Peptide Leucine Arginine, a Potent Immunomodulatory Peptide Isolated and Structurally Characterized from the Skin of the Northern Leopard Frog, *Rana pipiens*  

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On the basis of histamine release from rat peritoneal mast cells, an octadecapeptide was isolated from the skin extract of the Northern Leopard frog (*Rana pipiens*). This peptide was purified to homogeneity using reversed-phase high performance liquid chromatography and found to have the following primary structure by Edman degradation and pyridylethylation: LVRGCWTKSYPKPCFVR, in which Cys⁵ and Cys¹⁵ are disulfide bridged. The peptide was named peptide leucine arginine (pLR), reflecting the N- and C-terminal residues. Molecular modeling predicted that pLR possessed a rigid tertiary loop structure with flexible end regions. pLR was synthesized and elicited rapid, noncytolytic histamine release that had a 2-fold greater potency when compared with one of the most active histamine-liberating peptides, namely melittin. pLR was able to permeabilize negatively charged unilamellar lipid vesicles but not neutral vesicles, a finding that was consistent with its nonhemolytic action. pLR inhibited the early development of granulocyte macrophage colonies from bone marrow stem cells but did not induce apoptosis of the end stage granulocytes, i.e. mature neutrophils. pLR therefore displays biological activity with both granulopoietic progenitor cells and mast cells and thus represents a novel bioactive peptide from frog skin.

Studies on the array of biologically active substances present in amphibian skin have been reported in the scientific literature for almost 40 years (1). In particular, skin is a rich source of bioactive peptides, many of which are present in copious amounts (mg/g wet weight skin) (2). Many peptides occur at concentrations that are several orders of magnitude higher than the circulating levels in the frog, indicating that a major source of biosynthesis is in the skin (3). These peptides are putative components in the defense of the frog against predators or invading microorganisms (1).

Several peptides have been isolated from the skins of ranid frog species, which have structural analogs in mammalian neuroendocrine systems. Bradykinin and related family members have been identified in several *Rana* species (4–8) and others include the bombesin-like peptide, ranatensin (9), thyrotropin-releasing hormone (10, 11), caerulein (12), xenopsin-related peptide, marginatensin (13), and the tachykinin ranamargin (14). The hemolytic/chemoactive ranid frog peptides (7, 12) and the family of temporin peptides (15) demonstrate structural similarity to the mast cell-activating and phospholipase A₂-facilitating peptide, crabrolin, from the venom of the wasp, *Vespa crabro* (16).

Many peptides activate rat peritoneal mast cells; thus, histamine release can be utilized as a screen for putative bioactive peptides. There are several frog skin peptides that have been identified in this manner, namely, peptide XO-4 from *Kassina maculata* (17), granulinerin R from *Rana rugosa* (18), and the pipinins from *Rana pipiens* (19). A number of peptides have also been isolated from the venoms of hornets, wasps, and bees on the basis of histamine release (Refs. 16 and 20–26 and Table 1). Many mammalian peptides can activate mast cells, including substance P, neurokinin A, calcitonin gene-related peptide, neuropeptide Y and, neurotensin (27–31). Some of these mast cell-activating peptides also have modulatory effects on a variety of immune cells (32–35).

The present study describes the isolation and structural characterization of peptide leucine arginine (pLR) from the skin of the frog *R. pipiens*. We have shown that pLR is one of the most active, noncytolytic histamine-liberating peptides, which also inhibits granulocyte macrophage colony formation without induction of neutrophil apoptosis. This novel amphibian peptide may have an unrecognized counterpart with a regulatory function in the mammalian immune system.

**EXPERIMENTAL PROCEDURES**  

**Tissue Extraction**—The frogs were lightly anesthetized and killed by pithing. Dorsal skin (6.2 g wet weight) was removed from six adult specimens of *R. pipiens*, chopped into small pieces, and incubated in acidified ethanol (ethanol, 0.7 M HCl (3:1 v/v) and 8 ml/g of tissue) for 12 h at 4 °C. Tissue debris was removed by centrifugation (3000 × g, 4 °C, 30 min), and the ethanol was removed from the decanted supernatant prior to lyophilization.

