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Pituitary Adenylate Cyclase-Activating Polypeptide is a Potent Broad-Spectrum Antimicrobial Peptide: Structure-Activity Relationships

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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a naturally occurring cationic peptide with potent immunosuppressant and cytoprotective activities. We now show that full length PACAP38 and to a lesser extent, the truncated form PACAP27, and the closely related vasoactive intestinal peptide (VIP) and secretin had antimicrobial activity against the Gram-

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AUTHORSHIP CONTRIBUTIONS CGS and JH designed and performed experiments. DHC synthesized and purified the peptides. CGS, WCW and JM analyzed data. JM, WCW and CGS wrote the manuscript.

Values listed are minimum inhibitory concentration (MIC) in units of μM peptide. Each value is the mean of at least three independent measurements. Most standard deviations are 10–40% of the mean, with an average of ~30%. “>30” means that MIC is greater than 30 μM in the broth dilution assay. N/A means that no activity was observed in radial diffusion with 20 μM peptide. EC: *E. coli*, SA: *S. aureus*, PA: *p. aeruginosa*, BC: *B. cereus*. When compared to PACAP38 by ANOVA, the natural PACAP38 analogs have lower activity in radial diffusion (**, p<0.01, ***, p<0.001).

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DISCLOSURES

DHC and JLM are co-inventors on multiple World (PCT) patents about the properties and uses of PACAP and PACAP analogs. All of these patents have been assigned to the Administrators of the Tulane Educational Fund.

negative bacteria *Escherichia coli* in the radial diffusion assay. PACAP38 was more potent than either the bovine neutrophil antimicrobial peptide indolicidin or the synthetic antimicrobial peptide ARVA against *E. coli*. PACAP38 also had activity against the Gram-positive bacteria *Staphylococcus aureus* in the same assay with comparable potency to indolicidin and ARVA. In the more stringent broth dilution assay, PACAP38 had moderate sterilizing activity against *E. coli*, and potent sterilizing activity against the Gram-negative bacteria *Pseudomonas aeruginosa*. PACAP27, VIP and secretin were much less active than PACAP38 in this assay. PACAP38 also had some activity against the Gram-positive bacteria *Bacillus cereus* in the broth dilution assay. Many exopeptidase-resistant analogs of PACAP38, including both receptor agonists and antagonists, had antimicrobial activities equal to, or better than PACAP38, in both assays. PACAP38 made the membranes of *E. coli* permeable to SYTOX Green, suggesting a classical membrane lytic mechanism. These data suggest that analogs of PACPAP38 with a wide range of useful biological activities can be made by judicious substitutions in the sequence.

Keywords

Antibiotic Resistance; Antimicrobial Peptides; Innate Immunity; Pituitary Adenylate Cyclase-Activating Polypeptide; Vasoactive Intestinal Peptide

Introduction

Antimicrobial peptides (AMP) are an indispensable component of the innate immune system of multicellular animals and plants [1]. Small changes in the amino acid sequence of AMP can sometimes result in very large changes in activity against different microbial species. The clinical use of AMP has been proposed as a strategy to overcome the frequent resistance of many common microbes to conventional antibiotics [2]. The usefulness of AMP as drugs has usually been limited by residual toxicity, host cell inhibition [3], and their short half-life in circulation due to rapid proteolysis and filtration by the kidney. The few AMP that have been approved by the Food and Drug Administration (FDA) are only used for topical applications [2]. Therefore, there is a need for AMP analogs that have fewer impediments in order to effectively treat certain infections. AMP with other useful biological activities, especially potent immunosuppressive or cytoprotective activity, could have a wide range of medical applications [4,5].

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a member of the secretin/growth hormone-releasing hormone (GHRH)/vasoactive intestinal peptide (VIP) family, with potent anti-inflammatory and potent cytoprotective properties [6–13]. PACAP exists as two amidated peptides with 38 or 27 amino acids, differing in the C-terminus. PACAP27 is homologous to VIP and secretin. PACAP is most abundant in the brain, but there are significant levels in other organs, including the thymus, spleen, lymph nodes, and duodenal mucosa [10]. The usefulness of PACAP as a drug, however, is limited by its very short half-life in the circulation following systemic administration due, in part, to rapid proteolysis, especially rapid exopeptidase activity at the amino terminus by the enzyme DPP IV [14]. There is a need for analogs of PACAP38 with increased circulation half-life and altered

receptor activity/selectivity, goals that we have approached with rational design of PACAP analogs [15].

