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Stimulation of murine peritoneal macrophage functions by neuropeptide Y and peptide YY. Involvement of protein kinase C

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SUMMARY

The peptides neuropeptide Y (NPY) and peptide YY (PYY) at concentrations from 10^{-12} M to 10^{-8} M have been shown in this study to stimulate significantly, *in vitro*, several functions of resting peritoneal macrophages from BALB/c mice: adherence to substrate, chemotaxis, ingestion of inert particles (latex beads) and foreign cells (*Candida albicans*), and production of superoxide anion measured by nitroblue tetrazolium reduction. A dose–response relationship was observed, with a maximal stimulation of the macrophage functions studied at 10^{-10} M. These effects seem to be produced by specific receptors for the neuropeptides studied in peritoneal macrophages. Whereas the two peptides induced no change of intracellular cyclic AMP, they caused a significant stimulation of protein kinase C (PKC) in murine macrophages. These results suggest that NPY and PYY produce their effects on macrophage function through PKC activation.

INTRODUCTION

The idea of a functional connection among the nervous, endocrine and immune systems is now accepted. This connection has been supported by the observation that peptides from nervous or endocrine systems have receptors on immune cells and modulate the function of such cells.

Neuropeptide Y (NPY) and peptide YY (PYY) are two regulatory peptides from the pancreatic polypeptide family. They are 36 amino acid residues long, most of them homologous, and contain a C-terminal tyrosylamide and a N-terminal tyrosine amide.[1,2]

NPY, originally isolated from porcine brain,[2] has an important role as neurotransmitter or neuromodulator in the nervous system and it has been suggested as a hypophysiotropic neurohormone that regulates the secretion of various pituitary hormones such as luteinizant hormone (LH) in mammals.[3] Studies in some animal species have established the widespread distribution of NPY in both central and peripheral neurons, so that it is considered as a chemical messenger and an ubiquitous marker of the nervous system tissue.[3,4] In addition, NPY has been found in nerve endings in immunocompetent organs.[5,6] NPY is present in noradrenergic neurons and causes mainly increased vascular resistance.[7] In addition, NPY has an important role in the regulation of cardiovascular functions[8] and also exerts a great variety of physiological and pharmacological actions, including effects on food and water intake.[4]

PYY, which has a 70% sequence homology with NPY, is a gut regulatory peptide produced in endocrine cells from the lower intestine.[9] This peptide plays a regulatory role on pancreatic secretion,[10] smooth muscle tone, local blood flow[11] and food intake.[12]

NPY and PYY often although not always share common binding sites, which are distinct from the receptor for pancreatic polypeptide.[13] Studies *in vitro* have indicated that the C-terminal portion of NPY is primarily responsible for the biological activity of the peptide, while the N-terminal part would be involved in the affinity of NPY for its putative receptors.[14]

The effects of these peptides on the immune system have been very scarcely studied. NPY has been hypothesized to regulate the immune system, because this neuropeptide has been found in spleen nerve terminals.[6] Moreover, circulating concentrations of NPY have been shown to be inversely correlated with natural killer (NK) activity in depressed patients.[15] The effect of this peptide on immune cells has only been investigated on proliferation of lymphocytes *in vitro*, producing no change on spontaneous lymphoproliferation[16] or a decrease in the proliferative response to mitogen PHA.[17] The PYY to our knowledge has not been investigated in the immune system.

Therefore, the aim of the present work was to study for the first time the effects *in vitro* of NPY and PYY on the macrophage function. We show here the effect of a wide range of concentrations of these peptides on several representative

Abbreviations: NBT, nitroblue tetrazolium; NPY, neuropeptide Y; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; PKC, protein kinase C; PYY, peptide YY.

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activities of peritoneal macrophages: adherence to substrate, chemotaxis, ingestion of foreign cells and inert particles, and production of superoxide anion. In addition, since NPY has been shown to inhibit adenylate cyclase activity in the central nervous system[18] and increase intracellular Ca^{2+} and inositol phosphate concentrations in human erythroleukaemia cells,[19] we have studied the effect produced by NPY and PYY on intracellular levels of cAMP and protein kinase C (PKC) activity in murine peritoneal macrophages.

