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The Biological Activity of Selected Cyclic Dipeptides

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Abstract

Cyclic dipeptides are widely used as models for larger peptides because of their simplicity and limited conformational freedom. Some cyclic dipeptides have been shown to be antiviral, antibiotic and anti-tumour. The aim of this study was to determine the biological activity of four cyclic dipeptides synthesized in this laboratory: cyclo(L-phenylalanyl-L-prolyl), cyclo(L-tyrosyl-L-prolyl), cyclo(L-tryptophanyl-L-prolyl) and cyclo(L-tryptophanyl-L-tryptophanyl).

The enhancement or inhibition of calcium channels in ventricular myocytes from rats and delayed-rectifier potassium channels in ventricular myocytes from guinea-pigs were determined by use of the whole-cell patch-clamp technique. The induction of differentiation in HT-29 cells was assessed by assaying for an increase in the expression of alkaline phosphatase. Antibiotic properties against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilus* and *Streptococcus* sp. were determined by use of the Kirby–Bauer disc-diffusion assay. Results from these assays indicate that the cyclic dipeptides have biological activity in both prokaryotes and eukaryotes. Three of the dipeptides block cation channels in ventricular myocytes and all increase the expression of alkaline phosphatase. All the dipeptides have concentration-dependent antibacterial properties.

These results suggest that with increased solubility the cyclic dipeptides might have potential as muscle relaxants, anti-tumour compounds and antibiotics.

Conformationally restricted peptides are simple and valuable models for gaining conformational insight into the properties of larger peptides and proteins and their three-dimensional structure–bioactivity relationships (Anteunis 1978). During the past two decades cyclic peptides prepared by short-range cyclizations have attracted considerable attention because of their limited conformational freedom and higher probability of conformational homogeneity when compared with their linear analogues (Toniolo 1990). Proline is an important imino acid of many proteins and neuropeptides and imposes certain conformational restraints on these biomolecules (Ashida & Kakudo 1974). In addition, the conformational aspects of the pyrrolidine ring system are of particular interest as they reveal different modes of puckering of the five-membered ring system (Chacko et al 1983). Secondly, proline is the only residue which leads to an *N*-alkylamide bond when incorporated into a peptide via natural biochemical pathways. Numerous peptides with important biological activity (e.g. didemnin and cyclosporin) contain *N*-methyl amino acids (Smith et al 1991). Thirdly, the *cis–trans* isomerism of the *N*-alkylamide bond involving the amino group of proline has been implicated in the biological activity of peptides. Brandl & Deber (1986) have proposed that *cis–trans* isomerism of proline residues might play a role in transduction of transmembrane proteins.

The inclusion or incorporation of essential aromatic amino acids offers a model system of limited complexity for studying the influence of solvents and solvent mixtures on intramolecular interactions during the excited lifetime of the chromophore (Edelhoch et al 1968).

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Tryptophan contains an indole ring (a benzene ring fused to a pyrrole ring) which is found in many pharmacologically active compounds, including hallucinogens and other drugs which have mental or emotional effects, e.g. lysergic acid diethylamide (LSD), dimethyltryptamine (DMT), psilocybin, harmaline and strychnine. Bromocriptine, ergotamine, indapamide, indomethacin, sumatriptan and ondansetron are examples of commercially available drugs containing an indole ring.

Although cyclic dipeptides (2,5 diketopiperazines, DKPs, or 2,5 dioxypiperazines, DOPs) have been known since the beginning of this century (Fischer 1906), only during recent years have they attracted considerable interest (Sammes 1975). They do not exist as zwitterions and the simple members of this group of peptides are often neutral compounds. Their general structural formula is shown in Figure 1, where R_1 and R_2 are the side-chains of the different amino acid residues. They are easily formed from unprotected linear dipeptides under basic conditions, especially if the necessary head-to-tail folding is not prevented by steric constraints (Fischer 1906). Such compounds seem of interest in studies on the thermodynamic behaviour of non-ionic compounds in water because they share the capability of establishing hydrogen-bonds with the solvent (via the two *cis* amide groups in the DKP ring) and of giving rise to hydrophobic interactions (to an extent determined by the R substituents) (Crescenzi et al 1973). Because of their simplicity and limited conformational freedom, cyclic dipeptides are readily used as model compounds in studies aimed at the development and refinement of criteria for conformational insights into larger peptides (Bovey 1974; Anteunis 1978).