PACAP38 has physicochemical properties that are similar to many natural and synthetic antimicrobial peptides (AMP). For example, PACAP38 carries a highly basic charge of +10 at neutral pH. It has a significant compliment of hydrophobic residues with a structurally amphipathic arrangement, both hallmarks of canonical AMP [16]. Furthermore, there is evidence that other peptide hormones also have antibacterial activity [17,18]. Thus, here we examined the antimicrobial activity of PACAP and some analogs. PACAP38 and many analogs have potent antimicrobial activity against a panel of Gram-positive and Gram-negative pathogens. Activity was comparable to that of known synthetic and natural AMPs. These observations, combined with the potent physiological effects of PACAP, make it an intriguing candidate for development into a treatment for antibiotic resistant infections.

Materials and Methods

Peptide Synthesis

All peptides were synthesized using FMOC chemistry, purified by HPLC and verified by MALDI mass spectrometry [19].

Proteolysis Assay

Each peptide for the proteolysis assay was incubated in phosphate buffer at a concentration of 50 μM peptide with DPP IV at a concentration of 0.5 mg/ml at 25°C. Proteolysis was monitored by high-performance liquid chromatography (HPLC) over the course of a 72-hour incubation. The amount of proteolysis at the specified time was calculated as the area under the curve of the peptide peak for the peptide incubated with DPP IV divided by the area under the curve of the peptide peak for the same peptide incubated without DPP IV.

Microbiological assays

Escherichia coli (ATCC 25922), *Pseudomonas aeruginosa* (PA01, ATTC 47085) and *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* were used in these assays. Broth dilution sterilization and radial diffusion assays were performed as described elsewhere [3]. The minimum inhibitory concentration was determined to be the lowest concentration at which a zone of inhibition can be observed (radial diffusion), or the lowest peptide concentration at which no bacterial proliferation was observed (broth dilution).

Red Blood Cell Hemolysis Assay

Fresh human O+ human red blood cells (RBC) were used in hemolysis assays. Lysis was measured by hemoglobin release as described in detail elsewhere [3,20].

Bacterial Membrane Disruption Assay

Peptides, at 20 μM were incubated with bacteria in the presence of 5 μM SYTOX Green. Entry of SYTOX Green was measured by fluorescence [20].

Statistical Analyses

All measurements of minimum sterilizing/inhibitory activity were performed as serial dilution experiments where the minimum effective concentration is noted. Experiments were repeated at least 3 time and the minimum concentrations were averaged. The results were expressed as the mean ± SD. Multiple comparisons were analyzed using a one-way ANOVA, with Tukey-Kramer *post-hoc* tests.

Results

Antimicrobial Activity

We performed two classical antibacterial assays for this work. The radial diffusion assay measures inhibition of bacterial growth in a zone around where an antibiotic has been applied to an agar gel. The minimum inhibitory concentration (MIC) in a radial diffusion experiment is the lowest concentration at which any inhibition is detectable. The broth dilution assay is a much more stringent, all-or-none sterilization assay. In the broth dilution assay only complete sterilization is noted as positive activity. The MIC for this assay is the lowest concentration that completely sterilizes bacteria in media.

All four of the naturally occurring peptides, PACAP38, PACAP27, VIP and secretin had antimicrobial activity against the Gram-negative bacteria *E. coli* in the radial diffusion assay, with PACAP38 having the highest potency, i.e. the lowest minimum inhibitory concentration (MIC) (Table 1). Similarly, PACAP38 has potent sterilizing activity against *P. aeruginosa* in the more stringent broth dilution assay. PACAP38 also had antimicrobial activity against the Gram-positive bacteria *S. aureus* in the radial diffusion (Table 1). PACAP27, VIP and secretin did not have any detectable effect against *S. aureus* in either assay (Table 1). El Karim et al. [17] have also reported that VIP had activity against *E. coli*, but not against *S. aureus*.

For comparison, we measured, in parallel, the activity the bovine neutrophil AMP indolicidin [21,22] and the potent broad spectrum synthetic AMP ARVA (Arg-Arg-Gly-Trp-Ala-Leu-Arg-Leu-Val-Leu-Ala-Tyr-NH_2; [20,23]. These two peptides have activities that are very typical for the most active families of AMPs [3]. In the radial diffusion assays PACAP38 was more potent than either of these two control AMPs against *E. coli* and had very similar MIC against *S. aureus*. In the broth dilution assay, PACAP38 is much better than the controls against *P. aeruginisa*.

