membrane changes that expose proteases. Cell-associated—as opposed to secreted—proteases could be subject to tight local control by the cell. Degradation of the basement membrane by such proteases would occur only at the point of contact of the cell surface with the matrix. This hypothesis is consistent with ultrastructural studies of early angiogenesis (*1, 2*). The endothelial metalloproteinases identified for the first time in this study may be of importance in the physiologic turnover of the subendothelial matrix in the established microvasculature. Control of such enzymes may be altered in pathologic processes that exhibit a defective or altered basal membrane such as diabetes and cancer invasion.

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14. The tertiary structure of the native type IV and type V collagen substrates was examined by metal shadow-casting and electron microscopy (*5*). Type IV collagen molecules appeared as ropelike structures and retained the globular region at one end and the 7-S domain at the other end. Type V collagen appeared as intertwined ropelike filaments. α-Thrombin degrades denatured but not native type IV and type V collagen at 30°C [L. A. Liotta, R. H. Goldfarb, V. P. Terranova, *Thromb. Res.* **21**, 663 (1981)]. α-Thrombin degraded less than 5 percent of the native substrates bound to nitrocellulose. In contrast, if the substrates were heat-denatured, α-thrombin degraded 48 and 64 percent of the type IV and type V collagen, respectively.
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Murine I-Aβ Chain Polymorphism: Nucleotide Sequences of Three Allelic I-Aβ Genes

Abstract. *The polymorphism of immune response genes plays a critical role in determining the immune capabilities of a particular individual. The molecular nature of this polymorphism was studied by examining the structure of the coding portions of three alleles of the I-Aβ chain gene, an immune response gene whose protein product constitutes a subunit of the I-A molecule. Comparison of the I-Aβ chains encoded by these alleles revealed an amino acid sequence divergence of 5 to 8 percent. The differences were found to be a series of short alterations clustered in the amino terminal half of the polypeptide.*

The major histocompatibility complex (MHC)-linked immune response (Ir) genes determine the phenotypic ability of an animal to develop a high level of humoral or cell-mediated immunity to a defined antigen (*1, 2*). Recent studies have revealed that the class II, MHC-encoded, cell-surface glycoproteins (Ia in the mouse and DR or DR-like in man) are the structural products of these Ir genes, whose function is involved in complex regulatory interactions among