Many derivatives of these compounds with the DKP ring have antiviral properties—the gliotoxins (Ottenheijm et al 1976) and others, such as bicyclomycin, are, for example, powerful antibiotics (Bodansky et al 1973) and anti-tumour agents (Jensen et al 1973). Dioxopiperazines are used by nature to hold small peptide links together, as in the growth factor rhodotorulic acid (Atkins & Neilands 1968). The biological implications of DKPs are further demonstrated by their spontaneous formation from higher linear peptides containing imino acid residues (Goodman & Steuben 1962). Some DKPs are metabolic intermediates or provoke the destruction of the secondary globular protein structure (Crescenzi et al 1973).

In this paper we report the biological activity of cyclo(L-phenylalanyl-L-prolyl) (cyclo(Phe–Pro)), cyclo(L-tyrosyl-L-prolyl) (cyclo(Tyr–Pro)), cyclo (L-tryptophanyl-L-prolyl) (cyclo(Trp–Pro)) and cyclo(L-tryptophanyl-L-tryptophanyl) (cyclo(Trp–Trp)).

Materials and Methods

Chemicals and solutions

The linear dipeptides were synthesized in our laboratory by the method of Milne et al (1992). The *N*-protected dipeptides were treated with TFA or formic acid to remove the amino-protecting groups. Cyclization was achieved by use of saturated sodium hydrogen carbonate or by boiling in a neutral medium (*sec*-butyl alcohol–toluene, 4 : 1). Verification of structures was accomplished by X-ray crystallography, infrared spectroscopy, nuclear magnetic resonance spectroscopy, mass spectrometry and melting-point determination. The structures of the cyclic dipeptides are shown in Figure 2. The cyclic dipeptides were dissolved in chloroform to furnish a $1\,mg\,mL^{-1}$ solution and were then further diluted in chloroform to the required concentrations. The dipeptides and solutions were stored at 4°C. Other compounds used were of analytical grade.

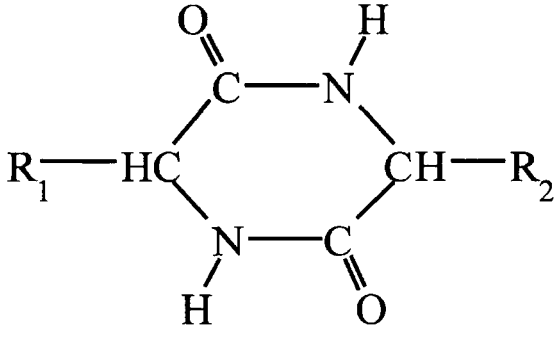

Figure 1. The general structural formula of cyclic dipeptides. R_1 and R_2 are the side-chains of the amino acids in the cyclic compound.

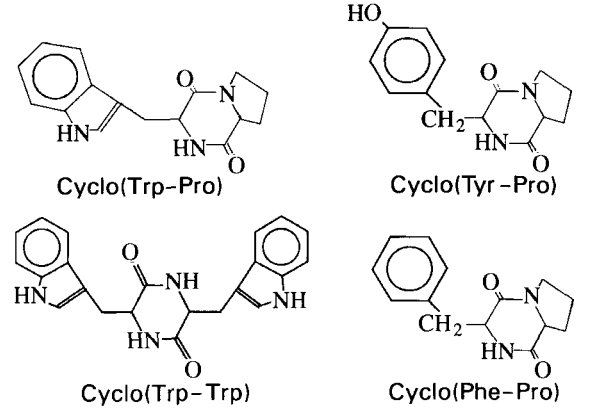

Figure 2. Structural representation of the cyclic dipeptides used in this study.

Cell cultures and microorganisms
The HT-29 cell line was obtained from Highveld Biologicals (Johannesburg, South Africa). The cells were routinely maintained in Dulbecco's modified Eagle's minimal medium (DMEM) supplemented with 10% foetal calf serum, 20 mg L^{-1} benzylpenicillin and 100 mg L^{-1} streptomycin. The bacterial cultures used were from the culture collection in the Department of Biochemistry and Microbiology at the University of Port Elizabeth. The identities of the cultures were confirmed by the method of Holt et al (1994).