MATERIALS AND METHODS

Animals
Male BALB/c mice (*Mus musculus*) (Iffa Credo, St Germain Sur L'Arbrese, France), aged 20 ± 2 weeks, were maintained at a constant temperature ($22 \pm 2°$) in sterile conditions inside an aseptic air negative-pressure environmental cabinet (Flufrance, Cachan, France) on a 12-hr light/dark cycle and fed Sander Mus and water *ad libitum*. Mice were checked periodically to assess their pathogen-free condition.

Collection of peritoneal exudate cells
Each animal was killed by cervical dislocation, the abdomen cleansed with 70% ethanol, the abdominal skin carefully dissected without opening the peritoneum, and 4 ml of Hanks' medium (Flow, McLean, VA), adjusted to pH 7·4, injected intraperitoneally. Then, the abdomen was massaged and the peritoneal exudate cells (PEC), containing 40% of the macrophages and 60% of the lymphocytes, removed. A recovery of 90–95% of the injected volume of Hanks' medium was obtained. Resting macrophages, determined by morphology and non-specific esterase staining, were counted and adjusted in the same medium at 5×10^5 macrophages/ml. Cellular viability was routinely measured before and after each experiment by the trypan blue exclusion test, and experiments in which cell viability was less than 95% were discarded.

All the incubations were performed at 37° in a humidified atmosphere of 5% CO_2. In the different assays performed with peritoneal macrophages, a positive control with the calcium ionophore A23187 (10^{-7} M; Sigma, St Louis, MO) was included.

Assay of adherence capacity
For the quantification of substrate adherence capacity, we have used the adherence to a smooth plastic surface because it resembles adherence to animal tissue.[20] The method was carried out as previously described by us.[21] Briefly, aliquots of the peritoneal suspension were placed in Eppendorf tubes and incubated with NPY or PYY (Sigma) at a final concentration ranging from 10^{-8} to 10^{-12} M, or Hanks' medium (controls). At 5, 10, 20 or 60 min of incubation, after gently shaking, the number of non-adhered macrophages was determined. The adherence index, AI, was calculated according to the following equation:

$$AI = 100 - \frac{\text{macrophages/ml supernatant}}{\text{macrophages/ml original sample}} \times 100.$$

Assay of chemotaxis
Chemotaxis was evaluated according to a modification[21] of the original technique described by Boyden,[22] which consists basically of the use of chambers with two compartments separated by a Millipore filter with a pore diameter of 3 μm. Aliquots of PEC were deposited in the upper compartment of the Boyden chambers containing NPY or PYY at final concentrations ranging from 10^{-8} to 10^{-12} M, or Hanks' medium (controls). Then, f-met-leu-phe (10^{-8} M; Sigma), as a positive chemotactic agent, was put into the lower compartment. The chambers were incubated for 3 hr, then the filters fixed and stained, and the chemotaxis index, which represents the total number of macrophages counted in the lower face of the filter, was determined.

Phagocytosis assay
Phagocytosis assay of inert particles (latex beads; Sigma; 1·09 μm diluted 1% in PBS) was carried out according to the technique described by De la Fuente *et al.*[21] The incubation of macrophages with each of the two peptides studied (ranging from 10^{-8} to 10^{-12} M), or medium (controls), was performed for 30 min. Afterwards, the number of particles phagocytized per 100 macrophages was counted.

Phagocytosis of *Candida albicans* was carried out by the method previously described.[21] Aliquots of cell suspension were incubated in MIF (migration inhibitory factor) plates (Sterilin, Teddington, U.K.) for 30 min. At the adherent monolayer were added *C. albicans* (10×10^6 cells/ml medium), incubated previously for 30 min with a pool of human serum obtained from 10 healthy men. Immediately, NPY or PYY at final concentrations ranging from 10^{-8} to 10^{-12} M, or medium (controls), were added. After 60 min of incubation, the plates were washed, fixed and stained, and the number of *C. albicans* ingested per 100 macrophages counted.

Assay of nitroblue tetrazolium reduction test
The quantitative nitroblue tetrazolium (NBT) reduction test was carried out according to the method described by De la Fuente *et al.*[21] Macrophage suspensions were mixed with NBT (Sigma; 1 mg/ml Hanks' solution) and the different concentrations of neuropeptides (10^{-8}–10^{-12} M) or medium (controls). To the stimulated samples, latex beads were added, and to the non-stimulated samples, PBS was incorporated. After 60 min of incubation, the absorbance of the reduced NBT was determined in a spectrophotometer at 525 nm. Data obtained were expressed as μmoles of NBT reduced by 10^8 macrophages by extrapolating in a standard curve different concentrations of NBT reduced with 1,4-dithioerythritol (Boehringer, Mannheim, Germany).

