+
capn13	NM_001035360	2442		NP_001030437	516	11	Atypical	ubiquitously	n.r.	+	+	-	-	+
capn14	NM_001192425	2178		NP_001179354	685	11	Typical	ubiquitously	n.r.	+	+	-	+	+
capn15	XM_005224584	5216	SOLH	XP_005224641	1139	25	Atypical	ubiquitously	n.r.	+	-	-	-	+
Regulatory subunits														
capns1	NM_174261	1315	css1 30K	NP_776686	263	18	[d]	ubiquitously	Embryonic lethal	-	-	-	+	-
capns2	XM_002694786	2150	css2	XP_002694832	246	18	[d]	ubiquitously	n.r.	-	-	-	+	-

[a]+ or - indicates that the molecule has, or does not have, a corresponding domain. For acronyms, see Figure 1, [b]+ or - indicates the presence or absence of splice variants □ Not yet reported [d] CAPNS1 and CAPNS2 are not calpains according to the definition adopted in this review

Calpastatin. Calpastatin (CAST) is the only known endogenous protein inhibitor for calpains. Although only a single calpastatin gene exists in humans, pigs, mice and bovine, more than eight calpastatin isoforms have been identified in the tissues of those organisms, suggesting different levels of translation start at the promoter or alternative splicing mechanisms (6,17).

The genomic sequence of bovine CAST contains 35 exons spanning nearly 130 kp (18). Based on amino acid sequences, six different domains can be recognized. The XL domain, presents three protein kinase A

(PKA) phosphorylation sites, the L domain, which varies in size due to alternative splicing and has been reported to be involved in binding calpastatin to biological membranes having a central role in the regulation of Ca2+ channel (19).

Domains I, II, III and IV can inhibit proteolytic activity of either CAPN1 or CAPN2. Each domain has three conserved amino acid sequences, termed subdomains A, B and C respectively. Subdomain A is a 14-amino acid sequence which binds specifically to domain IV of calpain in a Ca2+-dependent manner (7). Subdomain B is a 12-amino acid sequence essential for inhibitory activity. Subdomain C is also a 14-amino acid sequence that binds specifically to domain VI of calpain (Figure 2). The intact protein is capable of simultaneously binding to and inhibiting four calpain molecules (20).

Figure 2. Schematic structure of calpastatin.

Domain structures are defined according to the text. The XL domain contains three protein kinase A (PKA) phosphorylation sites; the L domain, which varies in size due to alternative splicing and has a central role in Ca2+ channel regulation; domains I, II, III and IV have three conserved amino acid sequences, termed subdomains A, B and C respectively. Subdomain A binds specifically to domain IV of calpain in a Ca2+-dependent manner; subdomain B is essential for inhibitory activity and subdomain C binds specifically to domain VI of calpain.

FIGURE 2
Figure 2

The variation in meat tenderness between species, breeds and individuals is partly responsible for the variation in calpastatin expression and differential transcriptional activity of calpastatin gene promoters (21), therefore it is very important to determine its concentration.

MOLECULAR APPROACH

In this section we highlight data collected about the calpain system polymorphisms at DNA level, expression at RNA level and activity at protein level, and their involvement in the tenderization process.

DNA polymorphisms. There are many single nucleotide polymorphisms (SNPs), however in this review, we will focused in those that are used in commercial tenderness test.

In 2000, the bovine capn1 gene was mapped to the telomeric end of BTA29 (Bos taurus autosome 29) (22) and a QTL (quantitative trait locus) for tenderness was found to segregate in this region (23,24). The markers CAPN1 316, a guanine to cytosine transversion in exon 14 (CC, CG or GG genotype) and CAPN1 530, an adenine to guanine transition in exon 9 (AA, AG, or GG genotype) were identified and associated with shear force values in Bos Taurus (25)(Figure 3). Animals homozygous for the C allele at marker 316 had lower shear force than animals of the CG or GG genotype while animals with homozygous G genotype at marker 530 had lower shear force than animals of the AG or AA genotype (26,27). The groups of animals, which were also analyzed with both markers fitted simultaneously, showed that specimens homozygous for the C/G haplotype are associated with the most favourable shear force phenotype. Nevertheless, these markers

were not validated in Bos indicus animals (28,29). In Argentinean breeds, contrary to previous studies, steers inheriting the AG genotype at marker CAPN1 530 had lower shear force (30).