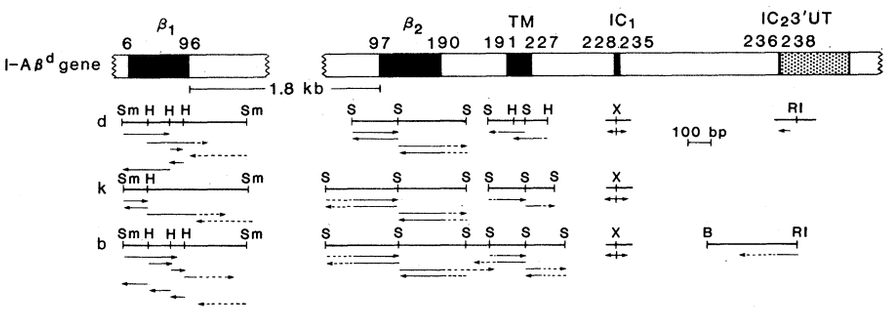

Fig. 1. The organization and sequencing strategy of I-Aβ genes in the mouse. The exons of the I-$A\beta^d$ gene are represented by solid bars in the coding regions and by a stippled bar in the 3′ untranslated (3′ UT) region. The exons encoding the two extracellular domains are labeled β_1 and β_2. The distance between the β_1 and β_2 exons is 1.8 kb in the I-$A\beta^d$ gene, 2.0 kb in the I-$A\beta^b$ gene, and 2.7 kb in the I-$A\beta^k$ gene. The exon encoding the transmembrane region is labeled TM, and the two exons encoding the intracytoplasmic region are labeled IC_1 and IC_2. The numbers above the solid bars indicate the amino acid residues encoded by that exon. Where an intron divides a codon between base positions 1 and 2, the amino acid residue for that codon is assigned to the 5′ exon. The 3′ UT region is contiguous with IC_2, and the sequence of the first 220 nucleotides of the 3′ UT region does not show an intervening sequence (data not presented). Genomic clones were mapped with restriction enzymes, and the locations of the exons encoding the TM, IC, and 3′ UT regions were determined by hybridization to the mouse I-Aβ cDNA clone pI-Aβ-1 (*7*). The locations of the exons encoding the β_1 and β_2 domains were determined by hybridization to a human HLA-DR-like β-chain cDNA clone (*10*). The appropriate restriction fragments from the three genomic clones were subcloned into the plasmid vectors pBR322 or pBR327. The subclones were digested with the restriction enzymes Sau 3AI, Hpa II, or Sma I and further subcloned into M13 mp9 for nucleotide sequencing by the dideoxy chain termination method (*19*). The M13 recombinants were screened with the human HLA-DR-like β-chain cDNA probe. The inserts in these M13 recombinants were sequenced and aligned according to their homology with either the human HLA-DR-like β-chain cDNA sequence or the mouse pI-Aβ-1 cDNA sequence. The sequencing strategies for the d, k, and b haplotypes are presented below the organization of the gene. The restriction sites are designated as follows: *Sm*, Sma I; *H*, Hpa II; *S*, Sau 3AI; *X*, Xho I; *RI*, Eco RI; and *B*, Bam HI. Arrows represent the extent of reading from a restriction site. The sequences from the Xho I site in IC_1 in all three haplotypes and from the RI site in the 3′ UT region in the d haplotype were determined by the chemical degradation method of Maxam and Gilbert (*20*). All other arrows represent sequences derived from M13 subclones. The dashed portions of the arrows represent sequences determined but not presented in Fig. 2. For all M13 subclones from the three haplotypes which had inserts starting at analogous restriction sites, the products of the sequencing reactions were subjected to electrophoresis side by side on the sequencing gels in order to facilitate comparisons between the haplotypes.

T lymphocytes, B lymphocytes, and macrophages. In particular, Ia molecules are critical to antigen-specific, MHC-restricted activation of T cells by macrophages and of B cells by T cells. These structures also play a major role in stimulating the allogeneic mixed-lymphocyte reaction.

A striking feature of Ia molecules is their high level of intraspecies polymorphism. This polymorphism is of functional significance, since recognition of antigen by many T lymphocytes requires simultaneous recognition of allele-specific determinants on the Ia glycoprotein. Immunogenetic, serologic, and biochemical studies have demonstrated that, in mice, Ia molecules consist of two major types of heterodimeric glycoproteins termed I-A and I-E. Each molecule contains an α (or heavy) chain of molecular weight 33,000 and a β (or light) chain of molecular weight 28,000, noncovalently associated on the plasma membrane. In the cytoplasm, these two chains are associated with a third, non-MHC-linked gene product, the invariant chain (Ii; molecular weight, 31,000), whose function is unclear (*3, 4*). Both the α and β polypeptides are transmembrane molecules (*4, 5*). Peptide mapping has revealed extensive polymorphism of the I-Aβ, I-Eβ, and I-Aα chains and less polymorphism of the I-Eα chains (*2*). However, conventional protein analytic methods are inadequate for rapid, complete determination of protein sequence of numerous allelic forms of these genes and cannot approach the critical issue of the underlying genetic mechanism for the generation and maintenance of the extensive polymorphism seen in this gene family. For these reasons, we undertook to analyze these genes using recombinant DNA technology. We report the overall organization of the I-Aβ gene and the nucleotide sequences of the coding regions of three alleles (b, d, and k) of this gene. This analysis reveals a striking concentration of amino acid substitutions in the NH_2-terminal domain of this molecule in a pattern consistent with multiple independent mutational events.