Effect of cyclo(Trp–Pro) and cyclo(Trp–Trp) on calcium channels in the ventricular myocytes of rats
Rats were killed by a sharp blow to the base of the skull. The heart was removed and perfused, by means of a constant perfusion system, with 6 mg collagenase (Type II, Sigma, St Louis, MO) and 4·5 mg protease (Type 14, Sigma) per 40 mL to effect the dispersion of the ventricular cells. The cells were resuspended in (mM): 137 NaCl, 5·4 KCl, 0·5 $MgCl_2$, 11·6 HEPES NaOH, 1·8 $CaCl_2$ and 10 Glucose (pH 7·2). The whole-cell patch-clamp technique (Hamil et al 1981) was used to record ionic currents under voltage-clamp conditions with the pipette solution containing (mM): 125 CsCl, 5 $MgCl_2$, 15 EGTA, 10 HEPES, 20 TEA-Cl and 5 Na_2ATP (pH 7·2). Inward calcium currents were recorded after changing the holding potential from −80 mV to −45 mV for 3 s to inactivate the sodium channels. The cells were then exposed to the cyclic dipeptides (100 μM) and the test potentials were increased in 5-mV steps from −35 mV to +20 mV. The dipeptides were washed out of the system for 3 min in the suspension solution.

Effect of cyclo(Trp–Pro), cyclo(Trp–Trp), cyclo(Phe–Pro) and cyclo(Tyr–Pro) on the delayed-rectifier potassium channel in ventricular myocytes from guinea-pig
Guinea-pigs were killed by a sharp blow to the base of the skull. The heart was removed and perfused, by means of a constant perfusion system, with calcium-free solution containing 16 mg collagenase A and 4·8 mg protease per 40 mL to effect the dispersion of the ventricular cells. The cells were resuspended in a solution containing (mM) 137 NaCl, 5·4 KCl, 0·5 $MgCl_2$, 11·6 HEPES NaOH, 1·8 $CaCl_2$ and 10 Glucose (pH 7·2) at 32°C. The whole-cell patch-clamp technique (Hamil et al 1981) was used to record ionic currents under voltage-clamp conditions with the pipette solution containing (mM) 125 KCl, 5 $MgCl_2$, 4 EGTA, 10 HEPES, 0·154 $CaCl_2$ and 5 Na_2ATP (pH 7·2). A holding potential of −40 mV was applied to the cells to inactivate the sodium channels while 0·2 μM nisoldipine was used to block calcium channels (Sanguinetti & Jurkewiecz 1990). The cells were then exposed to the cyclic dipeptides (100 μM). From the holding potential the cells were subjected to depolarization 225 ms at −30 mV to 50 mV in 10 mV steps. The tail currents between 250 ms and 900 ms were measured at −40 mV (Sanguinetti & Jurkewiecz 1990, 1991). At the end of the process the dipeptides were washed out of the system for 3 min.

Intestinal-like differentiation induction by cyclo(Trp–Pro), cyclo(Trp–Trp), cyclo(Phe–Pro) and cyclo(Tyr–Pro) The human colon carcinoma cell line, HT-29, is a valuable experimental model for determination of cellular differentiation in respect of proliferation, cytology, biochemistry, morphology and ultrastructure. The cells have been included in the NCI Preclinical Anti-tumour Drug Discovery Screen (Boyd 1989). In their original form HT-29 cells are undifferentiated and can, by use of differentiation-inducing agents, be induced to differentiate into gastrointestinal epithelium-like cells (Reynier et al 1991). Undifferentiated HT-29 cells express only baseline levels of alkaline phosphatase activity (Pinto et al 1982), whereas differentiated HT-29 cells express elevated levels of the enzymes as is found in-vivo in the gastrointestinal tract in man (Pinto et al 1982).

HT-29 cells were seeded into 96 well plates at a concentration of 50 000 cells/well and left to recover for 24 h. The cells were then exposed to solutions of the cyclic dipeptides in DMEM (125 μg mL^{-1}; 200 μL) and cultured for a further 10 days. The medium was replaced every 48 h. On day 10 the cells were washed twice with phosphate-buffered saline (0·1 M; pH 7·4) and glycine buffer (pH 10·5; 225 μL) was added to each well. At time 0 *p*-nitrophenyl phosphate (40 μL) was added to each well. After 5 min the extinction was read at 412 nm against a blank reaction mixture containing buffer (225 μL) and *p*-nitrophenyl phosphate (0·03 M; 40 μL). A standard curve was determined using buffer (200 μL), *p*-nitrophenyl phosphate (0·03 M; 40 μL) and commercial alkaline phosphatase (25 μL; Boehringer Mannheim, Mannheim, Germany) resulting in 5, 10, 20, 40, and 80 milliunits enzyme.