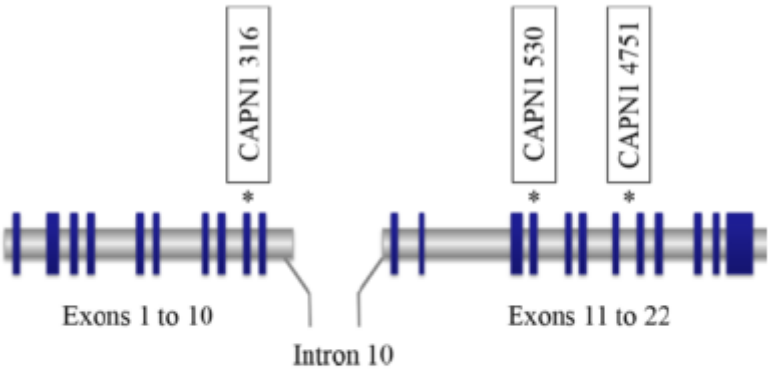

Figure 3. Genomic locations of SNP markers used to predict meat tenderness in capn1 gene.

Blue boxes represent exon sequences and the grey connective line represents the intron sequence. The SNP encoding meat tenderness is shown as asterisks. The CAPN1 316 marker is a cytidine/guanosine (C/G) transversion in exon 9 (base 5709 of AF252504) found by Page et al. (25). The CAPN1 530 marker is an adenosine/guanosine (A/G) transition in exon 14 (base 4558 of AF248054) found by Page et al. (25). CAPN 4751 is a cytidine/thymine (C/T) transition in intron 17 (base 6545 of AF248054) found by White et al. (31).

FIGURE 3
Figure 3

The new marker CAPN1 4751 was useful in populations of Bos taurus, Bos indicus, or Bos indicus x Bos taurus crossbred cattle (28,31,32) (Figure 3). Several studies in animals from different breeds demonstrated that those with the CC genotype at CAPN1 4751 had more tender meat than those inheriting the TT genotype (32,33,34). However, other authors found that the most favourable genotype in Bos indicus and its crosses with Bos taurus were CT alleles (35).

The cast gene, mapped to BTA7, is considered a candidate gene for beef tenderness. Several SNPs were found in calpastatin gene (29,36,37,38). One adenine to guanine transition in the exon 30/3 ′UTR region, termed as CAST T1; one guanine to cytosine transversion intron 5, termed UOGCAST, and a cytosine to thymine transition in exon 3 called WSUCAST (Figure 4). Schenkel et al. found a relationship between meat tenderness, measured as WBSF, and genotype in Bos taurus (38). Genotype CC yielded beef that was more tender than GG while CG showed intermediate tenderness. All these results indicate that the effects of the markers are breed-specific and cannot be extended to all breeds (29).

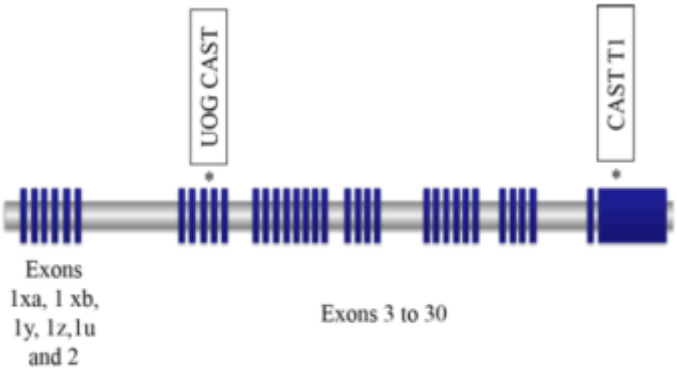

Figure 4. Genomic locations of SNP markers used to predict meat tenderness in cast gene.

