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The effect of two bovine β-casein peptides on various functional properties of porcine macrophages and neutrophils: differential roles of protein kinase A and exchange protein directly activated by cyclic AMP-1

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The effects of two bovine β-casein peptides on the urokinase plasminogen activator (u-PA) system and superoxide anion (SA) production by porcine macrophages and neutrophils activated by phorbol myristate acetate (PMA) were investigated. Macrophages and neutrophils were obtained from fourteen weaned piglets and were cultured *in vitro* for 24 h with or without one of two chemically synthesised peptides: tripeptide leucine–leucine–tyrosine (residues 191–193 of β-casein) (LLY) and hexapeptide proline–glycine–proline–isoleucine–proline–asparagine (residues 63–68 of β-casein). Following incubation, cells were stimulated with 80 μM-PMA. Total cell-associated u-PA, membrane-bound u-PA, free u-PA binding sites along with SA production were determined after stimulation with PMA. Both peptides suppressed the u-PA system and SA production of PMA-stimulated macrophages isolated from piglets during weeks 1–2 after weaning. Only the tripeptide LLY suppressed the u-PA system and SA production of PMA-stimulated neutrophils during the same time period. None of the peptides tested had any effect ($P > 0{\cdot}05$) on the u-PA system and SA production of PMA-stimulated macrophages and neutrophils isolated from the same piglets during weeks 5–6 after weaning. Thus, peptides are effective only in the early post-weaning period. Using cyclic AMP analogues that are highly specific activators of protein kinase A (PKA) or exchange protein directly activated by cyclic AMP-1 (Epac-1), we found that activation of PKA, but not Epac-1, was responsible for the downregulation of the u-PA system, whereas activation of PKA and/or Epac-1 was responsible for the downregulation of SA system in both macrophages and neutrophils.

Milk peptides: Phagocytes: Protein kinase A: Exchange protein directly activated by cAMP-1

The beneficial effect of milk on the implementation of a balanced nutrition as well as on the prevention of infections has been recognised for many years. Milk contains components that provide critical nutritive elements, immunological protection and biologically active substances (Clare & Swaiswood, 2000). Many biologically active peptides of milk are latent in that they are inactive within the parent protein sequence, requiring enzymatic proteolysis (e.g. during gastrointestinal digestion or food processing) for the release of bioactive peptides from their milk protein precursors (Meisel, 2005). Bioactive peptides are able to exert a wide range of effects, such as antimicrobial, antihypertensive and antithrombotic, opioid properties, aiding in the absorption of Ca and finally immunoregulatory properties (LeBlanc *et al.* 2002). Bioactive peptides are generated from all major milk proteins. However, two specific areas of β-casein (residues 60–70 and 191–202), known as the 'strategic zones', generate several peptides serving multifunctional capacities (opioid-like properties, immunomodulatory; Clare & Swaiswood, 2000).

Milk proteins represent the exclusive protein supply for the newborn. The evidence accumulated in the past few years led us to hypothesise that the natural *in vivo* role of certain milk protein peptides is to guide local immunity until it develops its full functionality (Baldi *et al.* 2005). The first few months of life constitute a critical period because the immune system should downregulate responses to nutrient molecules and upregulate responses towards pathogen-derived antigens. A great number of studies reviewed by Baldi *et al.* (2005) demonstrate the ability of milk protein peptides either to enhance or suppress the function of the immune system. Matar *et al.* (2001) showed that peptides generated by the action of proteases are responsible for the increased number of IgA (+) B-cells in the small intestine and bronchial tissues in mice fed with milk fermented with *Lactobacillus helveticus*. Perdigon *et al.*

Abbreviations: cAMP, cyclic AMP; Epac-1, exchange protein directly activated by cAMP-1; H89, N-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinoline sulfonamide dihydrochloride; HA1004, N-(2-guanidinoethyl)-5-isoquinoline sulfonamide hydrochloride; HBSS, Hanks' Balanced Salt Solution; KT5720, (9S,10S,12R)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1h-diindolo [1,2,3 fg:3′,2′,1′-kl] pyrrolo [3,4-i] [1,6] benzodiazocine-10 carboxylic acid hexyl ester; LLY, leucine–leucine–tyrosine (residues 191–193 of bovine β-casein); PKA, protein kinase A; PKI_{14-22}, protein kinase A inhibitor fragment 14-22 myristoylated trifluoroacetate salt; PMA, phorbol myristate acetate; SA, superoxide anion; u-PA, urokinase-plasminogen activator.

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(1999) demonstrated that feeding mice with milk fermented by lactic acid bacteria resulted in increases in IgA-positive cells in the gut, macrophage activity and specific antibody responses during infection. In contrast, Pessi *et al.* (2001) investigated the effects of digests of bovine casein by enzymes derived from *Lactobacillus rhamnosus* GG on T-cell activation. They found that the digests reduced the expression of IL-2 and inhibited protein kinase C translocation, effects that indicate a suppression of T-cell activation by casein digests. Furthermore, a synthetic peptide corresponding to residues 142–149 of a_{s1}-casein was proven to be an effective inducer of CD8-positive T-cells that recognised the parent protein and secreted interferon-γ, a potent inhibitor of T-helper-2-dependent events, including IgE production (Totsuka *et al.* 1998).

Cyclic AMP (cAMP) is the prototype 'second messenger', playing an important role in mediating the actions of extracellular signals (Qiao *et al.* 2002). cAMP has been implicated in regulating many cellular functions, such as gene expression, as well as having largely inhibitory effects on various functional properties of macrophages (Aronoff *et al.* 2005). An elevation of intracellular cAMP in macrophages, i.e. by prostaglandin E_2, has been shown to inhibit lipopolysaccharide-stimulated TNF-α, IL-1, IL-12, IL-6 and NO production (Kunkel *et al.* 1986; Tetsuka *et al.* 1994; Mustafa & Olson, 1998; Petrova *et al.* 1999; Procopio *et al.* 1999). Although these inhibitory actions against the stimuli-evoked formation of various immunoregulatory substances have been demonstrated, it is not known whether cAMP is involved in the downregulation of the urokinase plasminogen activator (u-PA) system and superoxide anion (SA) production in porcine macrophages and neutrophils. The u-PA is an important enzyme for phagocyte diapedesis and the overall ability of these cells to resist various pathogens (Politis *et al.* 2003).

