Role for VPAC2 Receptor–Mediated Signals in Pancreas Development

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Mature pancreatic cells develop from progenitors that proliferate and differentiate into endocrine and exocrine cells. This development is thought to be controlled by secreted soluble factors acting on their target cells after binding to membrane receptors. Here, we analyzed the impact on embryonic pancreatic development of ligands that bind to protein G–coupled receptors and increase cAMP accumulation. We found that embryonic pancreatic epithelial cells were sensitive to vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide. These factors generate signals after binding to the VPAC2 receptor, which is expressed by immature pancreatic epithelial cells between embryonic days 12 and 16. Finally, in vitro, VIP exposure increased the survival and proliferation of immature pancreatic cells, leading to an increase in the number of endocrine cells that will develop. *Diabetes* 52:85–92, 2003

The pancreas originates from the dorsal and ventral regions of the foregut endoderm immediately behind the stomach. The endoderm evaginates to form epithelial buds that subsequently enlarge. During this period, exocrine and endocrine cells that will constitute the functional pancreatic unit undergo differentiation.

Inductive signals have been shown to be important for normal pancreatic development. Such signals derive from various sources, such as the primitive streak mesoderm, notochord, blood vessels, cardiogenic mesoderm, septum transversum mesenchyme, mesoderm, and mesenchyme (1–6). Research has been conducted to define the exact nature of the inductive signals involved in pancreatic development, and several secreted factors have emerged as likely candidates. These factors belong to a variety of families. A large number of ligands for receptor tyrosine kinases such as fibroblast growth factor-1, -2, -4, -7, and -10; vascular endothelial growth factor; epidermal growth factor; and hepatocyte growth factor have been shown to be involved at various steps of pancreatic development (2,6–13). Several members of the transforming growth factor (TGF)-β superfamily (e.g., activin, bone morphogenetic proteins, TGF-β) have also been implicated in pancreas development (3,8,14,15).

Various ligands of protein G–coupled receptors that increase cAMP accumulation have been shown to exert an important influence on pancreatic function and regeneration during postnatal life (16–19). In other tissues, these ligands are important for normal development (20,21). However, to our knowledge, few data are available on their role in prenatal pancreas development. In the present study, we used a recently described in vitro system of immature embryonic pancreatic epithelium to look for evidence that ligands of protein G–coupled receptors that increase cAMP accumulation may contribute to the control of pancreas development.

Our data indicate that embryonic pancreatic epithelial cells are sensitive to vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase–activating polypeptide (PACAP). These factors signal via the VPAC2 receptor, which is highly expressed in the pancreatic epithelium between embryonic days 12 and 16. They increase the proliferation and survival of immature pancreatic epithelial cells, leading to a final increase in the number of insulin-expressing cells.

RESEARCH DESIGN AND METHODS

Animals. Pregnant Wistar rats were purchased from the Janvier breeding center (Le Genet, France). The morning of the discovery of the vaginal plug was designated embryonic day 0.5 (E0.5). The animals had free access to food pellets and water. Pregnant rats at different stages of gestation were killed by CO_2 asphyxiation.

Dissection of pancreatic epithelium. E13.5 embryos were harvested, and their dorsal pancreatic buds were dissected. The pancreatic epithelium was separated from its surrounding mesenchyme as described previously (22), with minor modifications. Briefly, the stomach, pancreas, and a small portion of the intestine were dissected together and incubated with 0.5 mg/ml collagenase A (Boehringer-Mannheim, Mannheim, Germany) at 37°C for 30 min. They were then washed several times with Hanks balanced salt solution (HBSS; Gibco Brl) at 4°C. The epithelium was then stripped mechanically from the surrounding mesenchyme using needles on a 0.25% agar, 25% HBSS, 75% RPMI (Gibco Brl) gel in a Petri dish.

Measurement of cellular cAMP formation. Batches of four epithelial buds were cultured for 2 h in 500 μl of RPMI 1640 (Gibco BRL) containing penicillin (100 units/ml), streptomycin (100 μg/ml), HEPES (10 mmol/l), L-glutamine (2 mmol/l), nonessential amino acid (1×; Gibco BRL), and 1% heat-inactivated FCS (Hyclone) at 37°C in a humidified 95% O_2/5% CO_2 atmosphere. Then, the medium was changed for identical medium containing 0.5 mmol/l 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich) and further supplemented with the factor to be tested: forskolin (Sigma-Aldrich); VIP and PACAP 38 (Neosystem); Ro 25-1553, a specific VPAC2 agonist (provided by Dr. P. Robberecht ULB, Brussels, Belgium); glucagon (Bachem); and exendin-4, CCK, gastrin, and isoproterenol (Sigma-Aldrich). Forskolin was dissolved in DMSO, whereas the other factors were dissolved in PBS containing 0.1% BSA (Sigma-Aldrich). Comparable concentrations of DMSO or PBS containing 0.1% BSA were used as controls. In some experiments, the effect of the PAC1/VPAC2-antagonist $PACAP_{6-38}$ (Bachem) was also tested. After 15 min of