A standard approach to cloning eukaryotic genes is to identify a complementary DNA (cDNA) clone constructed from messenger RNA (mRNA) of a cell making the relevant gene product and then to use this cDNA clone as a probe to isolate a bacteriophage or cos-

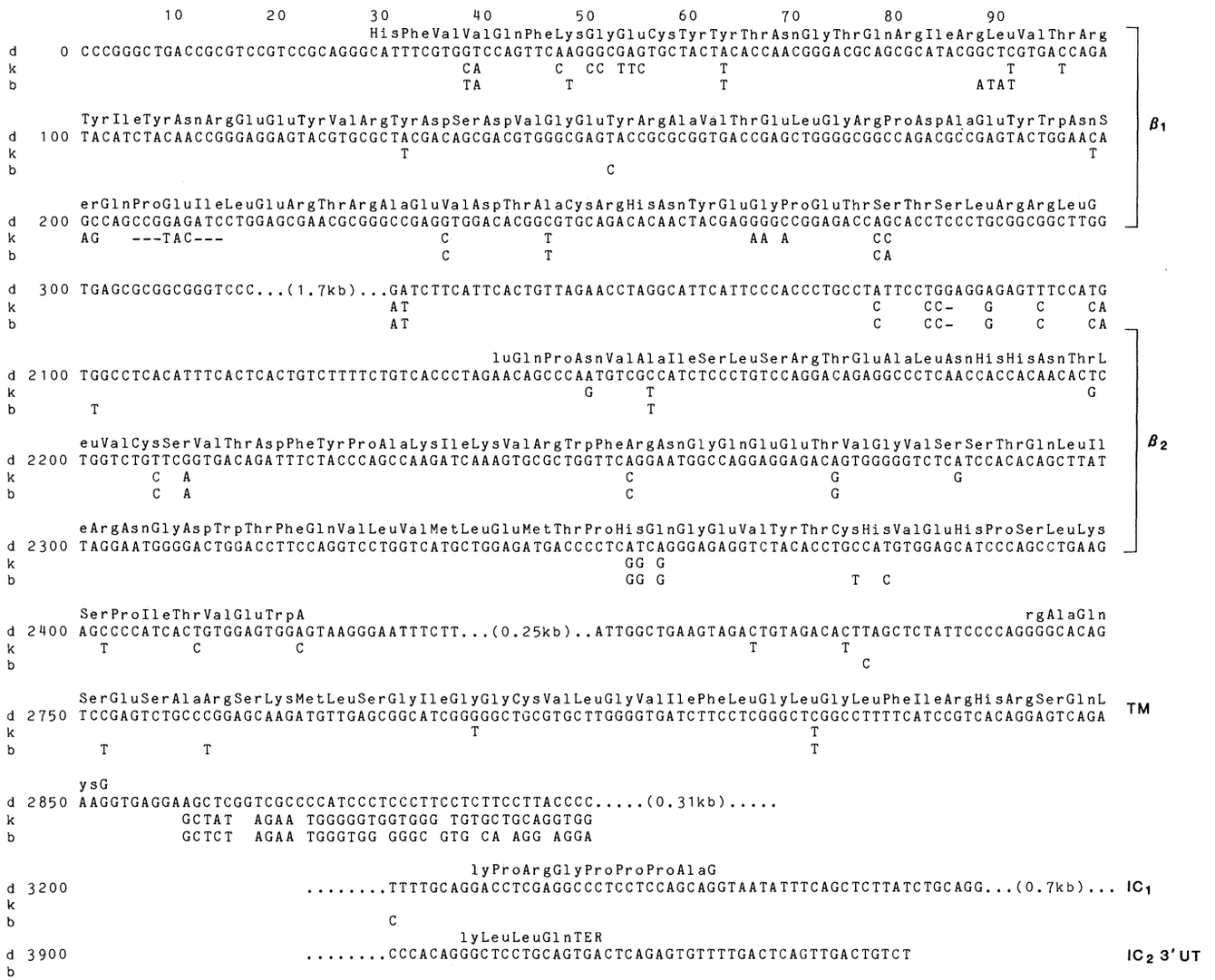

Fig. 2. Nucleotide sequence of the I-Aβ exons and portions of the flanking introns for the d, k, and b haplotypes of the mouse. The translation of the exons from the d haplotype is indicated above the sequence. Only those nucleotides that are different from the d haplotype sequence are shown below for the k and b haplotypes. Dashes represent gaps inserted into a sequence in order to maintain maximal alignment. The nucleotide sequence of $IC_2$3′UT has not been determined in the k haplotype. The abbreviations for nucleic acid residues are: A, adenine; C, cytosine; G, guanine; and T, thymine. Abbreviations for amino acid residues are: Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Glu, glutamic acid; Gln, glutamine; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; and Val, valine.

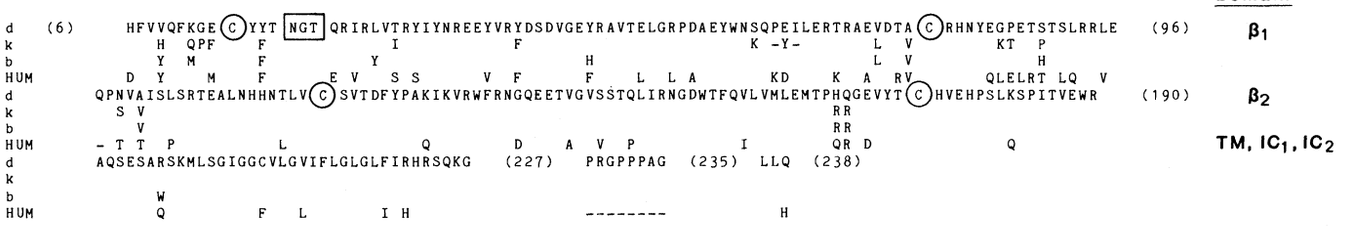

Fig. 3. Amino acid sequence comparison of the I-Aβ chains of the d, k, and b haplotypes and a human β chain (*10*). The protein sequence of the d haplotype I-Aβ chain as determined from our nucleotide sequence is shown with the one-letter amino acid code (A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; and V, valine). Amino acid residues that are different from those of the d haplotype sequence are shown for the other three β chains. Dashes represent gaps inserted into a sequence to maintain maximal homology. The cysteine residues thought to be involved in the formation of intrachain disulfide loops in both the β_1 and β_2 domains are circled. The boxed residues indicate a putative glycosylation site.