Binding sites
Binding sites were performed following the method by Segura *et al.*[23] Peritoneal macrophages ($1{\cdot}5 \times 10^5$ macrophages/ml) were resuspended on 0·5 ml of 35 mM Tris-HCl buffer (pH 7·5) containing 50 mM NaCl, 1·4% (w/v) bovine serum albumin and 45 pM ^{125}I-NPY alone or with increasing concentrations of unlabelled NPY or PYY (from 10^{-12} M to 10^{-7} M). After 90 min of incubation at 15°, the macrophage-bound peptide was separated by centrifugation (2000 ***g***, 5 min) and the cell sediment washed three times. The radioactivity of the cell sediment was measured in a LKB gamma-counter. The specific binding to macrophages was calculated subtracting the non-specific binding, which was determined from binding of tracer in the presence of unlabelled NPY at a 10^{-6} M concentration. This non-specific binding was $3{\cdot}8 \pm 0{\cdot}5\%$ of the total radioactivity added. The

degradation of ^{125}I-NPY was quantified by precipitation in 10% trichloroacetic acid, being only about 10%.

Isolation of peritoneal macrophages for cAMP and PKC measurements

PEC were obtained as indicated above, but with medium 199 (Flow) that was used in all the steps of this schedule. Aliquots of pooled peritoneal suspensions containing 5×10^5 macrophages/ml medium were dispensed into Eppendorf tubes, and incubated for 60 min with the addition of trypsin (0·25%; Sigma) in order to remove non-macrophage cells and debris. Then, adherent macrophages were gently washed three times, and finally incubated with medium 199 for up to 24 hr.

Determination of cAMP

Macrophages prepared as indicated above were incubated with NPY and PYY at final concentrations of 10^{-9} M, 10^{-10} M and 10^{-11} M, or with medium 199 (controls), for 30, 60 and 120 seconds. In all cases, samples were accompanied by a non-incubated sample (basal) to determine the intracellular cAMP levels from which we started in each experiment. After the incubation times indicated, 10% cold trifluoroacetic acid was added to each Eppendorf tube. After vigorous shaking for 2 min, the samples were centrifuged for 10 min at 300 ***g***, and the supernatants collected and lyophilized. Finally, the samples were reconstituted with the corresponding assay buffer and subjected to radioimmunoassay according to the Amersham kit (Amersham, Bucks, U.K.) for cAMP.

Measurement of PKC activity

Purified macrophages ($2{\cdot}5 \times 10^6$ cells/ml) were incubated for 5 min at 37° with phorbol myristate acetate (PMA; 50 ng/ml; Sigma), or with NPY or PYY (10^{-9} M, 10^{-10} M or 10^{-11} M). The incubation was terminated by the addition of ice-cold PBS solution and the cells immediately centrifuged. Then, macrophages were resuspended and centrifuged twice with ice-cold PBS. Cells were resuspended in 1 ml of homogenization buffer (20 mM HEPES, pH 7·5, 250 mM sucrose, 2 mM EDTA, 2 mM EGTA, 10 mM 2-mercaptoethanol, 1 mM phenylmethyl sulphonylfluoride (PMSF), 1 mM aprotinin, 0·1 mg/ml of leupeptin; all from Boehringer), and frozen and thawed twice in liquid nitrogen. The homogenates were centrifuged at 1500 ***g*** for 10 min at 4° in order to discard unbroken cells. The lysates were subsequently centrifuged at 100,000 ***g*** for 60 min at 4°, and the supernatants harvested. The pellets were resuspended in 1 ml of homogenization buffer containing 0·1% Triton X-100 and sonicated for 5 seconds. After 30 min in an ice-cold bath with occasional shaking, the Triton-soluble fractions were centrifuged at 100,000 ***g*** for 60 min at 4°, and the supernatants collected. Both supernatants of particulate fractions, cytosol and membrane, respectively, were applied to 2 ml DEAE–Sephadex (Whatman Ltd, Maidstone, U.K.) ion-exchange columns (1·3 × 5 cm) equilibrated in 20 mM HEPES (pH 7·5), 2 mM EDTA, 2 mM EGTA, 1 mM aprotinin, 0·1 mg/ml of leupeptin, 10 mM 2-mercaptoethanol and 1 mM PMSF. PKC was eluted using a gradient of 0–0·25 mM NaCl.