Antibacterial activity of cyclo(Trp–Pro), cyclo(Trp–Trp), cyclo(Phe–Pro) and cyclo(Tyr–Pro)
The antibacterial activity of the cyclic dipeptides was assayed according to the Kirby–Bauer disc-diffusion method. Gram-negative bacteria

Escherichia coli, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and the Gram-positive *Staphylococcus aureus*, *Bacillus subtilus* and *Streptococcus pneumoniae* were used as test organisms.

The cyclic dipeptides were double-diluted in dimethylsulphoxide as follows: cyclo(Tyr–Pro) 0·13 μM to 8·125 nM, cyclo(Trp–Pro) 0·14 μM to 8·75 nM, cyclo(Phe–Pro) 0·12 μM to 7·5 nM and cyclo(Trp–Trp) 0·18 μM to 5·625 nM. Sterile filter discs were submerged in the solutions for 5 min and then left to dry in air. Pour-plate overlays from overnight cultures of the six organisms in nutrient broth were prepared on standard nutrient agar. The filter discs were placed on to the surface of the agar and the plates incubated at 37°C for 24 h. Antibacterial activity was determined by measuring the diameter of the zone of inhibition around the filter discs.

Statistical analysis

Results are expressed as means ± s.d. Differences between the effects of the dipeptides on the expression of alkaline phosphatase were assessed by analysis of variance. P values < 0.05 were considered to be indicative of significance.

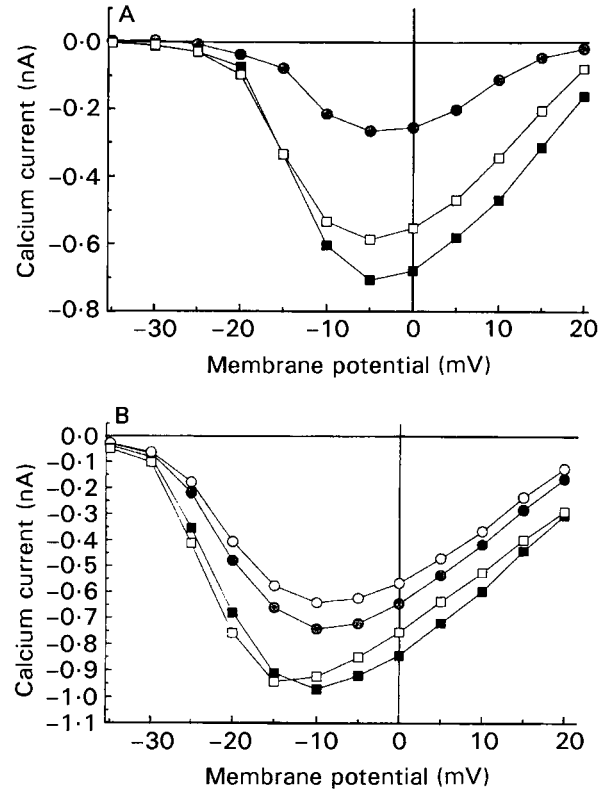

Figure 3. The influence of (A) 100 μM cyclo(Trp–Trp) and (B) 100 μM cyclo(Trp–Pro) on calcium channels in rat ventricular myocytes. A: ●, cyclo(Trp–Trp); □, wash-out; ■, control. B: ●, cyclo(Trp–Pro) replicate 1; ◍, cyclo(Trp–Pro) replicate 2; □, wash-out; ■, control.

Results

Effects on calcium channels

The two cyclic dipeptides assayed block calcium channels; the effect of cyclo(Trp–Trp) (n = 3) is faster than that of cyclo(Trp–Pro) (n = 4) (45% compared with 38% after 1 min exposure). After 3 min exposure the maximum effect was very similar—47% and 50%, respectively (Figure 3a, b). After 3 min wash-out the current affected by cyclo(Trp–Trp) returned to 80% of the control value. After the same period in the cyclo(Trp–Pro) system there was negligible wash-out and a significant return of the current was observed only after 14 min. Pre-drug controls were monitored in all experiments and only preparations that did not show a significant run-down of the calcium current were used for experiments on calcium currents.