In table 2 we show the antimicrobial activities of PACAP and VIP analogs that have agonist activity [19,24]. In Table 3 we show data for analogs that have antagonist activity [25,26], and in Table 4 we show data for the subset of analogs that have pipecolic acid (piperidine-2-carboxylic acid) or isonepecotic acid (piperidine-4-carboxylic acid) in position 3 [19]. Most of the 16 full length analogs studied had good antimicrobial activity against *E.* coli in the radial diffusion assay, Similarly, all of the full length PACAP38 analogs, except for [Lys^{38}-palmitoyl]PACAP38, had antimicrobial activity against the Gram-positive bacterium *S. aureus* in the radial diffusion assay.

To better assess sequence-structure-activity relationships, we used ANOVA on each radial diffusion column to determine which of the variants studied had statistically significant differences from PACAP38 in the radial diffusion assays. In each Table, variants with activity that is significantly different from PACAP38 are marked with asterisks indicating the significance level. Unmarked variants were not significantly different ($p > 0.05$).

In the broth dilution assay, all of the full length analogs have good activity against *P. aeruginosa*, similar to the activity of PACAP38 (Table 2). Further, some had improved activity against other microbes in the broth dilution assay against which PACAP38 has poor activity. For example four of the full length analogs have MIC < 10 μM against *E. coli* in the broth dilution assay, and seven have MIC < 10 μM against *B. cereus*. Sterilization of *S. aureus* in the broth dilution assay is especially challenging for AMPs [3], as shown here by the observation that only ARVA had a measurable MIC against *S. aureus* in the broth dilution assay. Despite the lack of sterilization, PACAP38 and many of its analogs have inhibitory activity against *S. aureus* in radial diffusion.

Exopeptidase Resistance

PACAP38 is thought to be rapidly cleaved and inactivated *in vivo* by the aminodipeptidase DPP IV [27,28]. PACAP analogs with non-natural amino acids near the N-terminus have been assumed to be resistant to DPP IV. Here we tested that assumption directly with purified DPP IV enzyme using PACAP38 analogs with non-natural amino acids in positions one, three or four. PACAP38 and PACAP27 were almost completely cleaved by DPP IV in less than 30 minutes (Fig. 1). On the other hand, there was no indication that any analog tested was cleaved at all by DPP IV even after 72 hours. The susceptibility of the analogs to DPP IV is negligible on any reasonable pharmacological or biological timescale.

Hemolysis

Hemolysis of red blood cells (RBC) has long been used as an index of toxicity of AMP against mammalian cells [29]. Here we measured the effect of PACAP38, some analogs, and control AMP, against human RBCs. PACAP38 was less toxic toward RBC than either the naturally occurring bovine neutrophil AMP indolicidin or the synthetic AMP ARVA (Fig. 2). Similarly, all four of the exopeptidase-resistant PACAP38 analogs tested (Fig. 1) were less toxic toward RBC than either indolicidin or ARVA (Fig. 2). The only exception was the analog of PACAP38 with a palmitate attached to the N-terminal lysine. This analog had dramatically increased toxicity toward RBC.

Membrane Permeabilization

Many synthetic and natural AMP sterilize bacteria by permeabilization of the bacterial cytoplasmic membrane [1]. Therefore, we tested PACAP38 and some analogs for membrane permeabilizing activity. PACAP38 and PACAP(6–38) made the membranes of *E. coli* permeable to SYTOX Green at a low concentration, consistent with this classical AMP mechanism [20,23]. The decrease of SYTOX Green fluorescence rate at high PACAP38 concentration is likely due to competition for DNA binding sites between the cationic peptide and cationic SYTOX Green. Interestingly, the shorter analogs PACAP27, VIP and secretin did not enable any entry of SYTOX Green suggesting differences in the size of the

membrane defects, or a different mechanism of action altogether at the concentrations tested (Fig. 3),

Discussion

We show here that PACAP38 and many full length, exopeptidase-resistant analogs, both agonists and antagonists, have potent antibacterial activity that is mechanistically similar to the action of other natural and synthetic AMP on the bacterial membranes. At the same time they have low toxicity against human RBCs. The potency and species selectivity of the antimicrobial activity of PACAP analogs can be changed independently of the receptor-dependent biological activity profile. Therefore, we conclude that PACAP38 analogs with a wide range of useful biological activities can be made by judicious substitutions to the PACAP38 template.

