Altered anti-inflammatory response of mononuclear cells to neuropeptide PACAP is associated with deregulation of NF-κB in chronic pancreatitis

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Michalski CW, Selvaggi F, Bartel M, Mitkus T, Gorbachevski A, Giese T, Di Sebastiani P, Giese NA, Friess H. Altered anti-inflammatory response of mononuclear cells to neuropeptide PACAP is associated with deregulation of NF-κB in chronic pancreatitis. Am J Physiol Gastrointest Liver Physiol 294: G50–G57, 2008. —Although it is recognized that neurogenic influences contribute to progression of chronic inflammatory diseases, the molecular basis of neuroimmune interactions in the pathogenesis of chronic pancreatitis (CP) is not well defined. Here we report that responsiveness of peripheral blood mononuclear cells (PBMC) to the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) is altered in CP. Expression of PACAP and its receptors in human CP was analyzed with quantitative RT-PCR, laser-capture microdissection, and immunohistochemistry. Regulation of PACAP expression was studied in coculture systems using macrophages and acinar cells. Responsiveness of donor and CP PBMC to PACAP was determined based on cytokine profiles and NF-κB activation of LPS- or LPS+PACAP-exposed cells. Although donor and CP PBMC responded equally to LPS, PACAP-mediated counteraction of LPS-induced cytokine response was switched from inhibiting TNF-α to decreasing IL-1β and increasing IL-10 secretion. The change of PACAP-mediated anti-inflammatory pattern was associated with altered activation of NF-κB: compared with LPS alone, a combination of LPS and PACAP had no effect on NF-κB p65 nuclear translocation in CP PBMC, whereas NF-κB was significantly decreased in donor PBMC. According to laser-capture microdissection and coculture experiments, PBMC also contributed to generation of a PACAP-rich intrapancreatic environment by upregulating PACAP expression in macrophages encountering apoptotic pancreatic acini. The nociceptive status of CP patients correlated with pancreatic PACAP levels and with IL-10 bias of PACAP-exposed CP PBMC. Thus the ability of PBMC to produce and to respond to PACAP might influence neuroimmune interactions that regulate pain and inflammation in CP.

Cytokines; inflammation; pituitary adenylate cyclase-activating polypeptide; peripheral blood mononuclear cells

Chronic pancreatitis (CP) is a progressive disease resulting in the destruction of the pancreas and is often associated with severe pain (38). Keith et al. (17) were the first to characterize perineural alterations in CP, with further studies showing a correlation between perineural accumulation of inflammatory cells and pain intensity, thus indicating a role of neurogenic inflammation in CP pathogenesis (5). Pathobiologically, the stimulation of sensory neurons by bradykinin or tryptase (secreted by degrading acinar cells) may lead to the secretion of bioactive substances such as calcitonin gene-related peptide (CGRP) or substance P, supporting an inflammatory reaction characterized by local tissue edema and vasodilatation (5).