Blue boxes represent exon sequences and the grey connective line represents the intron sequence. The SNPs encoding meat tenderness are shown as asterisks. The UOGCAST marker is a cytosine/guanosine (C/G) substitution in intron 5 (base 282 of AY008267) found by Schenkel et al (38). The CAST T1 marker is an adenosine/guanosine (A/G) transition in exon 30 (base 2959 of AF159246) of the 3 ´UTR region found by Barendse (36).

FIGURE 4
Figure 4

Epistasis studies to evaluate possible interactions between SNPs on the CAPN1 gene and SNPs on the CAST gene were conducted and demonstrated that marker CAPN1 4751 has a significant additive-by-additive interaction with the markers WSUCAST and UOGCAST (32). These studies suggest that WSUCAST and UOGCAST are linked and are probably heredity like haplotypes.

RNA expression. The expressions of calpains and calpastatin at the mRNA and protein levels have been often determined in an attempt to understand the role of the calpain system in myofibrillar protein metabolism and meat tenderization. Generally decreased calpain expression or increased calpastatin expression is associated with toughness meat (39).

Some authors suggest that muscle type and/or fiber type can influence calpain and calpastatin expression and/or activities and therefore could account for post mortem proteolysis and meat tenderization (39,40,41). Differences in the small subunit (CAPNS1) and variants in CAST expression were found in slow-type muscles compared to fast-type muscles (40). Calpastatin gene expression has an average of nearly 30% in high WBSF groups compared with low WBSF at 6 days postmortem (42). Moreover, calpain-1 mRNA abundance was slightly higher and calpastatin mRNA was lower in longissimus lumborum than infraspinatus (41), suggesting that the higher proportion of glycolytic fibers could improve the tenderness of certain muscles by accelerating post mortem aging due to the presence of a higher calpain/calpastatin ratio (43). Other researcher reveal that the slightly lower tenderness of meat from Zebu animals is probably not a result of lower expression of genes encoding proteases, but rather is due to the increased expression of CAST (44).

Some researchers found that gender alter mRNA levels of the calpain system. They show that bulls had lower WBSF values than heifers, which were accompanied by higher levels of CAPN1 and similar levels of CAST (45). Suggesting that variation in beef tenderness could be modulated through the differential expression of the members of the calpain system.

The maternal energy status during gestation alters meat tenderness. In a recent study, Jennings et al. found up-regulation of CAPN1 in longissimus muscle of high diet treatment foetuses (46). Feeding strategies have also impact in meat tenderness. Supplementation with vitamin D and zilpaterol hydrochloride had no significant impact on the expression of calpain-1 and calpastatin mRNA levels (47). Nevertheless, in previous

studies in ours laboratories, the supplementation with corn silage during finishing alter calpains (1 and 2) and calpastatin expression, and meat tenderness, suggesting that managing feeding system has a regulatory effect on biological processes that occur in the muscle, defining the final quality of the meat.

The relationship between polymorphism described above and gene expression was also evaluated. Niciura et al (48) found that CAST was expressed twice as much in muscle of homozygous GG as in heterozygous AG in the CAST T1 marker. Natrass et al (49) investigated this relationship in Bos indicus and Bos taurus cattle and found differences in CAPN1 and CAST gene expression between favourable and unfavourable allelic variants of these genes (CAPN1 4751 and CAST T1 markers), indicating a polymorphic effect on gene expression. These findings suggest that differences in tenderness explained by gene markers could be a consequence of the alteration in their mRNA levels, protein activity and rate and (or) the extent of postmortem proteolysis in skeletal muscle. Studies should try to identify genetic and epigenetic events that may control the differential gene expression attributed to polymorphism.