Structure-Activity Relationships

The structural diversity of AMP are very large, but all structural classes share a positive net charge and some amphipathicity which enable them to bind to and permeabilize anionic bacterial membranes [1,16]. The observations that PACAP38 and PACAP(6–38) make bacterial membranes permeable to SYTOX Green indicates that classical membrane permeabilization is the basis for their bactericidal activity, and that of all full-length analogs. PACAP38 is, in fact, both cationic and amphipathic. The first ~20 residues comprise a sequence with a high propensity to fold into an amphipathic α-helix when bound to membranes. In solution, residues 7–23 are α-helical [30,31]. These N-terminal 20 residues have a net charge of +5. The next 6 residues are strictly hydrophobic and provide amphipathicity along the sequence. The C-terminal 12 residues of PACAP38 are less amphipathic, but are highly cationic and contribute an additional charge of +6. The fact that PACAP27 and PACAP(6–38) have lower antimicrobial activity than PACAP38 in most assays indicates that both the central amphipathicity and terminal charges are needed for full activity. Similarly, the weaker activity, and lack of membrane permeabilization, for secretin and VIP (which are homologous to PACAP27) supports this conclusion. The changes to the N-terminal amino acids of PACAP38 that enable exopeptidase resistance cause generally small changes in antimicrobial activity, often improving it (See Tables 2–4). This is consistent with the structure-function relationship just described because these changes neither reduce positive charge nor decrease amphipathicity significantly.

Comparative Localization of AMP and PACAP38

The idea that PACAP38 evolved to have anti-infective activity in addition to its other activities is supported by the observation that PACAP38 also killed the bloodstream (infective) form of the protozoan parasite *Trypanosoma brucei* [32]. PACAP(6–38) was also effective against the infective form of *T. brucei*. However, the procyclic (noninfective) form of *T. brucei* was resistant to PACAP38. Furthermore, PACAP38 has been found in tissues and fluids that are known to contain high concentrations of other AMP. For example, PACAP38 has been found in the epithelial surface of the nose, tongue, larynx, and trachea [33]. PACAP38 has also been found in human breast milk [34] and tears [35]. Whether PACAP38 interacts synergistically with any of the AMP in any of these tissues or fluids

(e.g.,[36]) is currently unknown. PACAP and its analogs have an additional advantage over other AMP in that they are transported across the blood brain barrier [37].

Development of Clinical Applications

PACAP38 has been administered to healthy human volunteers by investigators in at least six academic laboratories in the European Union [38–45] and to a patient with multiple myeloma in the U.S. under a FDA-approved single-patient protocol [46] without any indication of serious side-effects. We expect that the PACAP38 analogs described above would have a similar high safety profiles and could be developed into therapeutics, including the example conditions described below.

Inflammation contributes significantly to the pathogenesis of many medical disorders. Immunosuppressive agents, such as corticosteroids, are among the most widely used drugs in modern medicine. However, the commonly used immunosuppressive agents also increase the risk of infection. PACAP38 and analogs with agonist activity have potent receptor-mediated immunosuppressive activity [6]. Unlike other immunosuppressive agents, PACAP38 and its analogs also have intrinsic broad-spectrum antibiotic activity which could offset some of the risk of infection caused by their receptor-mediated immunosuppressive activity and thus could possibly be developed into monotherapeutics and/or adjunctive therapeutics for a wide range of medical disorders [15]. Furthermore, PACAP38 and PACAP38 analogs with agonist activities have potent wound-healing properties [47,48], which may also act synergistically with antimicrobial activity.

The potent antimicrobial activity of PACAP38 agonists against *P. aeruginosa* in combination with its receptor-dependent effects could also be beneficial in the treatment of cystic fibrosis. *P. aeruginosa* is the most common pathogen in the lung of patients with cystic fibrosis and is the dominant cause of mortality [49]. PACAP has already been shown to have anti-inflammatory [8,50], antifibrotic [51], and bronchodilator [52] activities. Activation of the VPAC1 receptor by PACAP38 has been shown to stimulate the anion channel activity of the cystic fibrosis transmembrane conductance regulator (CFTR) in human epithelial cells [53] and to increase the insertion of functional ΔF508 CFTR protein into the apical membrane of human epithelial cells [54,55]. As a final example of the possibilities, we note that stimulation of mucus secretion from goblet cells via activation of VPAC2 receptors [56,57] may be dramatically reduced by replacing Tyr in position 22 of PACAP with Ala or Aib [19,58,59] and Table 2.