Imbalanced production of cytokines such as proinflammatory IL-1β, IL-6, and TNF-α or anti-inflammatory IL-10 by cells of the immune system may also contribute to the generation of inflammatory pain and neuroimmune interactions involved in the pathogenesis of chronic inflammatory diseases (25). Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic neuropeptide belonging to the vasoactive intestinal peptide (VIP)/secretin/glucagon family of peptides and occurring in precursor and two processed forms, PACAP-27 and PACAP-38, with the latter form being predominant (2). PACAP is produced by innervation and activated lymphoid cells, and it exerts a broad spectrum of biological activities, in particular in immune and nerve systems. Several animal pain models have suggested that PACAP plays a role in nociception. Although its exact involvement remains controversial, recent experiments with mice deficient in PACAP and its receptor PAC1R clearly identified PACAP as a nociceptive factor (16, 24). PACAP is also believed to function as an immunosuppressor, mostly because of its ability to inhibit production of proinflammatory cytokines (such as IL-1β, IL-6, IL-12, and TNF-α) and to increase secretion of anti-inflammatory effectors (such as IL-10) (2). PACAP’s effects are mediated through activation of a family of three receptors: VPAC1 and VPAC2 receptors, which are also activated by VIP, and PAC1R, which binds PACAP with at least 300-fold higher affinity than VIP (37). The receptors may activate different signaling pathways and affect different targets. For example, while VPAC1 and VPAC2 receptors predominantly activate the adenylate cyclase pathway, PAC1R also activates phospholipase C (2, 6). PAC1R is necessary for the attenuation of LPS-induced IL-6 secretion, but not for TNF-α (27). Requirements for transcription factors such as NF-κB, IFN regulatory factor-1 (IRF-1), and cAMP response (CRE)-binding protein (CREB) also differ among transduction pathways, indicating the intricacy of the mechanisms of the anti-inflammatory activity of PACAP (21). Adding to the complexity, human immune cells appear to differ from murine cells with regard to their PAC1R expression patterns and immunoregulatory activity. Therefore, the most prominent proof of PACAP’s anti-
inflammatory role, obtained in a septic shock model using PAC1R-deficient mice, may not hold true for human inflammatory diseases (27). Moreover, whereas the immunosuppressive potential of PACAP in mice was preferentially associated with interference in generation or execution of proinflammatory cytokine responses, other effector functions of rat or human inflammatory cells were instead stimulated by PACAP (3, 6, 13). In a mouse model of acute pancreatitis, VIP attenuated the severity of inflammation by modulation of cytokine production (18). The ability of PACAP to modulate cytokine secretion in normal or diseased primary human inflammatory cells has not yet been reported, prompting us to explore whether changes either in PACAP responsiveness or in PACAP expression may alter neuroimmune interactions, contributing to the protraction of inflammation and pain generation in CP.

MATERIALS AND METHODS

Patients and specimen collection. Pancreatic (n = 14) and lymph node (n = 7) tissue samples were collected either from individuals undergoing surgical resection of the pancreas due to CP or from normal pancreata (n = 9) resected within the organ donor program of the Departments of Surgery of the Inselspital (Bern, Switzerland) and the University of Heidelberg. Blood samples were taken from healthy donors (n = 14) or from CP patients (n = 18). The use of human tissues and blood probes for analysis was approved by the ethical committees of both universities, and written informed consent was obtained from the patients before the sampling/operation.

In all cases, the diagnosis was established by conventional clinical and histopathological criteria. Freshly removed tissue samples were immediately fixed in 4% paraformaldehyde solution for 12-24 h and paraffin embedded for routine histopathological analysis and immunohistochemistry. Tissue samples destined for laser-capture microdissection (LCM) or protein or RNA extraction were immediately snap-frozen in liquid nitrogen or placed in RNAlater (Ambion) and maintained at −80°C until use.

Cell isolation and cultures. Peripheral blood was taken from CP patients and healthy donors and used to isolate 1) leukocytes by ammonium chloride-potassium carbonate buffer lysis of whole blood; 2) peripheral blood mononuclear cells (PBMC) and granulocytes by the density centrifugation method using Histopaque-1077 solution (Sigma) according to the manufacturer’s protocol; 3) monocytes by 1-h adherence of PBMC to plastic; and 4) mature macrophages by 6-day in vitro culturing of monocytes (1, 7, 26). Cells were routinely cultured at 5% CO2 and 37°C in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS (PAN Biotech), nonessential amino acids, 2-mercaptoethanol, and penicillin-streptomycin. Single-cell suspensions of acinar and lymphoid cells were prepared by collagenase digestion of pancreatic tissues (20) or mechanical disintegration of lymph nodes, respectively.

Coculture experiments. For induction experiments, 2 × 10^5 PBMC were exposed to LPS, PACAP-38 or LPS plus PACAP-38 at final concentrations of 1 ng/ml for LPS (Sigma, Deisenhofen, Germany) and 10^-7 M for PACAP-38 (Calbiochem, Schwalbach, Germany). After an incubation period of 10 min (phosphorylation assays), 2 h (transcription factor assays) or 12 h (cytokine levels), samples were centrifuged for 5 min at 3,000 rpm. The supernatants were collected and stored at −80°C until further analysis by ELISA, while cell pellets were lysed in 300 μl of MagNA Pure lysis buffer (Roche Applied Sciences, Mannheim, Germany) until further analysis by real-time quantitative RT-PCR (QRT-PCR). For analysis of NF-κB or AP-1 family activation, stimulation experiments were terminated after 2 h by lysing cells with a Nuclear Extract Kit (Active Motif Europe, Rixensart, Belgium). For analysis of p38 and phospho-p38, cells were lysed as described previously (31).