1 The abbreviations used are: pLR, peptide leucine arginine; HPLC, high performance liquid chromatography; Fmoe, 9 fluoresinemethoxy-carbonyl; LDH, lactate dehydrogenase; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; C₁₂₅₀, concentration required to elicit 50% release of calcine or produced 50% hemolysis; C₂₀₀₀, concentration required to elicit release of 25% of the total cellular histamine content; C₉₆₀₀₅₀³, concentration of POPG or POPC in the large unilamellar vesicles.
Comparison of the primary sequence of peptide leucine arginine (pLR) with other known peptides that elicit histamine release from rat peritoneal mast cells. Single letter notations for amino acid residues have been used.

| Peptide | Primary sequence |
|---------|-----------------|
| pLR     | FIKQLLPHPGNDVANSFS-NH₂ |
| XO-4    | FGFLPquirrelPS-NH₂ |
| Granulin R | FLPIIAAKVVFPIFCA2SKC |
| Pipinin I | FLPIIAAKVVFPIFCA2SKC |
| Pipinin II | FLPIIAAKVVFPIFCA2SKC |
| Pipinin III | FLPIIAAKVVFPIFCA2SKC |
| Mastoparan C | INLAKIAALVKKUL-NH₂ |
| Crabolin | FLPLIRIKVITAL-NH₂ |
| Vespid chemotactic peptide L | FLPLIAKVLGG-NH₂ |
| Vespid chemotactic peptide X | FLPLIAKVLGG-NH₂ |
| Ropalinidan chemotactic peptide | IVPFLGLGLLTT-NH₂ |
| Vespid chemotactic peptide A | FLPIIAKLFGGL-NH₂ |
| Vespid chemotactic peptide M | FLPIIGKLLGG-NH₂ |
| Histamine releasing peptide T | FLPLILGKLGG-NH₂ |
| Bombolitin I | IKITMLAKGLKVLAAV |
| Bombolitin II | KITDILAKGLKVLAAV |
| Bombolitin III | IKMDILAKGLKVLAAV |
| Bombolitin IV | INIKDLALKVLKVLGH |
| Bombolitin V | INVLGLLGLRLAL |
| Mast cell degranulating peptide | IKCNKRRHIFKICRKICGKN-NH₂ |
| Magainin-2-amide | GIGKFLHSAKKFGKAFGVMINS-NH₂ |
| Melittin | GIGAVKVLITGLPALISWIKRKRQ-NH₂ |
| Vespid chemotactic peptide | GIGAVKVLITGLPALISWIKRKRQ-NH₂ |
| Pullulose | GIGAVKVLITGLPALISWIKRKRQ-NH₂ |

a References are given in parentheses.
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Automated microsequencing data of peptide leucine arginine before and after pyridylethylation

Initial sequence was obtained using 1.3 nmol of peptide by microsequencing with an Applied Biosystems 470A sequencer. Post-pyridylethylated peptide was used 60 pmol of peptide on an Applied Biosystems 491 Procise sequencer. “Before” and “after” refer to yields of amino acids before and after pyridylethylolation of the peptide.

| Cycle Number | PTH-amino acid | Yield Before | Yield After |
|--------------|----------------|--------------|-------------|
| 1            | Leu (L)        | 1099         | 49          |
| 2            | Val (V)        | 981          | 46          |
| 3            | Arg (R)        | 1307         | 14          |
| 4            | Gly (G)        | 628          | 43          |
| 5            | Cys (C)        | 20           |             |
| 6            | Trp (W)        | 118          | 14          |
| 7            | Thr (T)        | 519          | 21          |
| 8            | Lys (K)        | 226          | 19          |
| 9            | Ser (S)        | 111          | 14          |
| 10           | Tyr (Y)        | 240          | 24          |
| 11           | Pro (P)        | 160          |             |
| 12           | Pro (P)        | 163          | 25          |
| 13           | Lys (K)        | 35           | 13          |
| 14           | Pro (P)        | 93           | 14          |
| 15           | Cys (C)        | 6            |             |
| 16           | Phe (F)        | 44           | 8           |
| 17           | Val (V)        | 40           |             |
| 18           | Arg (R)        | 98           | 0.50        |

* Single letter notations for amino acid residues in parentheses.

b Cys indicates the presence of the pyridylethyl derivative.

release was measured. In a further series of experiments, the histamine release was induced by a range of pLR concentrations and various other known histamine-releasing peptides were compared.