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ABBREVIATIONS

AMP	antimicrobial peptide
ARVA	Arg-Arg-Gly-Trp-Ala-Leu-Arg-Leu-Val-Leu-Ala-Tyr-NH_2

CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony-forming unit
CNS	central nervous system
DPP IV	dipeptidyl peptidase IV
FDA	Food and Drug Administration
GHRH	growth hormone-releasing hormone
HPLC	high-performance liquid chromatography
MRSA	methicillin-resistant *Staphylococcus aureus*
PACAP	pituitary adenylate cyclase-activating polypeptide
PBS	phosphate-buffered saline
RBC	red blood cell
TSB	trypticase soy broth
VIP	vasoactive intestinal peptide

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Highlights

- PACAP38 has broad spectrum antimicrobial activity
- Full length agonist and antagonist variants retain antimicrobial activity
- Receptor activation and antimicrobial activity can be independently manipulated
- Antimicrobial activity results from bacterial membrane permeabilization

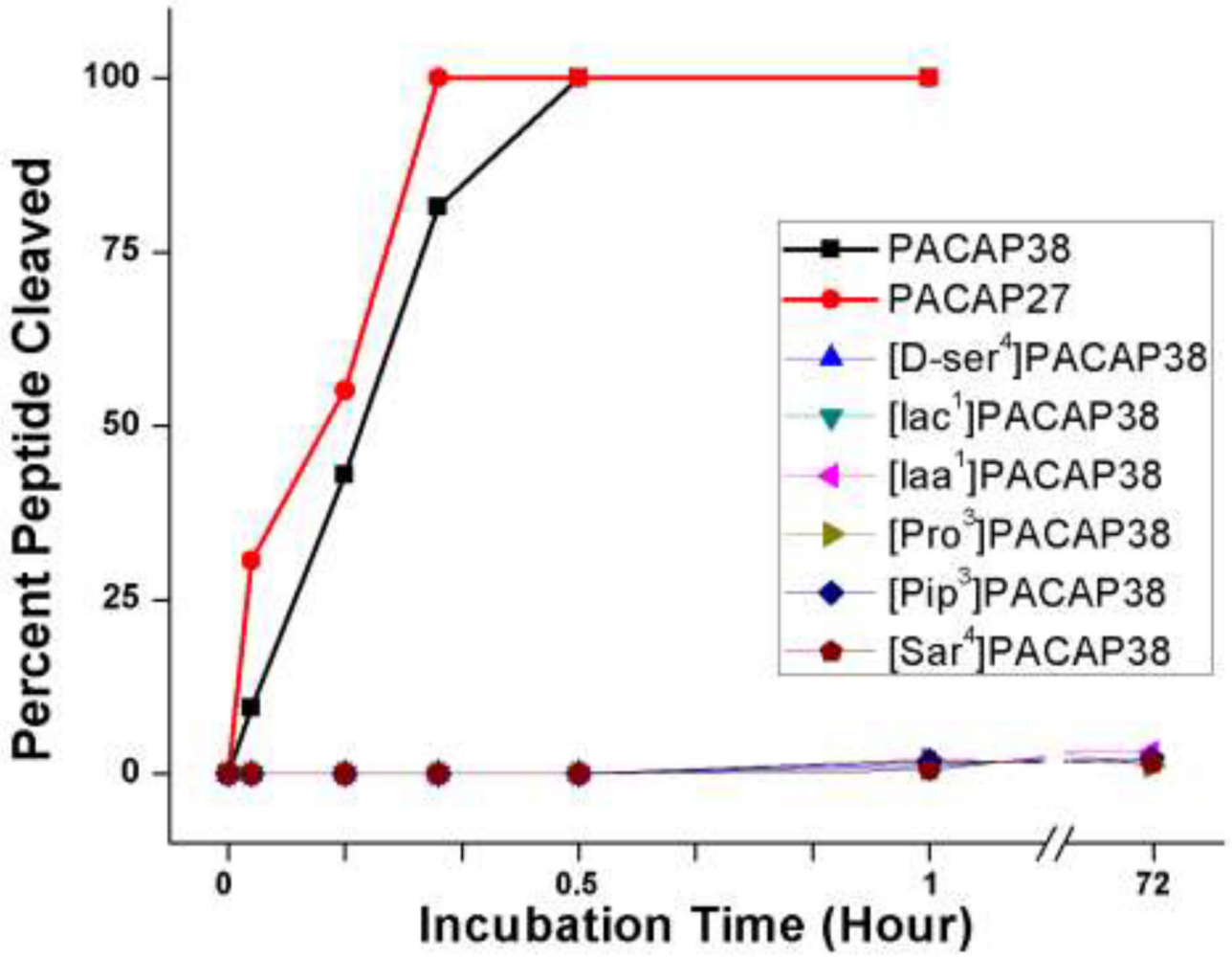

Fig. 1.