mid clone from a genomic library (*6*). This procedure was used to clone I-Aβ genes from three strains of mice. The isolation of a cDNA clone corresponding to a portion of the I-$A\beta^d$ mRNA (derived from BALB/c Ia^+ B-lymphoma mRNA) has been reported (*7*). This cDNA clone, called pI-Aβ-1, contains a 465–base pair insert that encodes the transmembrane, intracytoplasmic, and 3′ untranslated regions of the I-Aβ mRNA. Southern blot analysis of mouse DNA with the I-Aβ cDNA clone suggested that the I-Aβ sequence is present in only one copy in the mouse genome (*7*). Using this cDNA clone to screen genomic libraries, we isolated bacteriophage clones with DNA inserts encompassing the I-$A\beta^d$ (BALB/c) and I-$A\beta^k$ (A/J) genes as well as a cosmid clone containing the I-$A\beta^b$ gene (C57BL/6) (*8, 9*).

The overall structural organization (Fig. 1) and the nucleotide sequence of the coding portions (Fig. 2) of the I-Aβ chain genes on each of these genomic clones were determined. The gene consists of at least six exons, five of which are identified in this report. These five encode two extracellular domains (β_1 and β_2), the transmembrane region (TM), and the intracytoplasmic region (IC_1 and $IC_2$3′UT). The locations of the intervening sequences in this gene were determined primarily by comparing cDNA sequences with the genomic DNA sequences. However, since the mouse cDNA clone does not extend to the β_1 domain, the location of the intervening sequence between the β_1 and β_2 domains, as well as the 5′ intron-exon boundary of the β_1 domain, were inferred by comparison to a human HLA-DR-like β-chain cDNA sequence (*10*) and by observance of known splicing rules (*11*). The protein sequence of the mouse Aβ chain between amino acid residues 6 and 238 was deduced from the nucleotide sequence of these five I-Aβ exons (Fig. 3). The first five amino acids and a presumed leader peptide for the Aβ chain must be encoded on a separate exon.