Protein kinase A (PKA) is a primary target of cAMP in a diverse number of tissues and cell types. Recently, however, the expression of an alternative cAMP signalling target, exchange protein directly activated by cAMP-1 (Epac-1), has been described in various cell types, where it is known to modify cellular functions independently of PKA (Christensen *et al.* 2003; Aronoff *et al.* 2005). There is no study on whether cAMP and/or Epac-1 is implicated in the downregulation of various immune parameters including the u-PA system and SA production in porcine macrophages and neutrophils.

The objective of the present study was to determine the effect of two chemically synthesised peptides with specific amino acid sequences of bovine β-casein (tripeptide, leucine–leucine–tyrosine, residues 191–193 (LYY); hexapeptide, proline–glycine–proline–isoleucine–proline–asparagine, residues 63–68) on the dynamics of the u-PA system and SA production by phorbol myristate acetate (PMA)-activated macrophages and neutrophils obtained from piglets in the early post-weaning period. Whether PKA and/or Epac-1 is implicated in the mechanism by which these peptides affect the function of macrophages and neutrophils was examined in detail. The u-PA system and SA production by macrophages and neutrophils were selected as the outcome measures because both systems are altered as the immune system moves from immaturity to maturity in the early post-weaning period in piglets (Fragou *et al.* 2004).

Materials and methods

Animal and leucocyte isolation

Fourteen weaning piglets of the Pietrain × Dalant breed, housed within the premises of the experimental farm of the Agricultural University of Athens, were used to supply blood for monocyte–macrophage and neutrophil isolation. Monocytes–macrophages and neutrophils were isolated using methods previously described by Politis *et al.* (2003). Briefly, 15 ml heparinised (10 IU heparin/ml) venous blood were mixed with 15 ml Hanks' Balanced Salt Solution (HBSS; Sigma Chemical Co., St Louis, MO, USA), the mixture then being layered onto a 20 ml sodium metriozate (95 g/l)–polysaccharide (56 g/l) gradient with a specific gravity of $1{\cdot}077 \times 10^6$ g/m^3 (Sigma Chemical Co.) and centrifuged at 500 ***g*** for 45 min.

To isolate the monocytes, cells from the interface were collected, washed twice in RPMI-1640 (Sigma Chemical Co.) and resuspended in RPMI-1640 containing fetal bovine serum (100 ml/l) at a concentration of 1×10^7 cells/ml. For neutrophil isolation, cells from the bottom layer were washed twice in RPMI-1640 medium, and the erythrocytes were lysed by the addition of 20 ml sterile distilled water, followed by the addition of 10 ml sterile saline solution (27 g NaCl/l) to restore isotonicity. Cells were pelleted by centrifugation (200 ***g***, 10 min) and resuspended in RPMI-1640 medium containing fetal bovine serum (100 ml/l) at a concentration of 1×10^7 cells/ml. Cell viability was assessed by Trypan blue dye exclusion (Sigma Chemical Co.) and was always high (>95 %). The purity of the isolated monocytic and neutrophil cell populations was always over 95 %.

Treatment with milk peptides

Cells (monocytes or neutrophils; 2×10^6) were resuspended in 1 ml RPMI-1640 containing fetal bovine serum (100 ml/l) in the absence (control) or presence of one of two chemically synthesised peptides: tripeptide LLY, which corresponds to residues 191–193 of bovine β-casein (1 or 10 μM), or hexapeptide proline–glycine–proline–isoleucine–proline–asparagine, which corresponds to residues 63–68 of bovine β-casein (1 or 10 μM), and were incubated for 24 h. Preliminary experiments indicated that higher concentrations of the peptides (25–100 μM) were equally as effective as the 10 μM concentration, which was then considered optimal for both cell types. The hexapeptide and the tripeptide were purchased from Bachem Bioscience Inc. (Weil am Rhein, Germany). Both peptides were synthesised on a solid phase using 9-fluorenylmethoxycarbonyl chemistry and were then purified using standard HPLC methods (Scanlon & Finlayson, 2004).

Determination of urokinase-plasminogen activator activity

Total cell-associated urokinase-plasminogen activator activity. Total cell-associated u-PA activity (intracellular and membrane bound) was measured in activated monocyte–macrophages and neutrophils. Macrophages and neutrophils were activated following treatment with 80 μM-PMA for 30 min. This combination of concentration and time is sufficient to induce an optimal stimulation of neutrophils and macrophages (Politis *et al.* 2003).

Following treatment for 24 h with one of the two peptides, monocyte–macrophages or neutrophils (2×10^6/ml) were washed three times with HBSS and resuspended in 500 μl HBSS with 80 μM-PMA (Sigma Chemical Co.). After incubation for 30 min at 37 °C, cells were washed three times with HBSS. Cells were then lysed by the addition of 500 μl 1 mM-NaOH, centrifuged at 12 000 ***g*** for 3 min, divided into portions and stored at − 80 °C. Activities of u-PA in portions of lysed neutrophils or macrophages were determined following the procedure described by Politis *et al.* (2003). Briefly, reactions were performed in a total volume of 250 μl 100 mM-Tris buffer (pH 8·0) containing 100 mM-NaCl, 50 μg plasminogen/ml (Sigma Chemical Co.), 0·6 mM-valine–leucine–lysine–*p*-nitroanilide (Sigma Chemical Co.) and 10 μl lysed cells. The reaction mixture was incubated for up to 2 h, and absorbance at 405 nm was measured at 15 min intervals using a microtitre-plate reader. The rate of *p*-nitroaniline formation was calculated from the linear part of the curve for absorbance *v*. time. A sample without plasminogen served as a control.