The proteolysis of PACAP27, PACAP38 and PACAP38 analogs by dipeptidyl peptidase IV. Each peptide was incubated in phosphate buffer (pH 7.4) at a concentration of 50 μM with DPP IV at a concentration of 0.5 mg/ml at 22°C. Proteolysis was monitored by HPLC over the course of the 72-hour incubation. The lines for all of the PACAP analogs are overlapping. Iaa, imidazole-4-acetic acid; Iac, imidazole-4-acrylic acid; Pip, pipecolic acid; Sar, sarcosine.

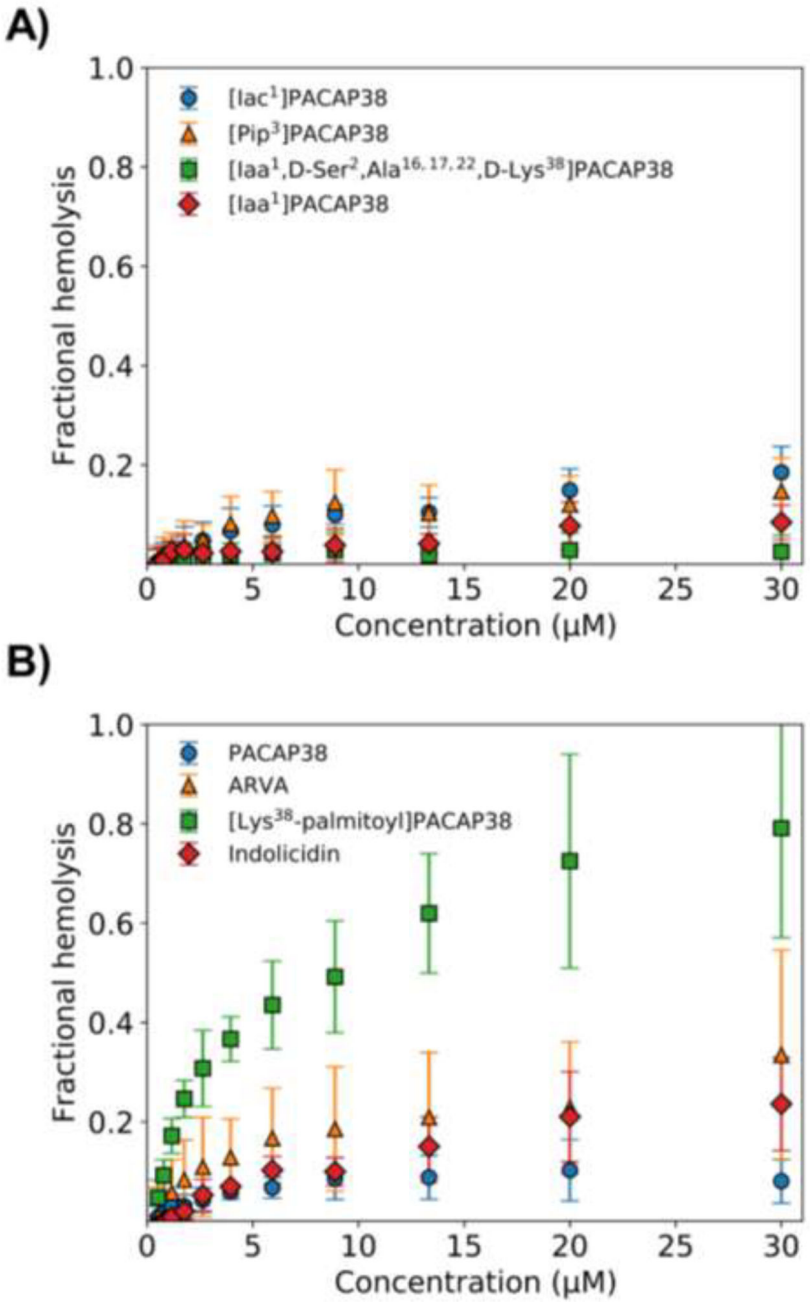

Fig. 2.