Independent confirmation that the genes described here do indeed correspond to the I-Aβ chain comes from comparison of the predicted amino acid sequences of the proteins encoded by these genes with the amino acid sequences available for the I-Aβ chain. Partial NH_2-terminal amino acid sequencing has revealed a total of 13 residues for the three I-Aβ chains of the b, d, and k haplotypes (*12*). The amino acid sequences predicted from the nucleic acid sequences (Fig. 3) are in good agreement with these partial amino acid sequences, with the exception that our sequence predicts a glutamine residue instead of proline at position 12 in the k haplotype. (We suggest that either an error has been made in the amino acid sequence at this position or the A/J allele of I-$A\beta^k$ differs from the C3H allele.)

Comparison of the amino acid sequences of the I-Aβ chain and the human HLA-DR-like β chain (*10*) reveals approximately 75 percent homology. The structure of β_1 and β_2 domains, as defined by intradomain disulfide loops between invariant cysteine residues, appears to be conserved between mouse and human, as does a potential glycosylation site in the β_1 domain (*13*) (Fig. 3). The region of sharpest nonhomology between the mouse and human sequences is at the juncture of the β_1 and β_2 domains, where six adjacent amino acids are different in the two sequences and where the mouse has one extra amino acid relative to the human (Fig. 3). As in the human sequence, the transmembrane region of the mouse sequence includes a stretch of amino acids from position 200 to 220 that is highly hydrophobic, with the exception of a cysteine residue at position 207 that is not found in the human. One striking difference between the mouse and human β chain sequences is that there are eight extra intracytoplasmic amino acids in the mouse I-Aβ chain. These eight amino acids are encoded separately by the IC_1 exon. This finding raises the possibility that a different form of the mouse I-Aβ mRNA may be created by splicing out the IC_1 coding block in a fashion analogous to the processing of class I antigen mRNA (*14*). Alternatively, the IC_1 coding block may be deleted from the human gene.

Protein biochemical studies on murine and human β chains have led to several conclusions concerning the structural basis for the known functional intraspecies polymorphism of the gene products. Uhr *et al.* (*2*) demonstrated that allelic I-Aβ chains differ by as much as 60 percent of their tryptic peptides, suggesting numerous differences in the amino acid sequences of these chains. Moreover, Kaufman and Strominger (*15*) pointed out that the polymorphism of HLA-DR-like β chains is largely confined to the amino terminal half of the polypeptide. The data presented here confirm and extend the conclusions of these earlier studies. Pairwise comparisons of the amino acid sequences of three alleles of the I-Aβ chain reveal that they differ markedly from one another—by 12 to 18 amino acid substitutions in 233 amino acid residues. Furthermore, there are three positions at which each of the three chains has a different amino acid. Of particular interest is the existence of a small deletion in the middle of the β_1 domain in the k haplotype, in which three amino acids have been replaced by a single tyrosine residue that could not have derived from any of the three by a simple point mutation. This pattern of polymorphism is different from that observed for other well-studied proteins expressed in these strains. For example, the two allelic forms of β_2-microglobulin that have been identified differ by only a single amino acid residue (*16*). The differences in the amino acid sequences are

sufficiently scattered over the length of the I-Aβ chain to account for the extreme variations that were noted in the tryptic peptide maps. However, the fact that most of the differences are confined to the β_1 domain suggests that mouse I-A and human HLA-DR-like β-chain genes have evolved along similar lines. Specifically, the Aβ chain of one individual is 5 to 10 percent different from that of another individual, and most of these differences are found in the β_1 domain. This clustering of variation in the NH_2-terminal extracellular domain of this polypeptide may be related to the function of Ia molecules as targets for T-cell recognition and cell-to-cell interactions.