Twenty-microlitre aliquots of each fraction eluted (cytosol and membrane) were assayed for PKC activity as follows. Cytosol and membrane extracts were incubated in 20 mM HEPES (pH 7·4), 20 mM $MgCl_2$, 2 mM $CaCl_2$, 0·02 mM adenosine triphosphate (ATP), 8 μCi/ml of [γ-^{32}P]ATP (New England Nuclear, Boston, MA), 2 mg/ml of histone type III-S (Sigma), and with or without 300 μg/ml of phosphatidylserine (Sigma) and 3·3 μg/ml of diacylglycerol (Sigma). Reactions were carried out at 30° for 3 min and then finished by the addition of 1 ml of 25% trichloroacetic acid. The precipitate was collected by filtration on Whatman glass fibre filters (GF/C). The filters were washed four times with 5 ml of phosphoric acid (75 mM), air-dried, placed in scintillation vials containing 10 ml of Betafluor (Packard Instrument Co., Lombard, IL), and counted for radioactivity in a LKB β-counter. PKC activity was calculated as the difference in the amount of radiolabel incorporated into histone in the presence of phosphatidylserine and diacylglycerol and the amount incorporated in the absence of phosphatidylserine and diacylglycerol. These values were transformed into pmol phosphate from ATP incorporated into histone per 10^6 cells per min.

Statistical analysis

All values are expressed as the mean ± SD of the number of experiments, performed in duplicate, as indicated in the corresponding tables and figures. The data were evaluated statistically by Student's *t*-test for paired observations of parametric data or by Wilcoxon signed-rank test for paired observations of non-parametric data, $P < 0{\cdot}05$ being the minimum significant level.

RESULTS

Figure 1 shows the adherence index of peritoneal macrophages incubated with NPY or PYY at 5, 10, 20 or 60 min. NPY and PYY stimulated significantly the adherence capacity of macrophages at concentrations between 10^{-8} M and 10^{-12} M, the 10^{-10} M and 10^{-11} M concentrations being the more efficient. The figure shows that PYY has a higher stimulative effect than NPY at 5 and 10 min of incubation. In order to discard the possibility of a non-specific effect via covering the surface of the tubes with positive charges, a group of control tubes was pretreated with NPY or PYY (10^{-9} M), obtaining no significant differences respect to controls: 45 ± 6 (5 min), 52 ± 7 (10 min), 60 ± 8 (20 min) and 73 ± 7 (60 min). To assess the results obtained with the two peptides a positive control with calcium ionophore A23187 (10^{-7} M) was included in the adherence assay, obtaining moderately greater adherence indexes (see legend in Fig. 1) than those of NPY and PYY. In addition, other peptides purchased from Sigma, such as choleystokinin 8-sulphated (CCK8s), were used as control peptides for the effects obtained with NPY and PYY. Thus, in an opposite way to the two peptides studied, CCK8s at 10^{-11} M did not show any stimulation of the adherence capacity of peritoneal macrophages (see results in legend to Fig. 1).

Once the stimulative effect of the two peptides on the adherence capacity was found, we studied their effect on the chemotaxis capacity. Figure 2 shows that NPY and PYY significantly stimulated the chemotaxis capacity of macrophages in a similar way. The more efficient concentrations were from 10^{-12} M to 10^{-10} M. In the same way as the adherence assay, peritoneal macrophages were incubated with calcium ionophore A23187 (10^{-7} M) as positive control, producing a significantly higher chemotaxis index (1368 ± 218) than those of NPY and PYY. As well as in the adherence capacity, CCK8s (10^{-11} M) did not affect the chemotaxis capacity of macrophages, showing a chemotaxis index of 437 ± 49.