The assay system utilises the enzymatically active u-PA present within the lysed macrophages or neutrophils to convert exogenously supplied plasminogen to active plasmin. Plasmin so produced is subsequently allowed to attack the chromogenic substrate valine–leucine–lysine–*p*-nitroaniline adjacent to the lysine and liberate the free chromophore *p*-nitroaniline. In this system, changes in colour are directly related to plasmin concentrations, and therefore indirectly related to u-PA activity (Politis *et al.* 2003).

Membrane-bound urokinase-plasminogen activator activity. Membrane-bound u-PA is catalytically active so its activity can be measured in 'live' PMA-stimulated monocyte–macrophages or neutrophils (Politis *et al.* 2003). Following treatment for 24 h with the peptide preparations, monocyte–macrophages or neutrophils (2×10^6/ml) were washed three times with HBSS and resuspended in 500 μl HBSS containing 80 μM-PMA. After incubation for 30 min at 37 °C, cells were washed three times with HBSS and finally resuspended in 250 μl 100 mM-Tris buffer (pH 8·0). Membrane-bound u-PA was determined following the procedure described in detail by Politis *et al.* (2003).

To eliminate the possibility that some u-PA was secreted into the medium in control wells, monocyte–macrophages or neutrophils were removed by centrifugation before each determination. No detectable activity was found in the medium in which the macrophages and neutrophils were cultured. This indicates that no u-PA was released during the incubation period. Therefore, any changes in colour were due to the conversion of plasminogen to plasmin by catalytically active u-PA present on the cell membrane. Cells remained viable and intact throughout this time period.

Free urokinase-plasminogen activator binding sites on cellular membranes. The u-PA binding sites present on the cell membranes of monocyte–macrophages or neutrophils may or may not be fully saturated (Politis *et al.* 2003). The free, unoccupied u-PA binding sites can be fully saturated following the incubation of monocyte–macrophages or neutrophils with purified u-PA. Thus, the difference in membrane-bound u-PA before and after incubation with u-PA reflects the presence of free u-PA binding sites on the cell membrane of macrophages or neutrophils.

Following treatment for 24 h with peptides, monocyte–macrophages or neutrophils (2×10^6/ml) were washed three times with HBSS and resuspended in 500 μl HBSS containing 80 μM-PMA. After incubation for 30 min at 37 °C, cells were washed three times with HBSS and then resuspended in 500 μl HBSS containing purified u-PA (Sigma Chemical Co.; 10 units/ml). After incubation for another 30 min at 37 °C, cells were washed three times with HBSS and then resuspended in 250 μl 100 mM-Tris buffer (pH 8·0) for determination of the membrane-bound u-PA as described earlier. Incubation of macrophages or neutrophils for 30 min at 37 °C in the presence of u-PA is sufficient to fully saturate all the u-PA binding sites on the cell membrane (Politis *et al.* 2003). The specificity of the binding of the exogenous u-PA on the cell membranes was verified using the competition assays described in detail by Politis (1996).

All determinations related to the u-PA system were carried out the day after the blood had been collected, following cell isolation and the 24 h incubation period with the peptides.

Determination of superoxide anion production

SA production was measured in macrophages and neutrophils as a direct indicator of respiratory burst activation by the reduction of ferricytochrome C, which can be inhibited by superoxide dismutase (Politis *et al.* 1995). Following treatment for 24 h with peptides, cells (2×10^6/ml) were washed three times with HBSS, resuspended in 1·4 ml HBSS and incubated in the presence or absence of 10 μl superoxide dismutase (3 g/l; Sigma Chemical Co.). After incubation for 2 min at 37 °C, 0·1 ml type-IV cytochrome C (35 g/l; Sigma Chemical Co.) was added, followed by addition of 1·5 ml 130 μM-PMA. After incubation for a further 10 min, the reaction was terminated by placing the tubes on ice, the cells then being centrifuged at 1500 ***g*** for 20 min. The supernatant was collected, and absorbance at 550 nm was measured at 15 min intervals using a microtitre-plate reader. The amount of SA produced was calculated as the difference between the amount reduced in the presence and absence of superoxide dismutase.

Assessment of the protein kinase A involvement

Following treatment for 24 h with or without one of two peptides (1 or 10 μM), monocytes–macrophages or neutrophils (2×10^6/ml) were washed three times with HBSS and resuspended in 500 μl HBSS containing 10 μM- (N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide dihydrochloride) (H89), (9S,10S,12R)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1h-diindolo [1,2,3-fg:3′,2′,1′-kl] pyrrolo [3,4-i] [1,6] benzodiazocine-10-carboxylic acid hexyl ester (KT 5720), (PKA inhibitor fragment 14–22 myristoylated trifluoroacetate salt) (PKI_{14-22}) or (N-(2-guanidinoethyl)-5-isoquinoline sulfonamide hydrochloride) (HA1004). All PKA inhibitors were purchased from Sigma Chemical Co. PKI_{14-22} is a highly specific PKA inhibitor, whereas H89, KT5720 and HA1004 are non-specific PKA inhibitors (Enserink *et al.* 2002; Aronoff *et al.* 2005). After incubation for 20 min at 37 °C, cells were washed with HBSS and resuspended in 500 μl HBSS containing 80 μM-PMA. After incubation for a further 30 min at 37 °C, the cells were washed and recovered, and membrane-bound u-PA activity was determined as described earlier.

To determine PKA involvement in SA production, macrophages and neutrophils, following treatment for 24 h with or without milk peptides (1 or 10 μM), were washed three times with HBSS, resuspended in 1·4 ml HBSS and incubated in the presence or absence of 10 μM-H89, KT5720, PKI_{14-22} or HA1004. After incubation for 20 min at 37 °C, cells were washed and recovered, and SA production was determined as described earlier.