Effects on delayed-rectifier potassium channels

Cyclo(Trp–Pro) and cyclo(Tyr–Pro) blocked delayed-rectifier potassium channels. The effect of cyclo(Tyr–Pro) was greater than that of cyclo(Trp–Pro), blocking 65% of the current after 2 min exposure in comparison with 38% (Figure 4B, C). Because there was no significant effect on the holding currents, these two cyclic dipeptides do not seem to influence other potassium channels. After 3 min wash-out the voltages in both systems returned to just over 50% of the control values.

Cyclo(Trp–Trp) and cyclo(Phe–Pro) had no effect on the delayed-rectifier potassium channels (Figure 4A, D).

Induction of differentiation

Although there was no visible inhibition of control culture growth during the treatment period, growth of the treated cells was retarded. This finding is in accord with the mutual exclusivity of proliferation and differentiation. Cyclo(Phe–Pro), cyclo(Tyr–Pro) and cyclo(Trp–Trp) all significantly ($P < 0.05$; univariate analysis of variance) enhanced the expression of the biochemical differentiation marker (Table 1). Cyclo(Trp–Pro) also increased the level of the marker, but not significantly ($P > 0.05$).

Because DMSO has been found to induce differentiation in HT-29 cells, the organic solvent was assayed separately to determine its effect on the expression of alkaline phosphatase; this was found to be negligible.