Protein quantification and activity. Although Ca2+ -dependent proteases were identified in 1964, until the 90s there were no details of the procedures for extraction and determination of calpain proteolytic system activities.

It has been demonstrated that pH and temperature cause dramatic effects on CAPN1 autolysis and activity (50,51). Increased temperature or decreased pH cause a more rapid decline in CAPN1 activity. Calpastatin activity also decreased at elevated temperatures (51,52) while its inhibition of CAPN1 was apparently uninfluenced by pH (50). It is important to note that different temperatures and pH were studied by these authors, but during postmortem meat tenderness these parameters vary together; studies to perform these analyses are needed to determine the overall effect generated during aging.

During the storage period, the evidence suggest that CAPN1 and CAST activities declined significantly, whereas CAPN2 activity remained stable (9,53,54). In a recent research, calpain-1 activity was detected up to 42 days of post-mortem, and autolyzed calpain-1 up to 70 days post-mortem. The calpain-2 activity increase during the aging period, peaking at day 42 before decreasing sharply at day 70 (55). Differences in activity could be ascribed to the type of muscle chosen or the cattle breed. Although some researchers suggested that loss of CAPN1 and CAST activities is due to the proteolytic degradation of these molecules, while others attributed it to extensive autolysis of CAPN1 and the breakdown of CAST possibly by calpains (39,56), it is uncertain whether the masking effect proposed by Kristensen et al. is the real cause of the decline in calpastatin activity (57). These authors demonstrated that the calpain system proteins are stable during the frozen storage period at -20°C or -80°C and that calpastatin activity is underestimated. These results suggest that calpain 1 contribute to early posmortem tenderization improvement and calpain 2 is responsible for additional tenderization during extended aging.

Calpastatin activity in muscle from older animals is more persistent postmortem than in muscle from growing animals (58). This difference may contribute to decreased protein degradation and increased toughness of beef from mature cattle, even after aging. These authors, also found that CAPN1 activity per unit of CAST activity in the muscle from growing animals is greater than in mature animals. Tissues with a greater CAPN1: total CAST ratio is expected to allow more postmortem protein degradation than tissues with a lesser CAPN1: total CAST ratio. Differences between young bulls and steers in CAPN1 and CAPN2 protein quantification were also detected (59). This effect could be the result of sex hormone action as previous studies have shown (60).

Feeding strategies during the growing and finishing phases have also impact in protein expression and meat tenderness (61). A recently study suggest that calpains, and most probably CAPN1, are more active in longissimus dorsi than in infraspinatus (63). In recently studies in our laboratories, finishing animals with corn-silage produced changes in CAPN1 and CAST activity (not published). Feeding strategies can modulate muscle protein turnover, muscle energy levels at slaughter and water holding capacity; and consequently, can modify meat tenderness, the pH/T decline curve and sensory characteristics of meats.

Thus, one of the goals to successfully implement a compensatory feeding approach is to establish the length of the compensatory period which results in the highest muscle protein degradation potential at the time of slaughter.

In conclusion Calpain system plays a central role in postmortem proteolysis and meat tenderization. Studies over the last decade indicate that CAPN1, CAPN2 and CAST plays an important role in postmortem degradation of myofibrillar proteins and tenderization of muscle during refrigerated storage. Polymorphisms in CAPN1 and CAST were evaluated as tools to predict meat tenderness, nevertheless, the results indicate that the effects of the markers are breed-specific. Muscle type, fiber type, gender, age and feeding strategies can influence calpain and calpastatin expression and/or activities. Muscle conversion involves several complex processes influenced by pre- and postmortem handling. New studies should try to identify the genetic and epigenetic events that may control the differential gene expression to explain these processes. New researches should focus on understanding how these polymorphisms/genes/proteins interact with the environment or with other genes, affecting economic traits.

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