Assessment of exchange protein directly activated by cyclic AMP-1 involvement

Following isolation, monocytes–macrophages or neutrophils (2×10^6/ml) were resuspended in 500 μl HBSS containing phosphodiesterase-resistant cAMP analogues that were highly selective in their activation of PKA and/or Epac-1: 10 μM-(S)-*p*-8-(4-chloro-phenylthio)adenosine-3′,5′-cAMP, 1 or 10 μM-N6-benzoyladenosine-3′,5′-cAMP or 1 or 10 μM-8-pCPT-2′-O-Me-cAMP. (S)-*p*-8-(4-chloro-phenylthio)adenosine-3′,5′-cAMP is a dual PKA/Epac agonist, whereas N6-benzoyladenosine-3′,5′-cAMP and 8-pCPT-2′-O-Me-cAMP are PKA and Epac-1 specific agonists, respectively (Christensen *et al.* 2003; Aronoff *et al.* 2005). All PKA and/or Epac-1 agonists were purchased from Sigma Chemical Co. After incubation for 30 min at 37 °C, cells were washed three times with HBSS and resuspended in 500 μl HBSS. Membrane-bound u-PA activity and SA production were determined as described earlier.

Statistical analysis

Values are reported as means and standard deviations. Differences between the means were determined using the Fischer's least significant difference procedure.

Results

Urokinase-plasminogen activator activities

Both the tripeptide and the hexapeptide caused decreases ($P<0·01$) in total cell-associated u-PA, membrane-bound u-PA activity and the free u-PA binding sites of monocytes–macrophages, compared with the control values, during weeks 1–2 after weaning (Table 1). The tripeptide LLY was more potent than the hexapeptide proline–glycine–proline–isoleucine–proline–asparagine. The effectiveness of both peptides was dose-related, and the optimal concentration for all peptides was 10 μM.

The tripeptide LLY, but not the hexapeptide proline–glycine–proline–isoleucine–proline–asparagine, at a concentration of 10 μM, caused a decrease in the total cell-associated u-PA and membrane-bound u-PA activity of neutrophils compared with the control values during weeks 1–2 after weaning. None of the peptides affected the free u-PA binding sites of neutrophils (Table 1).

The effect of the two peptides on total cell-associated u-PA, membrane-bound u-PA activity and the free u-PA binding sites of monocytes–macrophages and neutrophils, during weeks 5–6 after weaning, was examined. None of the peptides affected ($P>0·05$) total cell-associated u-PA, membrane-bound u-PA activity or the free u-PA binding sites of monocytes–macrophages and neutrophils (data not shown).

Superoxide anion production

The effect of the peptides on SA production by monocytes–macrophages and neutrophils during weeks 1–2 after weaning was examined. Both peptides caused a decrease ($P<0·01$) in SA production by monocytes–macrophages, compared with control values, during weeks 1–2 after weaning (Table 2).

Table 1. Effects of two bovine β-casein peptides on total cell-associated urokinase plasminogen activator (u-PA), membrane-bound u-PA and free u-PA binding sites of porcine macrophages and neutrophils activated by phorbol myristate acetate (80 μM) obtained from piglets during weeks 1–2 after weaning

(Mean values and standard deviations for ten observations per group)

	u-PA activity (ΔA/h)					
	Total		Membrane-bound		Free u-PA binding sites	
Treatment	Mean	SD	Mean	SD	Mean	SD
Macrophages						
Control	0·15[a]	0·03	0·15[a]	0·02	0·35[a]	0·05
Tripeptide (1 μM)	0·09[c]	0·02	0·09[c]	0·02	0·23[bc]	0·05
Tripeptide (10 μM)	0·06[d]	0·02	0·06[d]	0·02	0·16[d]	0·03
Hexapeptide (1 μM)	0·13[b]	0·02	0·12[b]	0·02	0·26[b]	0·04
Hexapeptide (10 μM)	0·09[c]	0·02	0·09[c]	0·02	0·21[c]	0·05
Neutrophils						
Control	0·27[a]	0·03	0·27[a]	0·03	0·37	0·05
Tripeptide (1 μM)	0·26[a]	0·04	0·26[a]	0·04	0·36	0·04
Tripeptide (10 μM)	0·19[b]	0·02	0·18[b]	0·02	0·33	0·07
Hexapeptide (1 μM)	0·26[a]	0·03	0·25[a]	0·03	0·37	0·06
Hexapeptide (10 μM)	0·27[a]	0·03	0·26[a]	0·03	0·38	0·05

Tripeptide, leucine–leucine–tyrosine (corresponding to residues 191–193 of bovine β-casein); hexapeptide, proline–glycine–proline–isoleucine–proline–asparagine (corresponding to residues 63–68 of bovine β-casein).

[a,b,c,d]Mean values with unlike superscript letters were significantly different according to the least significant difference multiple range test ($P\leq 0·01$).

For details of animals and procedures, see p. 554.

Table 2. Effects of two bovine β-casein peptides on superoxide anion (SA) production by porcine macrophages and neutrophils activated by phorbol myristate acetate (80 μM) obtained from piglets during weeks 1–2 after weaning

(Mean values and standard deviations for twenty observations per group)

	SA production (nmol/10^6 cells)			
	Macrophages		Neutrophils	
Treatment	Mean	SD	Mean	SD
Control	1·30[a]	0·28	2·08[a]	0·36
Tripeptide (1 μM)	0·88[c]	0·11	1·95[a]	0·32
Tripeptide (10 μM)	0·49[d]	0·11	1·50[b]	0·30
Hexapeptide (1 μM)	1·08[b]	0·28	2·03[a]	0·34
Hexapeptide (10 μM)	0·86[c]	0·18	1·99[a]	0·34

Tripeptide, leucine–leucine–tyrosine (corresponding to residues 191–193 of bovine β-casein); hexapeptide, proline–glycine–proline–isoleucine–proline–asparagine (corresponding to residues 63–68 of bovine β-casein).

[a,b,c,d]Mean values with unlike superscript letters were significantly different according to the least significant difference multiple range test ($P \leq 0{\cdot}01$).

For details of animals and procedures, see p. 554.