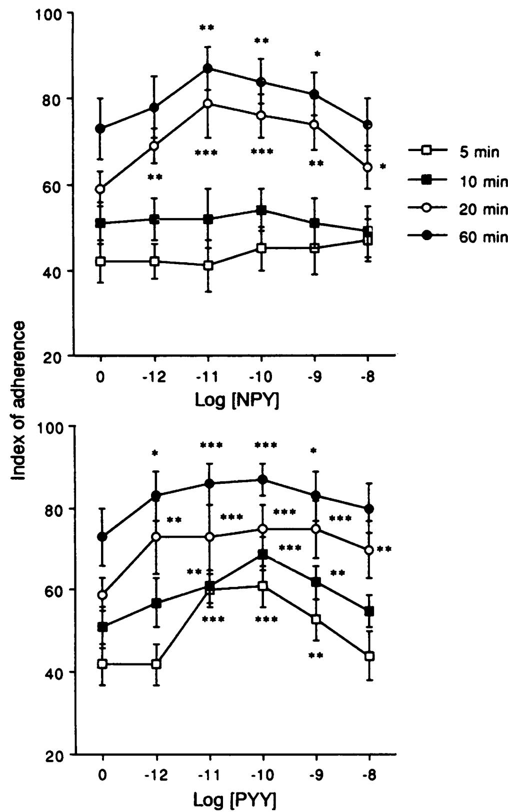

Figure 1. Effect of different concentrations of NPY and PYY on the adherence index of murine peritoneal macrophages. The results represent the mean ± SD of eight experiments performed in duplicate. $*P<0{\cdot}05$, $**P<0{\cdot}01$ and $***P<0{\cdot}001$ with respect to control values. A positive control with calcium ionophore A23187 (10^{-7} M) was made, with the following results: 64 ± 5 at 5 min, 75 ± 4 at 10 min, 87 ± 5 at 20 min and 89 ± 6 at 60 min. When 10^{-11} M CCK8s was added as a control peptide the following adherence results were obtained: 47 ± 8 at 5 min, 50 ± 7 at 10 min, 58 ± 6 at 20 min and 69 ± 9 at 60 min.

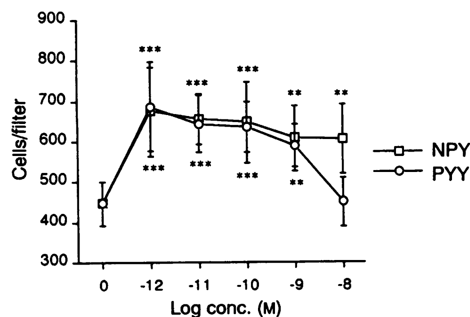

Figure 2. Influence of NPY and PYY on chemotaxis index of murine peritoneal macrophages. The results represent the mean ± SD of eight experiments performed in duplicate. $**P<0{\cdot}01$ and $***P<0{\cdot}001$ with respect to control values. A positive control, calcium ionophore A23187 (10^{-7} M), was included obtaining a chemotaxis index of 1368 ± 218. In addition, CCK8s (10^{-11} M), as control peptide, induced in peritoneal macrophages a chemotaxis index of 437 ± 47.

The results obtained in the phagocytosis capacity of *C. albicans* and latex beads are indicated in Figs 3 and 4. The two peptides increased significantly the phagocytosis function of

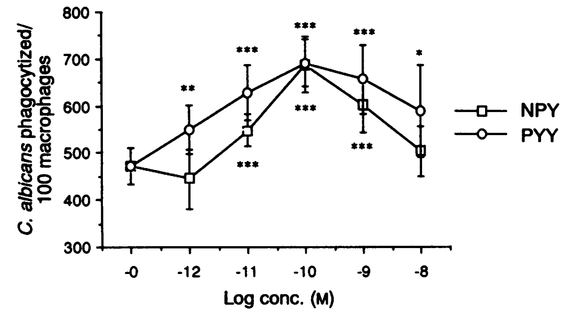

Figure 3. Effect of NPY and PYY on the number of *C. albicans* phagocytized per 100 peritoneal macrophages. Results represent the mean ± SD of eight experiments performed in duplicate. $*P<0{\cdot}05$, $**P<0{\cdot}01$ and $***P<0{\cdot}001$ with respect to control values. A phagocytosis index of 815 ± 83 was obtained with calcium ionophore A23187 (10^{-7} M) as a positive control, and 465 ± 48 with CCK8s (10^{-11}M) as control peptide.