For coculture experiments [conducted as described elsewhere (Refs. 1, 7; M. Bartel and N. A. Giese, unpublished observations)], in vitro-matured macrophages (5 × 10^4–10^5 per well) were cocultured with apoptotic pancreatic acinar cells or granulocytes in serum-free medium at a ratio of 1:2 for 90 min. To induce cell death, we used PKH26 labeling (Sigma), which was supplemented by ultraviolet exposure and overnight incubation in the case of granulocytes. Viability and apoptosis of cells were controlled by microscopic evaluation of Trypan blue-stained cells and by FACS evaluation of annexin V-FITC-stained cells. The Trypan blue inclusion index was below 10% and annexin V positivity was at 50–80%. After intensive washing, macrophage monolayers were collected into MagNA Pure lysis buffer and subjected to QRT-PCR analysis.

Conventional and real-time quantitative RT-PCR. All reagents and equipment for mRNA, cDNA preparation, and QRT-PCR were obtained from Roche. mRNA was prepared by automated isolation using a MagNA Pure LC instrument and isolation kits (for cells) and II (for tissue); cDNA was prepared with a first-strand cDNA synthesis kit; and QRT-PCR was performed with the Light Cycler Fast Start DNA SYBR Green kit, all according to the manufacturer’s instructions as described previously (12). The primers detecting housekeeping gene cyclophilin B (CPB) as well as IL-1β, IL-10, TNF-α, transforming growth factor-β, monocyte chemotactic protein (MCP)-1, VPAC1, and PAC1R were obtained from Search-LC (Heidelberg, Germany). The amount of detected specific transcripts was normalized to the level of a housekeeping gene and expressed as the number of target transcripts per 10,000 CPB copies. To validate expression data of PACAP receptors, we also performed conventional PCR using previously published primer pairs (15) and the THP-1 human monocytic cell line as positive control and visualized amplicons by electrophoretic separation using ethidium bromide-stained agarose gels.

ELISA. IL-1β, IL-6, IL-10, and TNF-α in supernatants were measured by ELISAs using BD OptEIA sets (BD Biosciences, Heidelberg, Germany), and concentrations were calculated with Ascent Software (Thermolabsystems, Waltham, MA). PACAP serum levels were measured by direct ELISA using goat-polyclonal PACAP antibody (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA) and peroxidase-labeled donkey anti-goat IgG (1:2,000, Santa Cruz Biotechnology).

Western blot analysis. After 10-min incubation with LPS, PACAP, or a combination of LPS and PACAP, PBMC were lysed and immunoblots were performed as described previously (31). Rabbit anti-p38 and rabbit anti-phospho-p38 were diluted 1:1,000, respectively [antibody concentration as provided by the manufacturer: 8 μg/ml (p38) and 92 μg/ml (phospho-p38)].

Determination of NF-κB and AP-1 family member activation. Twenty micrograms of the nuclear proteins extracted with the Nuclear Extract Kit from PBMC according to the manufacturer’s instructions was assayed for NF-κB p65 and AP-1 family members JunB, c-fos, Fra-1, and FosB activity with TransAM transcription factor activation kits (Active Motif).

Immunohistochemistry. Paraffin-embedded tissue sections (3-μm thickness) were subjected to immunostaining with the streptavidin-peroxidase technique. Sections were incubated with monoclonal mouse anti-human VPAC1 antibody or polyclonal goat anti-human PACAP antibody [Abcam (1:500) and SCBT (1:100), respectively] at 4°C overnight. After incubation with a streptavidin-phosphatase-coupled secondary goat anti-mouse antibody or a rabbit anti-goat antibody for 30 min at room temperature, the color reaction was performed by incubation with the DAKO + substrate. To ensure specificity of the primary antibodies, consecutive tissue sections were incubated with a mouse or goat IgG.