The effectiveness of the two peptides on SA production by monocytes–macrophages was dose-related, and the optimal concentration for both peptides was 10 μM. Only the tripeptide LLY, at the high concentration of 10 μM, caused a fall in SA production by the neutrophils, compared with the control values, during weeks 1–2 after weaning (Table 2). None of the peptides affected SA production by macrophages or neutrophils during weeks 5–6 after weaning (data not shown).

Exchange protein directly activated by cyclic AMP-1 involvement

The effect of three phosphodiesterase-resistant cAMP analogues that were highly selective in their activation of PKA and/or Epac-1 on the membrane-bound u-PA activity of PMA-activated monocytes–macrophages and neutrophils was examined. Addition of the dual PKA/Epac-1 agonist (10 μM) or the PKA specific agonist (10 μM), but not the Epac-1 agonist, caused a fall ($P < 0{\cdot}01$) in the membrane-bound u-PA activity of both monocytes–macrophages and neutrophils (Table 3). The dual PKA/Epac agonist was equally as effective as the PKA agonist. Thus, the dual PKA/Epac and PKA specific activator mimicked the effect of all peptide preparations on the membrane-bound u-PA activities of monocytes–macrophages and neutrophils.

The effect of the three cAMP analogues on SA production by monocytes–macrophages and neutrophils was examined. All activators (dual, PKA and Epac-1) reduced SA production by monocytes–macrophages. Furthermore, all activators caused a decrease in SA production by neutrophils only when used at the high concentration of 10 μM (Table 3). The dual PKA/Epac-1 agonist was a more potent inhibitor of SA production by monocytes–macrophages than either the PKA or the Epac-1 specific agonist. In contrast, all three activators were equally effective in reducing SA production by neutrophils.

Protein kinase A involvement

The effect of four different PKA inhibitors on membrane-bound u-PA activity and SA production by PMA-activated monocytes–macrophages and neutrophils was examined. Addition of the highly specific PKA inhibitor PKI_{14-22} or the non-specific PKA inhibitors H89, KT5720 or HA1004, at a concentration of 10 μM, completely abolished the effect of both peptides on the membrane-bound u-PA activity of monocytes-macrophages. Furthermore, all the inhibitors completely abolished the effect of tripeptide LLY on the membrane-bound u-PA activity of neutrophils (Table 4).

Conversely, the addition of the specific and non-specific PKA inhibitors partially inhibited the effect of both peptides on SA production by monocytes–macrophages. In the same way, all the inhibitors partially blocked the effect of the tripeptide LLY on SA production by neutrophils (Table 5).

Discussion

The first new finding emerging from the present study was that both peptides tested (hexapeptide and tripeptide) were effec-

Table 3. Effects of various cyclic AMP (cAMP) analogues on membrane-bound urokinase plasminogen activator (u-PA) and superoxide anion (SA) production by phorbol myristate acetate (80 μM)-activated porcine macrophages and neutrophils obtained from piglets during weeks 1–2 after weaning

(Mean values and standard deviations for ten observations per group)

	Membrane-bound u-PA activity (ΔA/h)				SA production (nmol/10^6 cells)			
	Macrophages		Neutrophils		Macrophages		Neutrophils	
Treatment	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	0·16[a]	0·02	0·24[a]	0·04	1·20[a]	0·10	2·04[a]	0·21
Dual PKA/Epac activity (10 μM)	0·08[b]	0·01	0·17[b]	0·02	0·59[f]	0·07	1·48[b]	0·11
PKA activity (1 μM)	0·15[a]	0·02	0·24[a]	0·03	1·06[b]	0·06	2·07[a]	0·20
PKA activity (10 μM)	0·09[b]	0·02	0·18[b]	0·02	0·77[d]	0·06	1·63[b]	0·09
Epac-1 activity (1 μM)	0·16[a]	0·02	0·24[a]	0·03	0·88[c]	0·07	1·10[a]	0·19
Epac-1 activity (10 μM)	0·16[a]	0·02	0·24[a]	0·03	0·68[e]	0·03	1·51[b]	0·16

Dual PKA/Epac activator, (S)-p-8-(4-chloro-phenylthio) adenosine-3′,5′-cAMP; PKA activator, 6-Bnz-cAMP (N6-benzoyladenosine-3′,5′-cAMP); Epac-1 activator, 8-pCPT-2′-O-Me-cAMP.

[a,b,c,d,e,f]Mean values with unlike superscript letters were significantly different according to the least significant difference multiple range test ($P \leq 0{\cdot}01$).

For details of animals and procedures, see p. 554.

Table 4. Effect of four protein kinase A inhibitors on membrane-bound urokinase plasminogen activator (u-PA) activities of phorbol myristate acetate (PMA; 80 μM)-activated porcine macrophages and neutrophils obtained from piglets during weeks 1–2 after weaning and cultured in the presence or absence of bovine β-casein peptides (10 μM)

(Mean values and standard deviations for eleven observations per group)

	Membrane-bound u-PA activity (ΔA/h)					
	Macrophages				Neutrophils	
	Tripeptide		Hexapeptide		Tripeptide	
Treatment	Mean	SD	Mean	SD	Mean	SD
PMA	0·20[a]	0·03	0·20[a]	0·03	0·30[a]	0·03
PMA + peptide	0·09[b]	0·02	0·11[b]	0·02	0·19[b]	0·02
PMA + peptide + H89 (10 μM)	0·21[a]	0·04	0·20[a]	0·03	0·30[a]	0·03
PMA + peptide + KT5720 (10 μM)	0·21[a]	0·04	0·20[a]	0·03	0·31[a]	0·05
PMA + peptide + PKI_{14-22} (10 μM)	0·21[a]	0·04	0·20[a]	0·03	0·30[a]	0·03
PMA + peptide + HA1004 (10 μM)	0·21[a]	0·04	0·20[a]	0·02	0·29[a]	0·04

Tripeptide, leucine–leucine–tyrosine (corresponding to residues 191–193 of bovine β-casein); hexapeptide, proline–glycine–proline–isoleucine–proline–asparagine (corresponding to residues 63–68 of bovine β-casein); H89, N-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride; KT5720, (9S,10S,12R)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester; PKI_{14-22}, protein kinase A inhibitor fragment 14–22, myristoylated trifluoroacetate salt; HA 1004, N-(2-guanidinoethyl)-5-isoquinoline sulfonamide hydrochloride.