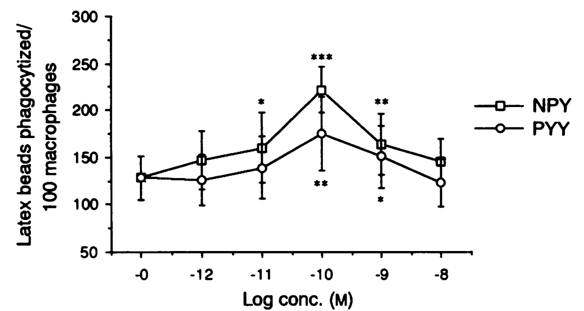

Figure 4. Influence of NPY and PYY on the number of latex beads phagocytized per 100 peritoneal macrophages. The results represent the mean ± SD of eight experiments performed in duplicate. $*P<0{\cdot}05$, $**P<0{\cdot}01$ and $***P<0{\cdot}001$ with respect to control values. Calcium ionophore A23187 (10^{-7} M) was included as a positive control, obtaining a phagocytosis index of 268 ± 28. In the case of CCK8s (10^{-11} M) as control peptide a phagocytosis index of 115 ± 28 was found.

murine peritoneal macrophages, presenting the highest effect at 10^{-10} M. NPY showed a higher capacity for latex bead phagocytosis, whereas in *C. albicans* phagocytosis PYY produced a higher effect. The calcium ionophore A23187 (10^{-7} M) was also included as positive control, showing scarcely higher values (see the legends to Figs 3 and 4) than those of NPY and PYY. The control peptide CCK8s (10^{-11} M) produced no stimulation of *C. albicans* and latex bead phagocytosis (see the results in the legends to Figs 3 and 4).

Table 1 shows the NBT reduction in macrophages non-stimulated (in absence of ingestion material) and stimulated (in presence of latex beads as ingestion material), and incubated in the presence of NPY and PYY. The two peptides at 10^{-10} M concentration produced a slight but significant increase in the amount of NBT reduced in the stimulated samples compared to the values obtained in controls. No differences were found for non-stimulated samples with respect to controls. When macrophages were incubated with PMA (50 ng/ml) as a positive control, the NBT reduction was significantly higher than those of NPY and PYY in both non-stimulated and stimulated samples.

Table 1. NBT reduction (μmol/10^8 cells) by peritoneal macrophages in the absence (non-stimulated samples) or presence (stimulated samples) of latex beads and with different concentrations of NPY or PYY

	0	10^{-12} M	10^{-11} M	10^{-10} M	10^{-9} M	10^{-8} M
NPY						
Non-stimulated	9·1±2·5	9·0±2·0	8·5±1·0	8·6±1·5	8·6±1·0	8·6±1·0
Stimulated	17·5±3·0	18·6±1·5	18·6±3·0	19·2±1·5**	19·7±3·0**	18·5±3·0
PYY						
Non-stimulated	9·1±2·5	9·0±2·5	8·5±1·5	8·7±1·5	8·5±1·5	8·6±1·5
Stimulated	17·5±3·0	18·0±3·5	18·0±1·5	19·1±1·5**	18·3±3·5	17·3±1·5

Each value is the mean ± SD of eight experiments performed in duplicate. ** $P<0·01$ with respect to control values. A positive control was made with PMA (50 ng/ml), obtaining a NBT reduction of 11·5±1·8 μmol/10^8 cells for non-stimulated and 21·8±2·1 for stimulated samples.

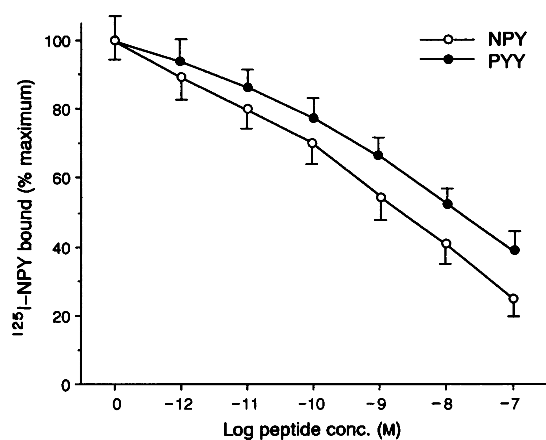

Figure 5. Competitive inhibition of ^{125}I-NPY binding to murine peritoneal macrophages by unlabelled NPY and PYY. Each point represents the mean ± SD of five experiments performed in triplicate.