Laser-capture microdissection of pancreatic specimens. To confirm PACAP expression in inflammatory infiltrates, CP tissue samples
were analyzed by QRT-PCR of LCM-dissected foci isolated from RNAlater-preserved tissues (CP pancreas, n = 9; normal lymph node, n = 1; CP lymph nodes, n = 4), as published previously (19). The collected cells were resuspended in MagNA Pure lysis buffer and stored at −80°C until further processing.

Pain scoring. CP patients were grouped according to their pain scores based on preoperative records reflecting duration, intensity, and frequency of pain, with each parameter being assigned 0–3 points (30). Addition of the single values resulted in a scale of 0–9 points. Patients scoring a sum of 0–4 points were merged into the low pain group, whereas patients scoring 5–9 points were assigned to the strong pain group.

Statistical analysis. Results are presented as means ± SE unless indicated otherwise. Comparisons were performed with Mann-Whitney U-tests, Wilcoxon signed-rank tests, or Kruskal-Wallis tests followed by Dunn’s multiple-comparison test, using the GraphPad Prism4 and SPSS 14.0 software for Windows. Correlation analyses were performed with a Spearman r-test. The minimal level of significance was defined as P < 0.05.

RESULTS

Differential activity of PACAP-38 toward normal and CP blood mononuclear cells. To date, the influence on cytokine production by PACAP-38 has been intensively studied in murine but not human immune cells. To determine the effects of exposure to PACAP-38, human PBMC were exposed to 10−7 M PACAP-38 for 12 h and supernatants were analyzed for cytokines (donor, n = 11; CP, n = 16). Constitutive and PACAP-38-stimulated release of IL-1β, TNF-α, and IL-10 by either donor or CP PBMC was undetectable for all of the cytokines. Donor and CP PBMC demonstrated similar responsiveness to proinflammatory signal LPS (1 ng/ml), which induced equally high levels of cytokines in culture supernatants (Fig. 1A). In contrast, PACAP’s ability to alter inflammatory response differed between donor and CP cultures. Although in both cases the response could be classified as anti-inflammatory, PACAP-38 upregulated IL-10 (P = 0.0353) and downregulated IL-1β (P = 0.0181) secretion by CP PBMC, whereas it downregulated TNF-α secretion by donor PBMC (P = 0.0186). However, IL-6 expression levels were comparable in donor and CP PBMC on PACAP treatment (data not shown).

To check whether all mononuclear cells in CP had this type of PACAP responsiveness, we analyzed cytokine production by lymph node-derived mononuclear cells (LNMC). As for PBMC, constitutive and PACAP-38-induced IL-10, IL-1β, and TNF-α levels were undetectable, LPS-induced accumulation was observed for all cytokines (Fig. 1C), and PACAP-38 reduced IL-1β without affecting TNF-α secretion (P = 0.040; Fig. 1D). In contrast to PBMC, PACAP-38 did not alter IL-10 production in LNMC. Thus PACAP-38 was able to downregulate proinflammatory IL-1β in CP mononuclear cells of both blood and lymph node origin, but only in PBMC did PACAP-38 upregulate anti-inflammatory IL-10.

Factors influencing PACAP responsiveness in normal and CP PBMC. The effects of PACAP on the immune system were shown to depend on the particular type of mediating receptor (PAC1, VPAC1, or VPAC2) and the activation status of the immune cells (4, 28).

In terms of receptor dependence, PBMC of both donors and CP patients bear only VPAC1 but not VPAC2 or PAC1 receptors (Fig. 2A). This finding contrasts with the pattern found in murine and human THP-1 cells constitutively expressing PAC1R but is in agreement with studies of primary human immune cells by El Zein et al. (6). Although basal levels of VPAC1 expression were lower in CP compared with donor PBMC, the difference was not statistically significant (P = 0.077; Fig. 2B). Nevertheless, this tendency may explain why the level of VPAC1 in LPS-exposed donor PBMC was signifi-
icantly decreased \((P = 0.0078)\). PACAP treatment did not alter LPS-mediated VPAC1 expression in either case but moderately upregulated constitutive expression in CP PBMC \((P = 0.0391)\). In pancreatic tissues, VPAC1 expression was significantly higher in CP samples, mostly because of expression on hypertrophic nerves and mononuclear cells (Fig. 2, C–F).