[a,b]Mean values with unlike superscript letters were significantly different according to the least significant difference multiple range test ($P \leq 0{\cdot}01$).

For details of animals and procedures, see p. 554.

tive at causing a downregulation of the critical parameters of macrophages obtained from piglets during weeks 1–2 after weaning. More specifically, both peptides caused a decrease in total cell-associated u-PA, membrane-bound u-PA activity, free u-PA binding sites and SA production by PMA-activated monocytes–macrophages. It is interesting to note that the tripeptide was a more potent immunoregulator than the hexapeptide. In marked contrast, none of the peptide preparations tested was effective on monocytes–macrophages during weeks 5–6 after weaning. All piglets are born with an immature immune system, and piglets move gradually towards maturity throughout the pre-weaning and the early post-weaning period (Hunter, 1986). The most reasonable explanation for the present findings is that peptides are effective at a time that coincides with immaturity of the immune system (weeks 1–2 after weaning) and that they are not effective 1 month later, at a time when the immune system has presumably gained its full functionality.

Further support for the idea that the effectiveness of the peptides was related to the maturity of the immune system is provided by the work of Fragou *et al.* (2004), who reported evidence related to the maturational development of porcine

Table 5. Effect of four protein kinase A inhibitors on superoxide anion (SA) production by phorbol myristate acetate (PMA; 80 μM)-activated porcine macrophages and neutrophils obtained from piglets during weeks 1–2 after weaning and cultured in the presence or absence of bovine β-casein peptides (10 μM)

(Mean values and standard deviations for eleven observations per group)

	SA production (nmol/10^6 cells)					
	Macrophages				Neutrophils	
	Tripeptide		Hexapeptide		Tripeptide	
Treatment	Mean	SD	Mean	SD	Mean	SD
PMA	1·46[a]	0·15	1·42[a]	0·14	2·36[a]	0·23
PMA + peptide	0·59[c]	0·06	0·78[c]	0·05	1·56[c]	0·12
PMA + peptide + H89 (10 μM)	1·17[b]	0·09	1·20[b]	0·12	1·89[b]	0·09
PMA + peptide + KT5720 (10 μM)	1·15[b]	0·06	1·21[b]	0·09	1·92[b]	0·14
PMA + peptide + PKI_{14-22} (10 μM)	1·16[b]	0·10	1·13[b]	0·14	1·96[b]	0·14
PMA + peptide + HA1004 (10 μM)	1·14[b]	0·07	1·19[b]	0·12	1·97[b]	0·19

Tripeptide, leucine–leucine–tyrosine (corresponding to residues 191–193 of bovine β-casein); hexapeptide, proline–glycine–proline–isoleucine–proline–asparagine (corresponding to residues 63-68 of bovine β-casein); H89, N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride; KT5720, (9S,10S,12R)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester; PKI_{14-22}, protein kinase A inhibitor fragment 14–22, myristoylated trifluoroacetate salt; HA 1004, N-(2-guanidinoethyl)-5-isoquinoline sulfonamide hydrochloride.

[a,b,c]Mean values with unlike superscript letters were significantly different according to the least significant difference multiple range test ($P \leq 0{\cdot}01$).

For details of animals and procedures, see p. 554.

phagocytes in the post-weaning period. More specifically, Fragou *et al.* demonstrated that total cell-associated u-PA, membrane-bound u-PA and SA production were lowest at day 0, increased gradually on days 12 and 24 and reached their maximum values on day 36 after weaning. Membrane-bound u-PA and SA production by macrophages on day 36 were 2–2·4-fold higher than the corresponding values on day 12 after weaning. Membrane-bound u-PA and SA production by neutrophils on day 36 were 87–108 % higher than the corresponding values on day 12 after weaning. The results of the present study, taken together with those of Fragou *et al.* (2004), strengthen the hypothesis that bioactive β-casein peptides are capable of guiding the immune system of piglets in terms of the process of maturational development of professional phagocytes.

The magnitude of the responses caused by the peptides is presumably of considerable importance from a biological point of view. In the present study, tripeptide at a concentration of 10 μM caused a fall in the membrane-bound u-PA of macrophages and neutrophils of 116 % and 50 %, respectively, compared with the corresponding control values. In a similar way, the tripeptide at the same concentration caused a decrease in SA production by macrophages and neutrophils of 165 % and 39 %, respectively, compared with the corresponding control values.

Two previous *in vivo* studies provide the biological basis from which to evaluate the magnitude of the present responses. More specifically, the SA production of fully mature neutrophils obtained from healthy adults was 51 % higher than that detected in human neonatal neutrophils, cells with a well-characterised developmental immaturity (Komatsu *et al.* 2001). Fragou *et al.* (2004) reported that the SA production of mature macrophages and neutrophils was 65–70 % higher than the corresponding value at weaning (immature state). Furthermore, membrane u-PA in macrophages and neutrophils was 50–65 % higher in mature macrophages and neutrophils compared with the corresponding value of their immature counterparts at weaning. Thus, the magnitude of the present responses is the same as or even higher than that observed in the two previous *in vivo* studies comparing SA production and the u-PA system in mature compared with immature immunological states.

Several studies have provided evidence that the hydrolysis of various milk proteins generates peptides capable of downregulating or upregulating various immune functions other than the u-PA system and the generation of free radicals. Otani & Hata (1995) reported that pancreatin and trypsin digests of β-casein inhibited cell proliferation in response to mitogens. Kayser & Meisel (1996) described how trypsin digests of bovine β-casein enhanced or inhibited the mitogen-induced proliferation of peripheral blood mononuclear cells. Elitsur & Luk (1991) similarly reported that β-casomorphins (fragments of β-casein, residues 60–70) inhibited the proliferation of human lamina-propria-derived lymphocytes *in vitro*. On the other hand, Migliore-Samour & Jolles (1988), using the same peptides that were used in the present study, found that both peptides (hexapeptide and tripeptide) promoted antibody formation and enhanced phagocytosis by murine peritoneal macrophages, whereas the tripeptide, but not the hexapeptide, enhanced antigen-dependent T-cell proliferation. Thus, the tripeptide was more potent than the hexapeptide in terms of its effect on T-cell proliferation. Politis (1995) reported that the hexapeptide upregulated IL-1 production, the expression of MHC class II molecules and SA production by macrophages obtained from lactating cows.