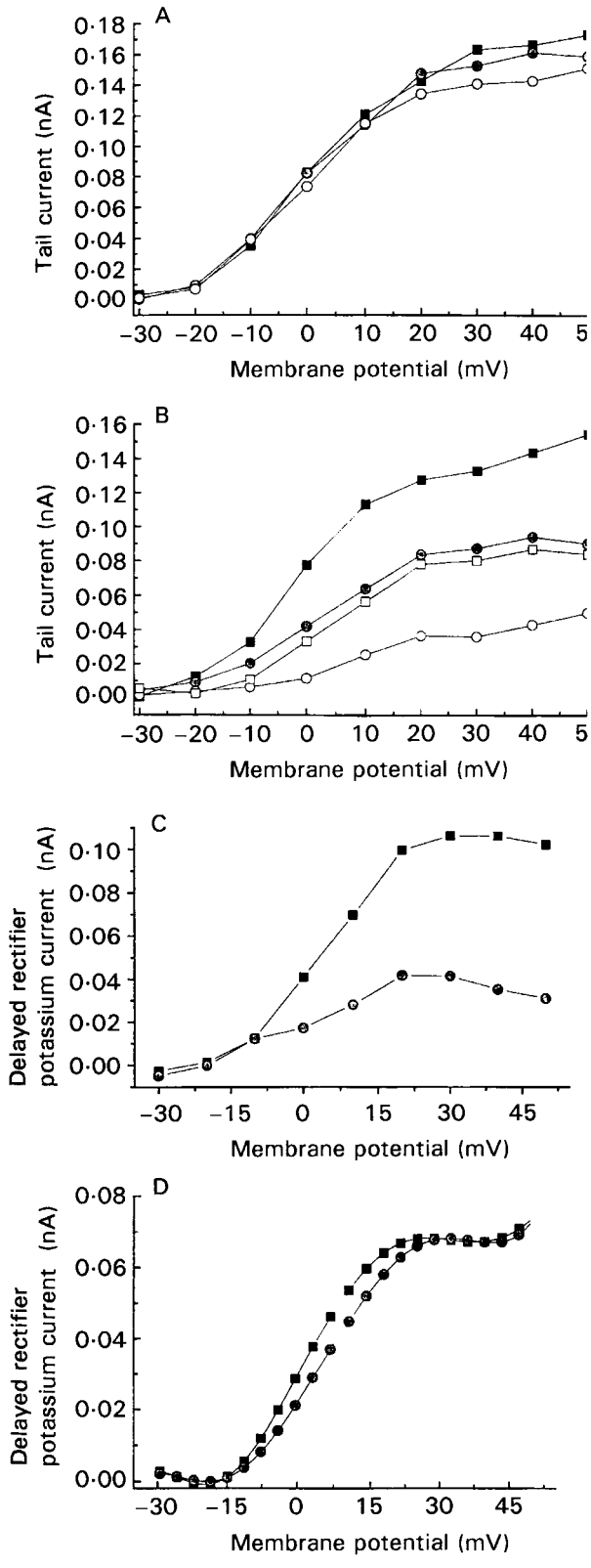

Figure 4. Current–voltage plots of the effects of 100 μM (A) cyclo(Trp–Trp), (B) cyclo(Trp–Pro), (C) cyclo (Tyr–Pro) and (D) cyclo(Phe–Pro) on the delayed-rectifier potassium channels in guinea-pig ventricular myocytes. A: ●, cyclo(Trp–Trp); ◍, cyclo(Trp–Trp); ■, control. B: ●, cyclo(Trp–Pro); ◍, cyclo(Trp–Pro); □, wash-out; ■, control. C: ●, cyclo(Tyr–Pro); ■, control. D: ●, cyclo(Phe–Pro); ■, control.

Table 1. Increases in the expression of the biochemical differentiation marker alkaline phosphatase induced by the cyclic dipeptides cyclo(Phe–Pro), cyclo(Tyr–Pro), cyclo(Trp–Pro) and cyclo(Trp–Trp) in HT-29 cells.

Cyclic dipeptide	Alkaline phosphatase activity (μM min^{-1})
Control Culture	3·52 ± 3·02
Cyclo(Trp–Pro)	7·54 ± 4·32
Cyclo(Trp–Trp)	13·67 ± 3·91
Cyclo(Phe–Pro)	14·81 ± 7·11
Cyclo(Tyr–Pro)	14·51 ± 4·52

Results are means ± s.d. (n = 15). The increases induced by cyclo(Phe–Pro), cyclo(Tyr–Pro) and cyclo(Trp–Trp) were significant at the 95% confidence level.

Antibacterial activity

The antibacterial activity of the cyclic dipeptides was concentration-dependent (Table 2). Cyclo(Trp–Pro), cyclo(Trp–Trp) and cyclo(Phe–Pro) were all active against Gram-positive and Gram-negative bacteria, whereas cyclo(Tyr–Pro) was effective against Gram-negative bacteria only.

The low levels of activity could possibly be ascribed to solubility. The cyclic dipeptides are more soluble in organic solvents than in water and would, therefore, not diffuse readily into the film of moisture which overlays all solid nutrient media. Thus, the detectable effect of the cyclic dipeptides might have been reduced. Because the filter discs were left to dry in air before being placed on the surface of the agar, the organic solvent did not effect the results found. This was confirmed by use of a set of controls using organic solvent only on the discs.

Discussion

The proposed biological action of cyclic dipeptides is currently still speculative. Within the confines of

Table 2. Concentration-dependent antibacterial effects of the cyclic dipeptides cyclo(Phe–Pro), cyclo(Tyr–Pro), cyclo(Trp–Pro) and cyclo(Trp–Trp).

Cyclic dipeptide	Minimum inhibitory concentration	
	Gram-positive bacteria	Gram-negative bacteria
Cyclo(Trp–Pro)	0·035 μM	8·125 nM
Cyclo(Trp–Trp)	22·5 nM	45 nM
Cyclo(Phe–Pro)	0·06 μM	0·03 μM
Cyclo(Tyr–Pro)	No effect	0·13 μM

The concentrations shown are the lowest concentrations at which significant growth inhibition was found for all the organisms tested. Results are means from three replicate experiments.

this study we intended to elucidate possible mechanisms of action of the cyclic dipeptides cyclo(Trp–Pro), cyclo(Trp–Trp), cyclo(Phe–Pro) and cyclo(Tyr–Pro). The summary of the activity of the dipeptides presented in Table 3 indicates that cyclo(Trp–Trp) is the most biologically active of the dipeptides investigated. In all cases it must be stressed that the activity observed might be highly important considering the poor solubility of the compounds in water (Young et al 1976), as all the amino acids used are hydrophobic.

Results from the study of the effects on Ca^{2+} and K^+ channels showed that all four dipeptides are capable of blocking cation channels in myocytes. Noise from other ion channels was avoided by use of a patch-clamp technique, enabling investigation of the effect of the cyclic dipeptides on individual groups of cation channels.