In terms of activation status dependence, we hypothesized that different mononuclear cell priming in the bloodstream of donors and CP patients might be reflected by differences in the levels of constitutive cytokine expression or transcription factor activation. Since constitutive and PACAP-stimulated cytokine release by PBMC was undetectable by ELISA, we used QRT-PCR to measure cytokine mRNA expression. Surprisingly, both IL-10 and TNF-\(\alpha\) detected in CP PBMC tended toward lower levels of expression compared with those in donor PBMC (Fig. 3A), but the difference reached statistical significance only for IL-10 \((P = 0.05)\). PACAP alone did not cause statistically significant changes in the levels of cytokine expression (data not shown).

As for transcription factor activation, constitutive levels of NF-\(\kappa B\) p65—a major target of PACAP signaling—were relatively similar among donor and CP PBMC (Fig. 3B). Furthermore, PACAP increased constitutive levels of nuclear NF-\(\kappa B\) in CP PBMC but not in donor PBMC (Fig. 3B). Comparable with murine and human THP-1 cells, PACAP inhibited LPS-induced NF-\(\kappa B\) translocation to the nucleus in donor PBMC by 32\% (Fig. 3B; \(P = 0.015\)). In contrast, LPS-induced translocation of NF-\(\kappa B\) p65 in CP PBMC was unchanged after treatment with a combination of LPS and PACAP. Furthermore, we analyzed transactivation of AP-1 family members JunB, c-fos, Fra-1, and FosB after LPS, PACAP, and LPS+PACAP treatment. However, there were no differences between these groups or between donor and CP PBMC (data not shown).

In a next step, we performed pairwise comparisons of the NF-\(\kappa B\) changes and cytokine levels (donor: \(n = 3\); CP: \(n = 2\); Fig. 3, C and D). These revealed that a low LPS-to-LPS+PACAP ratio for NF-\(\kappa B\) was associated with higher cytokine ratios, while in CP PBMC a higher LPS-to-LPS+PACAP ratio for NF-\(\kappa B\) was associated with lower cytokine ratios. Comparing the cytokine ratios of donor and CP PBMC, we found particular differences in MCP-1 (higher in donor than in CP) and TNF-\(\alpha\) (lower in donor than in CP) levels.

To elucidate whether MAP kinase signaling is involved in the observed PACAP-mediated effects, p38 phosphorylation was analyzed (Fig. 3E). Both in donor and CP PBMC, LPS induced phospho-p38, whereas PACAP (without LPS) had no effect on p38 phosphorylation. A combination of LPS and PACAP also showed no different results compared with LPS alone (Fig. 3E).

**PACAP is overexpressed in pancreata of CP patients.** To determine where VPAC1-expressing PBMC are most likely to be exposed to PACAP after leaving the bloodstream, we...
analyzed PACAP expression in peripancreatic lymph nodes and pancreatic tissues obtained from donors (n = 9) and CP patients (n = 14). Both pancreata and lymph nodes harbored low amounts of PACAP transcripts, which remained constant in peripancreatic lymph nodes but increased in pancreata of CP patients (Fig. 4A and Table 1). In accordance with previous reports, immunohistochemical analysis of pancreatic tissues located weak PACAP positivity in islets, in nerves, and around blood vessels, but not in other pancreatic structures (Fig. 4, B and C; Ref. 8). In contrast, CP specimens demonstrated a dramatic increase in PACAP immunolabeling, which was most prominent in the perineurium of hypertrophic nerves and in the areas of inflammatory cell infiltration (Fig. 4, D and E). Immunohistochemistry may reflect localization of protein produced elsewhere, with nerves being known for peripheral release of ganglia-synthesized PACAP.