The lysis of human red blood cells by PACAP38 and PACAP38 analogs. Serial dilutions of each peptide were incubated with human RBC in PBS (pH 7.4) for one hour at 37°C and then the cells were spun down at 2000 rpm for five minutes. The optical density of the supernatant was measured at 410 nm to determine the amount of hemoglobin released. Iaa, imidazole-4-acetic acid; Iac, imidazole-4-acrylic acid; Pip, pipecolic acid.

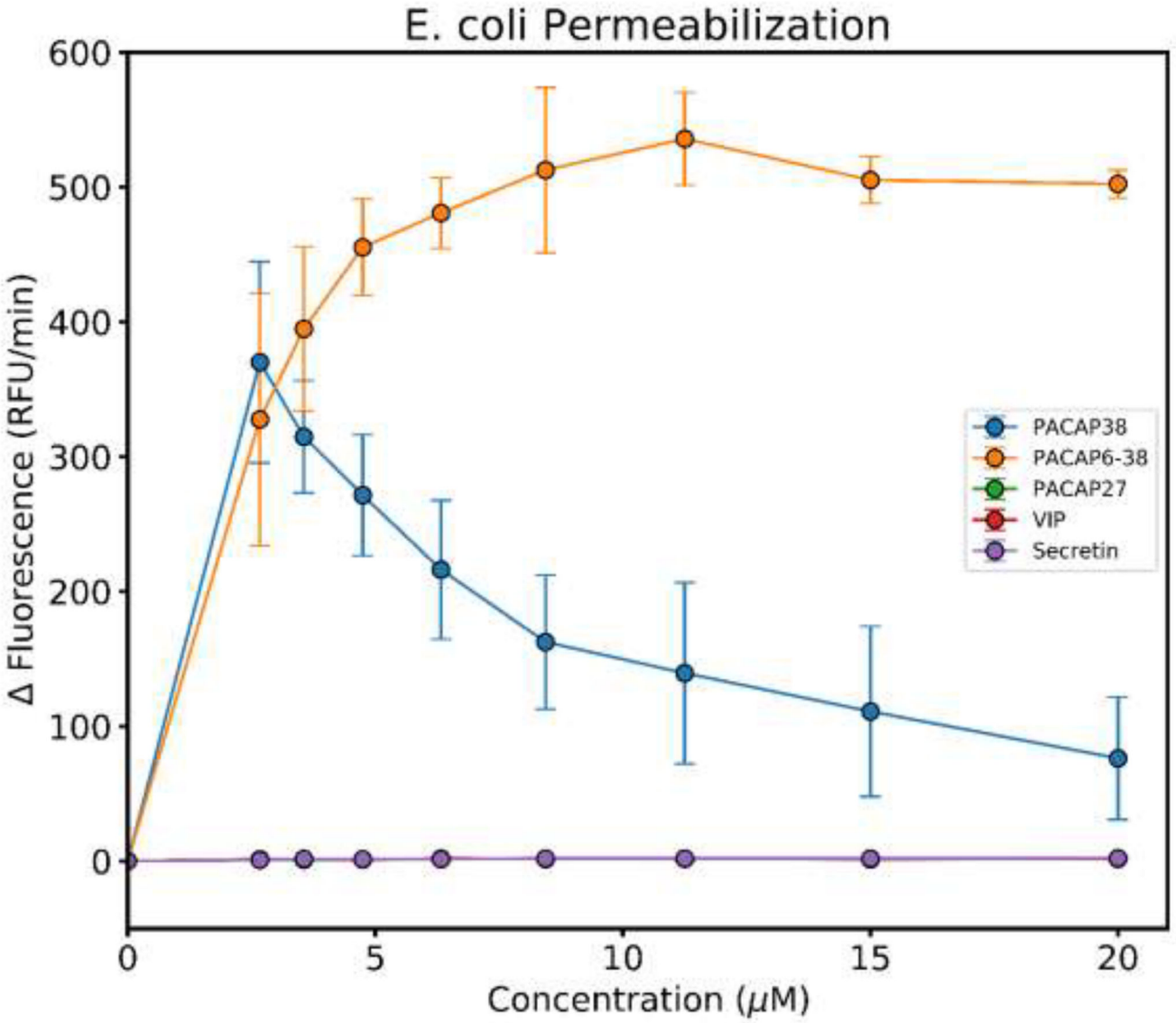

Fig. 3.