Membrane Permeabilizing Actions of pLR—Membrane perturbation was studied by synthetic peptide induced dye release from negatively charged 1-palmitoyl-2-oleoyl-phosphatidylinositol-glycerol (POPG) and neutral 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidylcholine (POPC) large unilamellar vesicles. Large unilamellar vesicles (diameter, 0.1 μm) containing calcine at self-quenching concentrations were prepared by extrusion as described (38). Calcine release was monitored fluorimetrically by measuring the decrease of self-quenching (excitation, 490 nm; emission, 520 nm) on a PerkinElmer LS 50B spectrofluorimeter after mixing peptide solution and vesicle suspension. pLR was tested over the concentration range of 1–40 μM for POPG and 1–110 μM for POPC vesicles; magainin-2 amide was used at concentrations between 0.1 and 0.5 μM for POPG and 1 and 50 μM for POPC vesicles, and melittin was tested at concentrations ranging from 0.01 to 1 μM for POPG and 0.01 to 0.1 μM for POPC vesicles. The fluorescence intensity corresponding to 100% dye release was determined by addition of Triton X-100. The percentage of leakage after 1 min was used to create dose response curves, and the concentration required to evoke 50% dye release (EC50) for each peptide was determined from duplicate experiments. Hydrophobicity of pLR, magainin-2 amide and melittin were determined from methods using the Consensus Scale of Hydrophobicity (39).

**Hemolytic Activity**—The hemolytic activity of the synthetic peptide was determined using human red blood cells (40). Suspensions containing 1.8 × 10⁸ cells/ml where incubated (30 min, 37 °C) with varying concentrations of peptides. Concentration ranges used were 1–40 μM, 1 μM to 1 mM, and 0.1–10 μM for pLR, magainin-2 amide, and melittin, respectively. After cooling in ice water (5 min) followed by centrifugation (2000 × g, 4 °C, 5 min), the supernatant (200 μl) was diluted with NH₄OH (1800 μl, 0.5%), and the optical density was measured at 540 nm. Peptide concentrations that produced 50% hemolysis (EC50) were determined from dose response curves.

**Measurement of Granulopoiesis by Colony Forming Units-Granulocyte Macrophage Formation (CFU-GM)—**Semi-solid agar cultures were performed utilizing normal human bone marrow obtained from thrombocytopenia rib specimens as previously described (41). In brief, bone marrow cells (1 × 10⁶) were cultured in the upper layer of triplicate cultures (1 ml), with human umbilical cord conditioned medium in the lower layer as the source of colony stimulating activity. Synthetic pLR was incorporated into both layers of the cultures in the range 0.1–100 μM. CFU-GM colonies (>20 cells) were scored using an inverted microscope after incubation (37 °C, 7 days, 5% CO₂/air). Colony inhibition in the
The presence of pLR was expressed as percentage inhibition of granulopoiesis compared with that observed in the absence of any stimuli. These experiments were carried out in triplicate.

**Measurement of Neutrophil Apoptosis**—Neutrophils were prepared by layering human peripheral blood on an equal volume of Histopaque 1119 and Histopaque 1077 (Sigma) (42). Cells were washed in phosphate-buffered saline (0.01 M, pH 7.4, Sigma), resuspended in RPMI 1640 (Life Technologies, Inc.) containing 10% (v/v) fetal calf serum (Life Technologies, Inc.), and counted by hemocytometer. A suspension of neutrophils (1 x 10⁶/ml) was cultured in the presence of synthetic pLR (0–4.7 μM) in 5% CO₂/95% air at 37 °C in 6-well multi-dishes (Nunc). After 24 h the neutrophils were removed, and the cell viability was estimated by trypan blue exclusion. Three cytospin slide preparations were made per peptide concentration. One slide was stained with Giemsa and examined for morphological signs of apoptosis. At least 500 cells were counted per slide, and the percentage of apoptotic cells was calculated. Two slides were labeled using the terminal deoxynucleotide triphosphate nick end labeling technique (43) for further evaluation of apoptotic status. All experiments were repeated three times.