To confirm that mononuclear cells may also represent a source of increased PACAP production in inflamed pancreatic tissue, we examined CP specimens with LCM and QRT-PCR (Table 1; CP pancreas, n = 9; normal lymph node, n = 1; CP lymph nodes, n = 4). Prominent accumulation of PACAP transcripts was found in LCM-dissected intrapancreatic inflammatory infiltrates but not in gradient-purified PBMC (with a cellular composition to similar that of tissue infiltrates). To exclude the possibility of PACAP production by contaminating stromal cells, we measured PACAP expression by QRT-PCR in LCM-dissected pancreatitis stroma and in primary cultures of pancreatic stellate cells (data not shown) and found none of these samples to be positive. Thus the emergence of a PACAP-rich environment in the pancreas with CP is probably created by nerves and by infiltrating mononuclear cells.

Uregulation of PACAP expression in macrophages by pancreatic acini. Since blood leukocytes and gradient-purified PBMC were PACAP negative (QRT-PCR analysis, Table 1), we hypothesized that local factors upregulate PACAP expression in inflammatory cells entering the chronically inflamed pancreas. Tissue residency status alone, simulated by 6-day-long maturation of monocytes into macrophages in vitro, was not associated with PACAP positivity (Table 1), indicating the necessity of an external signal. Indeed, exposure of mature macrophages to isolated apoptotic acinar cells led to the induction of PACAP mRNA expression in macrophages, independent of their belonging to the donor or CP group. Since coculturing of macrophages with dying acini was accompanied by a phagocytotic reaction (M. Bartel et al., unpublished observation), we excluded acinar remnants as a possible source of PACAP mRNA by analyzing the PACAP level in isolated primary human acinar cells (Table 1). Furthermore, acini appeared to represent a specific PACAP-inducing signal, because exposure of macrophages to apoptotic granulocytes induced a phagocytotic reaction but not PACAP expression (Table 1).

The experiments described above showed that after encountering dying acini in the pancreas macrophages are able to generate a PACAP-rich environment around inflammatory infiltrates. Furthermore, intrapancreatic accumulation remained a local event, since serum levels of PACAP were similar among donors and CP patients (data not shown).

Association of PACAP with pain in CP. To determine the clinical relevance of PACAP overexpression and altered responsiveness, we chose pain assessment as a parameter characterizing the outcome of neuroimmune interactions in CP. Pain was quantified in patients as low (pain score 0–4) or high (pain score 5–9), and constitutive, LPS-, and/or PACAP-induced expression (absolute levels and degree of response on PACAP or LPS exposure) were correlated with individual pain levels. This correlation analysis revealed that higher intrapancreatic PACAP mRNA expression and the ability of PACAP to

Fig. 3. Deregulated NF-κB activation but not level of basal cytokine expression is associated with altered PACAP responsiveness in CP. A: constitutive expression of IL-10 (lower in CP PBMC, *P = 0.05), IL-1β, and TNF-α was analyzed by QRT-PCR in normal and CP PBMC cultures without stimulation (experiments presented in Figs. 1 and 2. No. of cytokine transcripts is given per 10,000 CPB transcripts). B: activation of NF-κB was determined as the amount of nuclear NF-κB p65 detected by an ELISA-based TransAM kit in PBMC exposed or not to LPS, PACAP-38, and a combination of both for 2 h. The effect of PACAP in inhibiting LPS-induced NF-κB p65 translocation in donor PBMC was considered significant (*P = 0.015) whereas in CP PBMC, no such effect was observed. C and D: pairwise comparisons of the NF-κB changes and cytokine levels (donor: n = 3, CP: n = 2). Solid line, LPS-to-LPS + PACAP ratio for NF-κB nuclear activity; dashed line, ratio of 1. TGF-β, transforming growth factor-β; MCP-1 monocyte chemotactic protein-1. E: p38 MAP kinase signaling in donor and CP PBMC. PBMC were treated for 10 min with control (C), LPS (L), PACAP (P), or LPS + PACAP (L + P). Cell lysates were subjected to immunoblot analysis using total p38 and phospho-p38 antibodies. While LPS induced phospho-p38, there were no differences in LPS + PACAP-treated PBMC.
upregulated steady levels of IL-10 mRNA in PBMC were correlated with pain [Spearman $\rho$ coefficient (SRC) $= 0.612$ for higher expression ($P = 0.034$) and SRC $= 0.595$ for the ability to upregulate ($P = 0.025$)]. Furthermore, patients with high pain scores had significantly higher pancreatic PACAP levels than those with low pain scores (Fig. 5A; $P = 0.048$). In PBMC, the ability to upregulate IL-10 expression was evident only in the high pain group (low pain vs. high pain: $P = 0.036$; Fig. 5B). CP patients also demonstrated increased intrapancreatic expression of IL-10 ($2 \pm 1$ vs. $17 \pm 5$ transcripts; $P = 0.04$), which did not correlate with the PACAP expression (SRC $= 0.437$, $P = 0.119$) or the level of pain (SRC $= 0.442$, $P = 0.150$).