It is not clear why the same peptides are capable of causing a suppression of immune function in some instances and an induction in others. It is apparent that the effectiveness of the peptides may be species specific, that it is affected by sampling time and the developmental stage of the animals, and finally that it is probably related to the state of differentiation of these cells.

The second finding to emerge from the present study is that only the tripeptide LLY inhibited total cell-associated u-PA, membrane-bound u-PA activity, free u-PA binding sites and SA production by PMA-activated neutrophils obtained from piglets during weeks 1–2 after weaning. In contrast, the tripeptide was ineffective towards neutrophils when the immune system had reached full maturity (weeks 5–6 after weaning). It should be noted that neutrophils are affected less by the peptides than are the corresponding macrophages. Thus, the effectiveness of the peptides depends on the cell type, the time from weaning and/or the state of differentiation of these cells.

The cAMP signalling pathway is a functionally important mechanism in regulating immune function. More specifically, cAMP is known to inhibit a number of critical functional properties of alveolar macrophages, such as phagocytosis (Aronoff *et al.* 2004), the production of reactive oxygen species (Dent *et al.* 1994) and the production of various inflammatory mediators (Rowe *et al.* 1997). cAMP generated by adenylate cyclase acts as a second messenger capable of activating PKA, which phosphorylates numerous intracellular proteins possessing a specific serine/threonine-containing motif. Recently, a number of PKA-independent targets for cAMP have been described, affecting several cellular functions. These include cyclic nucleotide gated channels and the guanine exchange proteins directly activated by cAMP, such as Epac-1 and Epac-2. Aronoff *et al.* (2005) examined in detail the role of PKA and/or Epac-1 in alveolar macrophages. They found that the activation of Epac-1, but not PKA, suppressed phagocytosis. In contrast, the activation of PKA inhibited the production of leukotrienes and TNF-α. Finally, the activation of PKA or Epac-1 suppressed bactericidal activity and SA production.

The third finding from the present study is that the effect of both peptides on the u-PA system of porcine macrophages and neutrophils requires an activation of the PKA system. Two lines of evidence suggest that PKA alone is responsible for the downregulation of the u-PA system. First, in contrast with the effectiveness of the dual PKA/Epac agonist (10 μM) and the PKA specific agonist (10 μM), the Epac-1 specific agonist had no effect on the u-PA system of PMA-activated macrophages and neutrophils. Second, the addition of the highly specific inhibitor PKI_{14-22} or the three non-specific PKA inhibitors (H89, KT5720 and HA1004) completely abolished the effect of the peptides on the u-PA system of PMA-stimulated macrophages and neutrophils. Thus, the PKA system, but not Epac-1, is implicated in the mechanism by which the peptides affect the u-PA system.

The fourth finding of the present study was that the effect of both peptides on SA production by porcine macrophages and neutrophils required an activation of the PKA system and/or Epac-1. This conclusion is supported by two important

observations. First, all three cAMP analogues (dual, PKA specific and Epac-1 specific activator) reduced SA production by monocytes–macrophages and neutrophils. Second, the addition of the highly specific inhibitor PKI_{14-22} or the three non-specific PKA inhibitors (H89, KT5720 and HA1004) partially inhibited the effect of the peptides on SA production by monocytes–macrophages and neutrophils. These findings suggest that PKA and/or Epac-1 is implicated in the mechanism by which the peptides affect SA production by monocytes–macrophages and neutrophils.

In summary, the present experiments demonstrated that two chemically synthesised peptides with amino acid sequences of bovine β-casein suppressed the u-PA system and the production of SA by porcine macrophages during the early post-weaning period. The tripeptide was a more potent immunosuppressor than the hexapeptide or the crude preparation. In contrast, only the tripeptide LLY inhibited the u-PA system and SA production by porcine neutrophils during the same time period. The PKA system, and not Epac-1, was implicated in the mechanism by which the peptide preparations affected the u-PA system in both macrophages and neutrophils. By contrast, PKA and/or Epac-1 were implicated in the mechanism by which the peptide preparations affected SA production in the same cell types. Overall, the results of the present study provide support for the idea that peptides originating from β-casein are capable of guiding the immune system of piglets in the process of the maturational development of its phagocytes.

The putative benefits of downregulating the activity of professional phagocytes in the intestine during the early post-weaning period are not known with certainty. Future studies using cells obtained from piglets in the pre-weaning period, including suckling animals, will help us to elucidate the role of casein peptides throughout the whole period of the maturational development of professional phagocytes.

References

Aronoff DM, Canetti C & Peters-Golden M (2004) Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. *J Immunol* **173**, 559–563.

Aronoff DM, Canetti C, Serezani C, Luo M & Peters-Golden M (2005) Cutting edge. Macrophage inhibition by cyclic AMP: differential roles of protein kinase A and exchange protein directly activated by cAMP-1. *J Immunol* **174**, 595–599.

Baldi A, Politis I, Pecorini C, Fusi E, Chronopoulou R & Dell'Orto V (2005) Biological effects of milk proteins and their peptides with emphasis on those related to the gastrointestinal ecosystem. *J Dairy Res* **72**, 1–7.

Christensen AE, Selheim F, De Rooij J, *et al.* (2003) cAMP analog mapping of Epac-1 and cAMP kinase: discriminating analogs demonstrate that Epac-1 and cAMP kinase act synergistically to promote PC-12 cell neurite extension. *J Biol Chem* **278**, 35394–35402.