**Data Presentation and Statistical Analysis**—Unless otherwise stated all values are given as the means ± S.E. Statistical analyses were performed using Student’s t test. For multiple comparisons, an analysis of variance was first performed followed by the protected t test.

## RESULTS

**Isolation of Peptide Using Histamine-releasing Activity**—The maximal histamine release was detected in the pool of fractions 33–36 from the reversed-phase HPLC (Fig. 1A). Other histamine-releasing fractions were detected that were identified as related to the previously documented pipinins (44). The individual fraction 35 possessed all of the activity (data not shown). Fraction 35 was further resolved into two major peptides (Fig. 1B). The second, more hydrophobic peptide evoked all histamine-releasing activity, and 2.23 nmol of peptide demonstrated a highly effective histamine-releasing ability of 80.6%. There was negligible LDH release evoked with this peptide (data not shown). LDH release 0.2% ± 0.01%. Furthermore, significant histamine release could still be detected at a 1:100 dilution (223 pmol) and had a comparable dose response curve to the synthetic pLR (data not shown). Therefore, synthetic pLR was used for all subsequent functional studies.

**Structural Analyses**—²⁵²Cf plasma desorption mass spectroscopy of the isolated peptide indicated a molecular mass of 2137 Da [MH]+ or 2136 Da in nonprotonated native form. Automated Edman degradation established the identity of residues through 18 cycles with two blanks in positions 5 and 15. The most likely reason for this was the presence of an intramolecular disulfide bridge between two cysteiny1 residues. The calculated molecular mass of the peptide, taking this into account, was 2135.6 Da. However, the identity of the cysteiny1 residues at these sites, following reduction and pyridylethylolation of the natural peptide, was unequivocally confirmed (Table II). From automated sequence analysis, the initial yield of purified peptide was determined as 2.23 nmol, which enabled the quantity of pLR in R. piniens to be estimated as 45 μg/g wet weight of skin. This is an estimation of peptide quantity, which was achieved on the basis of peptide sequence analysis data and not the preferred method of amino acid analysis. Amino acid analysis has a reported accuracy of 5% compared with 20% by peptide sequence analysis, and therefore the reported quantification may be suboptimal but still is satisfactorily accurate to supply an estimated quantity.

**Conformational Studies**—The CD spectra of pLR in buffer or 2136 Da in nonprotonated native form. Automated Edman degradation established the identity of residues through 18 cycles with two blanks in positions 5 and 15. The most likely reason for this was the presence of an intramolecular disulfide bridge between two cysteiny1 residues. The calculated molecular mass of the peptide, taking this into account, was 2135.6 Da. However, the identity of the cysteiny1 residues at these sites, following reduction and pyridylethylolation of the natural peptide, was unequivocally confirmed (Table II). From automated sequence analysis, the initial yield of purified peptide was determined as 2.23 nmol, which enabled the quantity of pLR in R. piniens to be estimated as 45 μg/g wet weight of skin. This is an estimation of peptide quantity, which was achieved on the basis of peptide sequence analysis data and not the preferred method of amino acid analysis. Amino acid analysis has a reported accuracy of 5% compared with 20% by peptide sequence analysis, and therefore the reported quantification may be suboptimal but still is satisfactorily accurate to supply an estimated quantity.
the negative band. The minor differences in the CD characteristics of pLR in the different solvent systems point to pronounced conformational constraints in the peptide.

Molecular Modeling—pLR displayed no identity to sequences from any of the contemporary data bases nor with peptides that were isolated on the basis of histamine releasing ability or which demonstrate this activity (Table I). A number of proteins were found that contained cysteinyl and prolyl residues with identical positioning to those found in pLR. Proteins from the three-dimensional structure protein data base also demonstrated the motifs TKSXPPK and YPPKP, which are found within the cyclic portion of pLR and this polyproline consensus sequence PPKP resulted in near identical backbone conformations in the three-dimensional structure of all these proteins. A relatively rigid cyclic structure is therefore predominantly determined by the consensus polyproline motif Pro11-Pro12-Lys13-Pro14 and the disulfide bridge between Cys5 and Cys15. The resultant molecular model, from simulations with the PPKP conformation weakly constrained during simulated annealing procedures, also displays a β-strand-like conformation formed by Pro12-Lys13-Pro14-Cys15, a sharp turn between Ser9 and Pro12, and N- and C-terminal tails that appear to remain flexible (Fig. 2).