**DISCUSSION**

Chronic inflammatory processes are known to affect peripheral immunocompetent blood cells. PBMC of CP patients have been shown to acquire certain phenotypic and functional changes (10, 11), some of them correlating with the most prominent clinical symptom of CP, pain (32). Although neurogenic inflammation and inflammatory pain have been implicated in the pathogenesis of acute pancreatitis and CP (5, 22, 29), the full spectrum of neuroimmune interactions remains to be better elucidated.

According to murine models, the neuropeptide PACAP appears to exert anti-inflammatory effects, particularly with regard to cytokine responses, although it was also shown to promote other functions in human cells, for example, production of reactive oxygen species in monocytes and granulocytes (6, 9, 13). Analysis of PACAP expression and PACAP responsiveness in CP revealed that this neuropeptide may be involved in CP pathogenesis at multiple levels. Interaction of bloodstream-circulating PACAP with PBMC may provoke a CP-specific anti-inflammatory pattern of cytokine response associated with altered NF-kB signaling. Entering the pancreas through the blood or the lymphatic system, monocytes may create a PACAP-rich environment while contacting dying acini. Overexpressed PACAP may directly affect pain transmission or activity of various inflammatory cells. Then the most obvious intrapancreatic targets of PACAP in CP—peripheral nerves and immune cells—may simultaneously drive pronociceptive and anti-inflammatory responses. Although puzzling at first, this link, when interpreted in the context of studies dealing with mechanisms of chronic inflammation and chronic pain, extends our understanding of neuroimmune interactions governing chronic inflammatory disorders. In particular, with the issue of whether PACAP is nociceptive or anti-inflammatory remaining highly controversial, recent studies employing PACAP- and PAC1R-knockout mice clearly demonstrate the need for PACAP in the generation of chronic, but not acute, pain. Upregulation of PACAP was found in the superficial layer of the spinal cord 7 days after L$\alpha$ spinal nerve transection (24) and in dorsal root ganglion neurons 10 days after axotomy (39) or sciatic nerve injury (16). According to these studies, an inability to upregulate PACAP or to transmit a PAC1R-mediated signal results in reduced nociceptive responses. In addition, intrathecal injection of PACAP induces an early analgesic effect but subsequently lasting algesia (35).

Together with the recent report demonstrating the neuroprotective activity of PACAP, which is capable of preventing ischemic neuronal cell death and limiting neurological deficits (33), these data suggest that PACAP is an injury-induced neuropeptide designed to repair nerve damage. Mechanistically, the simultaneity of neuroprotective and pain-inducing repercussions of PACAP overexpression can possibly be attributed to the ability to activate different downstream pathways. Indeed, PACAP was shown to prevent neuronal cell death via an IL-6-dependent mechanism in an ischemic model but promoted pain generation through functional coupling of neuronal nitric oxide synthase (NOS) to N-methyl-D-aspartic acid (NMDA) receptors in a nerve damage model (24, 33).