The comparison of the amino acid sequences of the I-Aβ chain alleles suggests that the evolution of these genes has not involved gross insertions or deletions in the exons. By contrast, restriction map analysis of the sequences flanking the I-Aβ chain exons (*7, 17*) suggests that there have been large insertions and deletions in these regions. In particular, the intron between β_1 and β_2 differs in size (by as much as 0.9 kilobase) in all three of the haplotypes we examined (Fig. 1). Also striking is the complete sequence divergence among the three haplotypes just 3′ of the TM exon (Fig. 2). From the data presented here we cannot determine whether the large differences between the introns of these alleles are a result of the same mutational events that altered the exons or whether the introns have been affected by an independent series of mutational events. However, our data do allow us to exclude certain models for the genetic basis of I-A polymorphism. Earlier data on I-A polymorphism suggested that models involving mechanisms analogous to the mechanism by which trypanosomes vary their coat protein genes were plausible (*18*). Such mechanisms, involving the insertion and deletion of large stretches of DNA, are not consistent with the data presented here. The pattern of sequence differences presented in Fig. 3 is more consistent with multiple separate events that affect one or a few nucleotides. How these relatively small changes in amino acid sequence can have such a profound effect on immune responsiveness remains to be determined. Future studies on the structure of mutationally altered genes detected in laboratory strains of mice may determine the precise nature and frequency of the mutational events. Eventually these studies should determine whether the chromosomal region containing these genes is hypermutable or whether a selective advantage conferred by multiple forms of these genes leads to the maintenance of a large number of polymorphic alleles.

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19 April 1983

Peptide Cotransmitter at a Neuromuscular Junction

Abstract. *The neuropeptide proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH) is present in the nerve terminals of an identified slow skeletal motoneuron in the cockroach. Proctolin is released onto the target muscle, a coxal depressor, by neuron stimulation and by depolarization with potassium. The physiological action of the motoneuron suggests that proctolin acts as a cotransmitter. Proctolin and neural stimulation produce delayed and sustained contractile effects without muscle depolarization.*

Evidence that cotransmitters may act with classical transmitters at skeletal nerve-muscle junctions in both vertebrates and invertebrates is accumulating. Thus, although the vertebrate motor end plate has long been regarded as exclusively cholinergic (*1*), synthetic enzymes for neuroactive substances other than acetylcholine have now been found in mammalian motoneurons (*2*). Similarly, excitatory motoneurons in insects are generally regarded as L-glutaminergic (*3*), but we have shown that the insect gut neuropeptide proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH) (*4*) is associated with some excitatory motoneurons in the cockroach *Periplaneta americana* (*5*). In considering the role of this peptide at the insect neuromuscular junction, we studied the individually identified, proctolin-containing, slow coxal depressor or Ds motoneuron (*5, 6*) in the cockroach. We chromatographically characterized the proctolin-like immunoreactivity (PLI) in Ds nerve terminals and showed a calcium-dependent release of proctolin from Ds. The Ds motoneuron produces transient effects consistent with the recognized effects of L-glutamate in addition to sustained, delayed effects consistent with the action of proctolin. Our findings provide direct evidence for a peptide

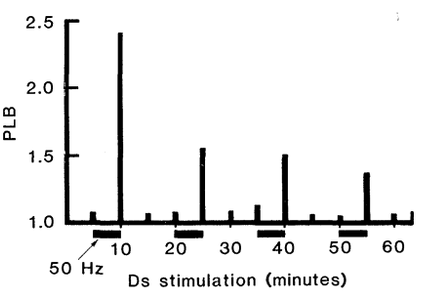

Fig. 1. The release of PLB from a single Ds coxal depressor muscle preparation (*7, 10*) by electrical stimulation of the Ds axon at 50 Hz for 5 minutes on four successive occasions (black bars). Bioassay of the perfusate (*10*) showed significant elevation of PLB after each stimulus.