Clare DA & Swaiswood HE (2000) Bioactive milk peptides: a prospectus. *J Dairy Sci* **83**, 1187–1195.

Dent G, Giembycz MA, Rabe KF, Wolf B, Barnes PJ & Magnussen H (1994) Theophylline suppresses human alveolar macrophage respiratory burst through phosphodiesterase inhibition. *Am J Respir Cell Mol Biol* **10**, 565–572.

Elitsur Y & Luk GD (1991) Beta-casomorphin (BCM) and human colonic lamina propria lymphocyte proliferation. *Clin Exp Immunol* **85**, 493–497.

Enserink JM, Christensen AE, De Rooij J, Van Triest M, Schwede F, Genieser HG, Doskeland OS, Blank JL & Bos JL (2002) A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nat Cell Biol* **4**, 901–906.

Fragou S, Fegeros K, Xylouri E, Baldi A & Politis I (2004) Effect of vitamin E supplementation on various functional properties of macrophages and neutrophils obtained from weaned piglets. *J Vet Med A* **51**, 178–183.

Hunter P (1986) The immune system of the neonatal and weaner piglet: a review. *J S Afr Vet Assoc* **57**, 243–245.

Kayser H & Meisel H (1996) Stimulation of human peripheral blood lymphocytes by bioactive peptides derived from bovine milk proteins. *FEBS Letts* **383**, 18–20.

Komatsu H, Tsukimori K, Hata K, Satoh S & Nakano H (2001) The characterization of superoxide production of human neonatal neutrophil. *Early Hum Dev* **65**, 11–19.

Kunkel SL, Chensue SW & Phan SH (1986) Prostaglandins as endogenous mediators of interleukin 1 production. *J Immunol* **136**, 186–192.

LeBlanc JG, Matar C, Valdez JC, LeBlanc J & Perdigon G (2002) Immunomodulating effects of peptidic fractions issued from milk fermented with *Lactobacillus helveticus*. *J Dairy Sci* **85**, 2733–2742.

Matar C, Valdez JC, Medina M, Rachid M & Perdigon G (2001) The implication of proteolysis during milk fermentation on enhancing the immune system and the regression of fibrosarcomas. *J Dairy Res* **68**, 601–609.

Meisel H (2005) Biochemical properties of peptides encrypted in bovine milk proteins. *Curr Med Chem* **12**, 1905–1919.

Migliore-Samour D & Jolles P (1988) Casein, a prohormone with immunomodulating role for the newborn? *Experimentia* **44**, 188–193.

Mustafa SB & Olson MS (1998) Expression of nitric-oxide synthase in rat Kupffer cells is regulated by cAMP. *J Biol Chem* **273**, 5073–5080.

Otani H & Hata L (1995) Inhibition of proliferative responses of mouse spleen lymphocytes and rabbit Peyer's patch cells by bovine milk caseins and their digests. *J Dairy Res* **62**, 339–348.

Perdigon G, Vintini E, Alvarez S, Medina M & Medici M (1999) Study of the possible mechanisms involved in the mucosal immune system activation by lactic acid bacteria. *J Dairy Sci* **82**, 1108–1114.

Pessi T, Isolauri E, Sutas Y, Kankaanranta H, Moilanen E & Hurme M (2001) Suppression of T-cell activation by *Lactobacillus rhamnosus* GG-degraded bovine casein. *Immunopharmacology* **1**, 211–218.

Petrova T, Alcama K & Van Eldik LJ (1999) Selective modulation of microglial activation by prostaglandin E_2. *J Biol Chem* **274**, 28823–28827.

Politis I (1995) Effects of milk peptides on the immune system of dairy cows. In *Reproduction and Animal Breeding: Advances and Strategy*, pp. 309–314 [G Enne, GF Greppi and A Lauria, editors]. Paris: Elsevier.

Politis I (1996) Plasminogen activator system: implications for mammary growth and involution. *J Dairy Sci* **79**, 1097–1107.

Politis I, Hidiroglou M, Batra TR, Gilmore JA, Gorewit RC & Scherf H (1995) Effects of vitamin E on immune function of dairy cows. *Am J Vet Res* **56**, 179–184.

Politis I, Voudouri A, Bizelis I & Zervas G (2003) The effect of various vitamin E derivatives on the urokinase-plasminogen activator system of ovine macrophages and neutrophils. *Br J Nutr* **89**, 259–265.

Procopio DO, Teixeira MM, Camargo MM, Travassos LR, Ferguson MA, AlmeidaI C & Gazzinelli RT (1999) Differential inhibitory mechanism of cyclic AMP on TNF-alpha and IL-12 synthesis by macrophages exposed to microbial stimuli. *Br J Pharmacol* **127**, 1195–1205.

Qiao J, Mei FC, Popov VL, Vergara LA & Cheng X (2002) Cell cycle-dependent subcellular localization of exchange factor directly activated by cAMP. *J Biol Chem* **19**, 26581–26586.

Rowe J, Finlay-Jones JJ, Nicholas TE, Bowden J, Morton S & Hart PH (1997) Inability of histamine to regulate TNF-(production by human alveolar macrophages. *Am J Respir Cell Mol Biol* **17**, 218–223.

Scanlon DB & Finlayson J (2004) Prep/Semiprep separations of peptides. *Methods Mol Biol* **251**, 191–210.

Tetsuka T, Daphna-Iken D, Srivastava SK, Baier LD, DuMaine J & Morrison AR (1994) Cross-talk between cyclooxygenase and nitric oxide pathways: prostaglandin E2 negatively modulates induction of nitric oxide synthase by interleukin 1. *Proc Natl Acad Sci U S A* **91**, 12168–12172.

Totsuka M, Kakehi M, Kohyama M, Hachimura S, Hisatsure T & Kaminogawa S (1998) Enhancement of antigen-specific IFN-gamma production from CD8 (+) T cells by a single amino acid-substituted peptide derived from bovine alpha s_1-casein. *Clin Immunol Immunopathol* **88**, 277–286.