Our study points to a novel mechanism associated with involvement of IL-10-biased immune cells. In contrast to exogenous IL-10, the level of endogenous IL-10 appears to determine and to increase nociception (36), indicating that PACAP anti-inflammatory activity toward sensitized immune cells might at the same time create a pain-promoting environment. Thus, in addition to neurons producing and responding to PACAP in an IL-6-dependent, NOS-dependent, or NMDA-dependent way, immune cells emerge as an essential source and an important mediator of IL-10-dependent PACAP activ-
PACAP, pituitary adenylate cyclase-activating polypeptide; CPB, cyclophilin B; CP chronic pancreatitis; PBMC, peripheral blood mononuclear cells; Mφ, macrophages; N/A, not available.

Table 1. PACAP expression in tissues and cells

<table>
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<tr>
<th>Sampling</th>
<th>Cells</th>
<th>Mean No. of PACAP Transcripts per 10,000 CPB Transcripts</th>
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<tr>
<td>Tissue</td>
<td>Pancreas total</td>
<td>27</td>
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<tr>
<td></td>
<td>Lymph node total</td>
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<td></td>
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<tr>
<td></td>
<td>PBMC</td>
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<tr>
<td></td>
<td>Mφ</td>
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<tr>
<td></td>
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<td>0</td>
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PACAP anti-inflammatory activity. Interestingly, in addition to reduced nociception (16), PAC1R-knockout mice showed reduced anti-inflammatory protection against endotoxic shock (27). Although observed species-specific differences in expression of PAC1R (murine immune cells are VPAC1/PAC1R+ and human immune cells are VPAC1/PAC1R−; Refs. 2, 6, 13) restrict interpretation of results, it would be interesting to see studies with PACAP- and PAC1R-knockout mice being extended to investigate involvement of IL-10 and immune cells.

It should be noted that although intrapancreatic IL-10 expression was upregulated in our study, it did not correlate either with PACAP or with pain. We ascribe this lack of correlation to the possible heterogeneity of pancreatic mononuclear cell sources, meaning that mononuclear cells entering pancreatic tissue through the blood or lymphatic system can be primed at different locations: in the bloodstream or in lymph nodes, respectively. In our LPS model, PACAP downregulated IL-1β in CP LNMC and PBMC but upregulated IL-10 in donor PBMC. Dissimilar priming of immune cells at these locations might have resulted in the differential sensitivity to PACAP. Variation in cell composition (a single-cell suspension of PBMC had more monocytes than a suspension of LNMC) could have influenced the outcome in our experimental setting. However, disease or organ specificity of priming seems to be an important factor determining PACAP responsiveness.

The most important observation of our study was that PACAP anti-inflammatory activity in CP PBMC was not accompanied by downregulation of NF-κB activation. Inhibition of NF-κB is a crucial part of PACAP signaling and is associated with changes in the composition of CRE binding complexes (while affecting TNF-α expression), ets complexes (IL-12), or reduction of IRF-1 binding (inducible NOS) (21). Increased CREB binding was shown to be important for PACAP-induced augmentation of LPS-mediated IL-10 expression. However, most recent studies have shown that LPS can activate transcription of the IL-10 promoter not only through transcription factors Sp1 and C/EBPβ and -δ but also through NF-κB and MAP kinase signaling pathways (23). In the presence of the NF-κB inhibitor, LPS-mediated IL-10 expression was completely blocked, in part because of inhibition of expression and binding of C/EBPδ. Thus, although it seems paradoxical at first, upregulation of NF-κB by PACAP may explain the loss of the TNF-α response and acquisition of the IL-10 response in CP PBMC. Moreover, it should be stressed that efficient NF-κB signaling also requires LPS-induced removal of NcoR/HDAC3/TBL corepressor complexes from inflammatory gene promoters (14, 34). Thus, if ubiquitylation-dependent clearance of NcoR and HDAC3 components of this complex from target genes is distorted in CP, it would alter NF-κB signaling. Altogether, although our results indicate that altered PACAP responsiveness of PBMC in CP is associated with deregulated NF-κB signaling, only further comprehensive analysis of mononuclear cell transcriptional activation in CP will allow elucidation of the exact mechanism.

To conclude, development of CP is associated with intrapancreatic accumulation of the neuropeptide PACAP around nerves and inflammatory infiltrates, with altered patterns of PACAP-mediated cytokine responses favoring chronic inflammation and nociception in CP patients.
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