In order to corroborate that the effects shown above by the peptides were specific, the existence of specific receptors for NPY were studied in peritoneal macrophages. Cells were incubated with NPY concentrations ranging from 10^{-12} M to 10^{-7} M in the presence of a fixed concentration (45 pM) of ^{125}I-NPY. A competitive binding to mice macrophages was found with a half-maximal inhibition (IC50) of 3·7±0·5 nM native NPY. The Scatchard study suggested the existence of two different types of NPY binding sites, one with high affinity (K_d = 2·7±0·4 nM) and low binding capacity (38·0±3·5 fmol/10^6 cells), and another type with low affinity (K_d = 64·2±7·1 nM) and high binding capacity (420·5±65·0 fmol/10^6 cells). The specificity of NPY receptors in peritoneal macrophages was investigated by determining the specific binding of ^{125}I-NPY in the presence of PYY (Fig. 5). PYY was able to inhibit tracer binding but with about an eight-fold lower potency than NPY.

To test the possibility of NPY and PYY actuation via cAMP, we measured the variations of intracellular cAMP in controls and samples with different concentrations of peptides incubated at times of 30, 60 and 120 seconds. No modification in cAMP levels in these cells was found at any time, or neuropeptide concentration, in comparison with control samples (15±4 pmol/10^8 cells).

Several agonists act on cells via the inositol phospholipid pathway. In addition to inositol trisphosphate production, inositol phospholipid hydrolysis also generates 1,2-diacylglycerol, which in turn activates PKC after translocation of the enzyme from the cytosol to the membranes. As shown in Table 2, stimulation with NPY or PYY induced a significant increase in the membrane fraction activity and a loss of PKC activity in the cytosolic fraction, the 10^{-10} M concentration producing the most effective stimulation (200%). Because cytosol and membrane extracts were subjected to DEAE–anion exchange chromatography before the eluted fractions were assayed for PKC activity, the stimulatory effect of the two peptides was due to alterations in the intrinsic activity of PKC rather than to changes in the activity of inhibitory or activating factors presenting in cell fractions. Macrophages incubated with PMA (50 ng/ml), a specific activator of PKC, showed a higher degree of membrane PKC stimulation (300%) than NPY or PYY.

Table 2. PKC activity (pmol/min/10^6 cells) in membrane and cytosol from peritoneal macrophages incubated with NPY or PYY

		PKC	
Substance	Con.	Membrane	Cytosol
None (control)		2·7±0·7	4·9±0·8
PMA	50 ng/ml	8·2±2·4***	1·8±0·5***
NPY	10^{-9} M	3·7±0·9**	3·3±0·7***
	10^{-10} M	6·0±1·0***	2·5±0·5***
	10^{-11} M	4·2±0·9***	4·3±0·4*
PYY	10^{-9} M	3·4±0·5**	4·0±0·8*
	10^{-10} M	5·7±1·0***	3·5±0·3***
	10^{-11} M	4·4±1·1***	3·7±0·3***

The results are the mean±SD of five experiments performed in duplicate. * $P<0·05$, ** $P<0·01$, *** $P<0·001$ compared to their respective controls.

DISCUSSION

To our knowledge, the present study is the first carried out in relation to NPY and PYY effects on macrophage function. Peritoneal macrophages were used because these immune cells are easily available and representative of other populations of macrophages, according to Unanue.[24] The results show that

these peptides stimulate, in mouse macrophages, the different functions studied: adherence, chemotaxis, ingestion of cells and inert particles, and production of superoxide anion, through a mechanism that seems to implicate functional receptors and activation of PKC. This stimulation was in some cases, such as adherence and phagocytosis, of a similar grade to that obtained with calcium ionophore A23187, used as a positive control. In general, 10^{-11} M, 10^{-10} M and 10^{-9} M were the more stimulant concentrations of NPY and PYY. The fall in the response at the highest concentration of 10^{-8} M could indicate a process of cell desensitization that is characteristic of receptor 'down-regulation'.[25]