Ca^{2+}-channel blockers have shown promise in the treatment of cardiovascular disorders (Cook 1988) and have consequently been researched extensively. The blocking effect caused by cyclo(Trp–Trp) was faster than that of cyclo(Trp–Pro) (Figure 3), suggesting that cyclo(Trp–Trp) has greater affinity for the receptor preventing Ca^{2+}-channel function. However, we propose that the strength of the interaction with cyclo(Trp–Trp) was lower than that with cyclo(Trp–Pro) because cyclo(Trp–Trp) was washed out after 3 min whereas significant wash-out of cyclo(Trp–Pro) was observed only after 14 min.

The delayed-rectifier potassium channels in guinea-pig myocytes (Figure 4) were blocked by both cyclo(Trp–Pro) and cyclo(Tyr–Pro). The initial effect of the cyclo(Trp–Pro) was only 58% of that of cyclo(Tyr–Pro), indicating that cyclo(Tyr–Pro) is a potent blocker of the delayed-rectifier potassium channel. The affinities of both dipeptides for the unidentified cell-surface receptor were equal, with wash-out after 3 min only reducing the effect of both dipeptides by 50%.

The modulation of K^+-channel opening encompasses most known intracellular secondary messengers including cAMP, cGMP, GTP-binding proteins, protein kinase C and ATP. Ca^{2+} is also a secondary messenger affected by K^+ (Cook 1988). Thus certain effects of K^+ on the cell are similar to those of Ca^{2+} and K^+ has been implicated in cell division, secretion, endocytosis, metabolism and cell movement (Galione 1993).

Although the metabolic effect of the cyclic dipeptides on gastrointestinal cells was not investigated, the results shown in Table 1 indicate that cyclo(Phe–Pro), cyclo(Tyr–Pro) and cyclo(Trp–Trp) are inducers of gastrointestinal epithelial-like differentiation, because they significantly increased the expression of the differentiation marker alkaline phosphatase (Pinto et al 1982). Multiple pathways control proliferation in eukaryotic cells. Changes in protein phosphorylation, ion fluxes and cyclic nucleotides all accompany cell-cycle progression. The triggering of secondary messengers by K^+ could explain the effect of the cyclic dipeptides on the expression of differentiation markers by HT-29 cells. Wonderlin & Strobl (1996) indicated that activation of K^+-channels is necessary for the progression of a cell through the G1 phase. Thus, blocking the channels would arrest the cells in G1 and could enable differentiation to occur (Cook 1988).

The activity of Ca^{2+} as a secondary messenger could also explain the effect of the cyclic dipeptides on the differentiation of HT-29 cells. Calmodulin, which is active in the presence of calcium, activates enzymes, controls protein phosphorylation and cyclic nucleotide levels, and is an important regulator of cellular signalling by second messengers. Calcium and calmodulin have long been implicated in growth regulation, but little is known about the molecular mechanisms involved.

Initiation of differentiation requires a change in the expression of the current set of transcribed genes to a different set. This change in gene expression will result in altered cellular metabolism leading to differentiation of the cell. Differentiation could, however, occur only if the signal triggered by the cyclic dipeptides can induce the correct sequence of gene expression required

Table 3. Summary of results from determination of the biological activity of the cyclic dipeptides and qualitative comparison of the results.

	Effect on:			
Cyclic dipeptide	Ca^{2+}channels	K^+ channels	Induction of differentiation	Antibacterial activity
Cyclo(Trp–Trp)	+	–	+	+
Cyclo(Trp–Pro)	+	+	–	+
Cyclo(Tyr–Pro)	ND*	+	+	+
Cyclo(Phe–Pro)	ND	–	+	+

*Not determined.

for differentiation rather than triggering a housekeeping response.

The concentration-dependent antibacterial effect of the cyclic dipeptides described in Table 2 could be explained by the hydrophobic nature of the compounds. Their hydrophobicity would enable the cyclic dipeptides to interfere with outer-membrane (Gram-negative) and plasma-membrane (Gram-positive) function. The loss of function could result in a loss of cellular integrity leading to cell death.

In conclusion, the results from this preliminary investigation suggest that the cyclic dipeptides cyclo(Phe–Pro), cyclo(Tyr–Pro), cyclo(Trp–Trp) and cyclo(Trp–Pro) are biologically active in prokaryotes and eukaryotes. The results suggest that with increased solubility the compounds might have potential as muscle relaxants, anticancer compounds and antibiotics.

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