Characterization of Histamine Release by Synthetic pLR—

**Fig. 4.** Time and temperature dependence of peptide leucine arginine-induced histamine release. A, kinetics of histamine release, from rat peritoneal mast cells, stimulated with peptide leucine arginine (500 nM, n = 4–6). B, DEPendence of temperature on histamine release induced by peptide LEUcine arginine (500 nM, n = 4–6). All data are presented as the means ± S.E. and expressed as the percentage release of total cellular histamine corrected for spontaneous release.

**Fig. 5.** Calcium and energy dependence studies of peptide leucine arginine-induced histamine release. A, the effect of different extracellular calcium concentrations on the ability of peptide leucine arginine (500 nM, n = 4–6) to induce histamine release from rat peritoneal mast cells. Asterisks indicate significant difference (p < 0.05) of histamine release compared with 0.1 mM extracellular calcium. B, solid bars indicate the effect of using metabolic uncoupling agents 2-deoxyglucose and antimycin A on the ability of peptide leucine arginine to evoke histamine release (500 nM, n = 4–6). Hatched bars indicate the histamine release in the absence of stimuli. Asterisks indicate significant difference compared with glucose-free peptide-induced histamine release. All data are presented as the means ± S.E. and expressed as the percentages of release of total cellular histamine corrected for spontaneous release.

The amino acid sequence of this peptide has been deposited in the SWISSPROT database under the accession number P82110.
TABLE III
Lytic activity of pLR compared to melittin and magainin-2 amide

| Peptide             | Hydrophobicity | POPG EC50 | POPC EC50 | l-(C = 100 μM RBC) |
|---------------------|----------------|-----------|-----------|-------------------|
| PLR                 | -0.184         | 15 ± 1    | NA        | 2.5 ± 0.6         |
| Magainin-2a         | -0.036         | 0.18 ± 0.01| 5.8*      | 25 ± 5            |
| Melittin            | -0.086 (+0.248)| 0.23 ± 0.005| 0.021 ± 0.002| 100               |

**Stimulation of rat peritoneal mast cells with synthetic pLR caused a significant and dose-dependent histamine release after correction for spontaneous release, which was less than 5% of total (Fig. 3). Maximal histamine release occurred at a concentration of 2.5 μM, and the concentration required to elicit release of 25% of the total cellular histamine content (EC25) was 330 ± 8 nM. The molar potency of the synthetic analog was comparable with that of natural pLR, except that the maximal release obtained with the natural pLR was 80% compared with 67.9 ± 2.4% for the synthetic peptide (data not shown).**

On comparing the histamine-releasing activity of pLR to other mast cell-activating peptides including substance P, neuropeptide Y, bradykinin, magainin-2 amide, and melittin, the cytolytic bee venom peptide (a very potent histamine liberator), pLR was the most potent of all of these peptides (Fig. 3).

pLR induced a rapid release of histamine, and within 15 s, maximal release had occurred (Fig. 4A). Histamine release was still maximal after 30 min of stimulation with pLR. pLR-induced histamine release was temperature-dependent with an optimum at 37 °C. Both increasing and decreasing temperatures significantly reduced the histamine-releasing potential of pLR, and at 4 °C there was negligible release (Fig. 4B).

Histamine release induced by pLR was also affected by extracellular calcium concentrations (Fig. 5A). The optimal extracellular calcium concentration was 0.1 mM. pLR-induced histamine release was significantly reduced by calcium concentrations higher than 1 mM (p < 0.05). In nominally free calcium medium, there was no significant difference in the histamine release by pLR. Preincubation of the mast cells with the metabolic uncouplers, 2-deoxyglucose and antimycin A, significantly inhibited pLR-induced histamine release, indicative that pLR induced histamine release is energy-dependent (Fig. 5B, p < 0.05). pLR was devoid of LDH releasing ability at all concentrations tested (0–5 μM), thus supporting a noncytolytic mode of activation (data not shown).