Disruption of bacterial cell membranes by PACAP38 and PACAP38 analogs. The fluorescence of the membrane impermeable, cationic DNA-binding dye, SYTOX Green was measured immediately after the addition of each peptide and then after 30 minutes. The change in fluorescence was plotted as ΔRFU/min for each concentration of each peptide. The decrease in the rate of SYTOX Green fluorescence increase at high PACAP concentrations is probably due to electrostatic competition between the cationic peptide and SYTOX Green for DNA binding sites. The rate of fluorescence change for PACAP27, VIP and secretin are all exactly zero, such that the points overlay.

TABLE 1

Antimicrobial activity of some members of the secretin/vasoactive intestinal peptide family

Peptide	EC Radial Diffusion	SA Radial Diffusion	EC Broth	PA Broth	SA Broth	BC Broth
PACAP38	0.76	3.54	28.3	1.2	>30	23.3
PACAP27	4.40**	N/A***	>30	23.6	>30	>30
VIP	6.90***	N/A***	>30	30.0	>30	>30
Secretin	14.75***	N/A***	>30	30.0	>30	>30
ARVA	1.57	3.46	2.3	4.3	5.1	6.4
indolicidin	2.78	3.66	6.24	>50	7.9	10.7

TABLE 2

Antimicrobial activity of some PACAP/VIP receptor agonists

Peptide	EC Radial Diffusion	SA Radial Diffusion	EC Broth	PA Broth	SA Broth	BC Broth
PACAP38	0.76	3.54	28.3	1.2	>30	23.3
[D-Ser^{2}]PACAP38	0.41	1.30	>30	6.1	>30	13.6
[Lys^{38}-palmitoyl]PACAP38	N/A***	N/A***	9.1	6.1	>30	4.9
[Iaa^{1}]PACAP38	2.11	24.75***	>30	1.8	>30	4.8
[Iac^{1}]PACAP38	0.95	1.89	16.6	2.7	>30	20.4
[Iac^{1},Nal^{6}]PACAP38	1.93	7.04	16.3	0.8	>30	5.9
[Pro^{3}]PACAP38	1.46	23.03***	>30	1.8	>30	8.9
[Ala^{22}]PACAP38	2.01	17.37***	2.15	0.52	>30	4.84
[Iaa^{1},D-Ser^{2}]PACAP38	1.65	9.72	>30	1.17	>30	>30
[Iaa^{1},D-Ser^{2},Ala^{16,17,22},D-Lys^{38}]PACAP38	1.26	7.47	>30	1.17	>30	>30
[Aib^{16,28},Ala^{17},Lys^{34},D-Lys^{38}]PACAP38	0.72	4.62	7.3	1.2	>30	13.3
PACAP27	4.40**	N/A***	>30	23.6	>30	>30
VIP	6.90***	N/A***	>30	30.0	>30	>30
[Ala^{2,8,9,16,19,20,21,24,25}]VIP	12.28***	N/A***	>30	5.9	>30	>30
ARVA (control AMP)	1.57	3.46	2.3	4.3	5.1	6.4

TABLE 3

Antimicrobial activity of some PACAP/VIP receptor antagonists

Peptide	EC Radial Diffusion	SA Radial Diffusion	EC Broth	PA Broth	SA Broth	BC Broth
PACAP38	0.76	3.54	28.3	1.2	>30	23.3
[Sar^4]PACAP38	0.91	8.95	24.5	1.2	>30	20.0
PACAP(6–38)	1.36	1.81	>30	30.0	>30	>30

TABLE 4

Antimicrobial activity of PACAP38 analogs

Peptide	EC Radial Diffusion	SA Radial Diffusion	EC Broth	PA Broth	SA Broth	BC Broth
PACAP38	0.76	3.54	28.3	1.2	>30	23.3
[Pip^{3}]PACAP38	1.01	2.70	>30	8.9	>30	8.9
[N-acetyl-His^{1},Pip^{3}]PACAP38	1.03	1.45	>30	20.0	>30	7.3
[Ini^{3},Aib^{16,28},Ala^{17},Lys^{34},D-Lys^{38}]PACAP38	1.69	6.11	24.5	0.96	>30	>30
[Pip^{3},Aib^{16,28},Ala^{17},Lys^{34},D-Lys^{38}]PACAP38	1.13	5.52	2.15	0.52	>30	3.95
[Pip^{3},Aib^{16,28},Ala^{17,21},Lys^{34},D-Lys^{38}]PACAP38	1.48	4.97	1.43	0.64	>30	7.26