Adherence, the first step in the immune response of phagocytic cells,[26] has been the last phenomenon studied and is the least known. The stimulating effect produced *in vitro* by NPY and PYY on adherence could represent an activation stage of macrophages. When comparing the effect of the two peptides we found that in adherence capacity at the earliest times of incubation (5 and 10 min), PYY had a higher stimulation capacity than NPY. This difference could be attributed to the distinct amino acid sequence of the two neuropeptides studied, which is implicated in the receptor affinity.[14] A similar fact has been found for related neuropeptide families, such as gastrin-releasing peptide with respect to bombesin,[21] and neurotensin with respect to neuromedin N.[27]

The chemotaxis capacity of peritoneal macrophages was also stimulated *in vitro* by the presence of NPY and PYY. The chemotaxis stimulation could signify *in vivo* an increased accumulation of phagocytic cells in the inflammatory area, with resulting improvement of the phagocytic function. An increase of macrophage chemotaxis capacity has also been observed with some neuropeptides such as bombesin-related peptides[21] and neurotensin[26] at similar concentrations to those used here. The neuropeptide substance P has been shown to inhibit[28] or stimulate[29] neutrophil chemotaxis induced by formylpeptide.

When comparing the effect of NPY and PYY on phagocytosis capacity we found that PYY was more active than NPY in ingestion of *C. albicans*, whereas NPY was so with respect to latex bead ingestion. This difference could be attributed again, as with the adherence results, to the distinct amino acid sequence of the two peptides. Moreover, the kind of receptors that mediated the ingestion of inert particles or opsonized cells is very distinct and, therefore, NPY and PYY could be implicated in a different way with each type of receptor.

The NBT reduction is a measure of O_2^- synthesis produced in the activation of the oxidative burst,[30] the formation of superoxide anion being the initial step in the killing of phagocytized organisms. The peritoneal macrophages incubated with NPY or PYY showed only a slightly increased, but significant, NBT reduction at 10^{-10} M when macrophages were stimulated with latex beads, and there was no increase in their absence. Several neuropeptides, such as met- and leu-enkephalin[31] and substance P[32] in human neutrophils and bombesin-related peptides,[21] neurotensin and neuromedin N[27] in murine macrophages, also activate the superoxide anion production.

As suggested by Scatchard analysis of the binding data for NPY, two different types of receptors may be present in peritoneal macrophages: a type with high affinity ($K_d = 2{\cdot}7 \pm 0{\cdot}4$ nM) and low binding capacity ($38{\cdot}0 \pm 3{\cdot}5$ fmol/10^6 cells), and another type with low affinity ($K_d = 64{\cdot}2 \pm 7{\cdot}1$ nM) and a high binding capacity ($420{\cdot}5 \pm 65{\cdot}0$ fmol/10^6 cells). When PYY was investigated with respect to the specificity of these receptors, the results showed that PYY was able to inhibit tracer binding but with about an eight-fold lower potency than NPY. These data are similar to those obtained in other cells such as chief cells from guinea-pig stomach,[33] and they corroborate that the peptide effects shown above are produced through specific receptors for NPY and PYY in murine peritoneal macrophages.

Although several authors have shown that other neuropeptides, such as substance P, vasoactive intestinal peptide (VIP) or somatostatin, act in immune cells through the cAMP pathway,[34] the measure of intracellular cAMP showed that in mouse macrophages there was no significant modification of cAMP levels by NPY or PYY.

PKC is a phospholipid/Ca^{2+}-dependent enzyme that regulates various cellular responses[35] such as the control of proliferation and differentiation in different immune cells. Since only the membrane-bound enzyme can express biological activity,[36] it is essential to know the correlation between the biological effects elicited by PKC and the subcellular distribution of the enzyme. Since NPY mobilizes intracellular Ca^{2+} and increases inositol phosphate production in human erythroleukaemia cells,[19] we studied the PKC activity in murine macrophages and we have found in this study that NPY and PYY induce a significant increase in the membrane fraction activity of PKC and a loss of activity in the cytosolic fraction, which seems to indicate an activation of the enzyme by translocation from cytosol to membranes.

There is no direct evidence of a correlation between activation of PKC and stimulation of phagocytic cell function, although it is known that phorbol esters, which specifically activate PKC by mimicking the requirements for diacylglycerol, are potent activators of the oxidative burst,[37] and chemotactic peptides increase PKC activity through an increase of diacylglycerol.[38] However, this study shows, for NPY and PYY, the first evidence of correlation between PKC activation and stimulation of several representative functions of peritoneal macrophages.

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