**Membrane Permeabilization and Hemolysis by pLR—**The EC50 of pLR from negatively charged POPG vesicles was determined as 15 μM (Table III). Complete lysis of POPG liposomes occurred at 40 μM. In contrast, pLR was completely inactive against neutral POPC liposomes up to a concentration of 110 μM. The low lytic activity of pLR against electrically neutral membranes is supported by low hemolytic activity. Although melittin (100 μM) and magainin-2 amide (100 μM) produced complete and 25% hemolysis, respectively, pLR was practically inactive (Table III).

**Effect of pLR on CFU-GM Formation and Neutrophil Apoptosis—**pLR exerted a dose-dependent inhibition of normal CFU-GM formation (Fig. 6). At a concentration of 46.8 nM, pLR evoked inhibition of granulopoiesis (34.3 ± 5.4%). Near maximal inhibition was achieved by a pLR concentration of 4.68 μM (92.8 ± 0.7%). pLR had no effect on either viability or neutrophil apoptosis up to concentrations of 4.7 μM (data not shown).

**DISCUSSION**

This study describes the isolation and characterization of pLR from the skin of *R. pipiens*. The peptide has potent immunomodulatory effects on mast cells and granulopoietic progenitor cells. This is the first documentation of a peptide that has dual effects on histamine release and on granulocyte/macrophage colony formation. Concentrations of pLR, which elicited the observed effects, are comparable with the estimated concentration of pLR within the skin of *R. pipiens*, therefore reflecting that the in vitro responses were generated using physiological concentrations.

pLR is one of the most potent naturally occurring, noncytolytic histamine-releasing peptides discovered to date. This peptide shows no known sequence similarity with other peptides, which have also been isolated on the basis of mast cell activation or with other histamine-releasing peptides, such as melittin and magainin-2 amide (Table I). The EC50 of pLR (330 ± 8 nM; Fig. 3) is 2-fold greater than that of melittin (600 ± 2 nM;
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Fig. 3) whose action is purely cytolytic. The rapid, energy/temperature-dependent characteristics of pLR-induced histamine release were similar to those of other classical mast cell-activating peptides (46); however, compared with other noncysteine mast cell activating peptides, pLR was 10–20 times more active on a molar basis, e.g. substance P (4.15 ± 1.65 μM; Fig. 3). This high potency can be related to its primary structure as pLR is highly cationic (pI = 10.2) with a net positive charge of +4. It has been previously documented that an increasing net positive charge increases the histamine-releasing ability of peptides (47). Mast cell activation involves initial electrostatic interaction between the polycationic peptides and the anionic sialic acid residues on the mast cell membrane (48). From the primary structure, because of the presence of the helical breaking residues, proline and glycine, and the disulfide-bridge at Cys1 and Cys15, pLR is predicted to be devoid of α-helical regions. This is consistent with previous reports that histamine-releasing ability is independent of helicity (36, 49). The very high potency of pLR has yet to be explained and may relate to the mechanisms by which pLR activates intracellular signaling mechanisms, which are currently being investigated.

Peptide membrane interaction can also be related to total hydrophobicity of the peptides and the distribution of hydrophobic and charged residues in the sequence (50). High cationic peptide charge favors the accumulation of peptides to negatively charged POPG bilayers by electrostatic interactions. The peptide charge favors the accumulation of peptides to negatively charged POPC and red blood cell membranes, whereas the helix domain (H1) activates intracellular signaling mechanisms, which are currently being investigated. Where pLR exerted a strong inhibitory effect on myeloid progenitor cell development, anti-proliferative effects, and the reported presence of high affinity binding sites merit further investigations for future development as a potential chemotherapeutic agent.

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Peptide Leucine Arginine, a Potent Immunomodulatory Peptide Isolated and Structurally Characterized from the Skin of the Northern Leopard Frog, *Rana pipiens*

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