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HBSS, Hanks balanced salt solution; IBMX, 3-isobutyl-1-methylxanthine; PACAP, pituitary adenylate cyclase–activating polypeptide; TGF, transforming growth factor; TUNEL, Tdl-mediated dUTP nick-end labeling; VIP, vasoactive intestinal peptide.

incubation at 37°C, the reaction was stopped by transferring the epithelial buds into 500 μl of cold perchloric acid. The buds were disrupted by sonication. Their cAMP content was measured by radioimmunoassay as previously described (23). Data are expressed as nanomoles per liter of cAMP per epithelial bud.

RNA extraction and RT-PCR. Total RNAs were extracted by the guanidinium isothiocyanate method (24). For RT-PCR, total RNAs first treated for 30 min at 37°C with RNase-free DNase (Gibco BRL) were used to prepare cDNAs with random hexamers as the primers. The reaction was performed with (RT+) or without (RT−) MuLV reverse transcriptase (Gibco BRL). The following sets of primers were used for the PCR: Cyclophilin-5′, 5′CAGGTCCTGGCATCTTGTCC 3′; Cyclophilin-3′, 5′TTGCTGGTCTTGCCATTCCT 3′; VPAC1–5′, 5′GCCCCCATCCTCCTCTCCATC 3′; VPAC1–3′, 5′TCCGCCTGCACCTCACCATTG 3′; VPAC2–5′, 5′ATGGATAGCAACTCGCCTTTCTTTAG 3′; VPAC2–3′, 5′GGAAGGAACCAACACATAACTCAAACAG 3′; PAC1–5′, 5′TTTCATCGGCATCATCATCATCATCCTT 3′; and PAC1–3′, 5′CCTTCCAGCTCCTCCATTTCCTCTT 3′.

Thirty amplification cycles were performed. Each cycle consisted of denaturation for 30 s at 96°C, annealing for 30 s at 57°C, and extension for 30 s at 72°C. The resulting fragments were separated by electrophoresis on 1.5% agarose gel then photographed. Each experiment was repeated at least three times.

Organ culture. Pancreatic epithelia were embedded in 500 μl of collagen gel (10% RPMI 10×; Sigma-Aldrich), 80% type I rat tail collagen (2.5 mg/ml; provided by Dr. B. Coulomb, Saint-Louis Hospital, Paris, France), 10% sodium bicarbonate in 0.1 mol/l NaOH, in four-well plates, as previously described (22,25). Once the gel was polymerized, 500 μl of RPMI 1640 (Gibco BRL) containing penicillin (100 units/ml), streptomycin (100 μg/ml), HEPES (10 mmol/l), L-glutamine (2 mmol/l), nonessential amino acid (1×; Gibco BRL), and 1% heat-inactivated FCS (Hyclone) was added. Cultures were maintained at 37°C in a humidified 95% air/5% CO_2 atmosphere. The medium was supplemented with 100 nmol/l of either VIP or RO 25-1553. The medium containing the relevant factor or its vehicle (PBS containing 0.1% BSA) was changed every day. At the indicated times, the pancreatic epithelia were photographed and fixed for immunohistochemistry, as described below.

Immunohistochemistry, surface quantification, and statistical analysis. Pancreatic rudiments were fixed in 3.7% formalin for 1 h, pre-embedded in agarose gel (4% of type VII low gelling-temperature agarose [Sigma] in Tris-buffered saline), and embedded in paraffin. Consecutive sections 4 mm thick were collected on gelatinized glass slides, deparaffinized, and microwaved for 12 min. Immunohistochemistry was performed as previously described (11,22) using the following antibodies: mouse anti–human insulin (1:2,000; Sigma), mouse anti-human glucagon (1:2,000; Sigma), rabbit anti-glucagon (1:2000; Incstar), rabbit anti-PDX1 (1:500; gift from Dr. Madsen), rabbit anti–carboxypeptidase-A (1:600; Biogenesis), and rabbit anti-porcine VIP (1:200; Incstar). The fluorescent secondary antibodies obtained from Jackson Immunoresearch Laboratories were fluorescein anti-rabbit antibodies (1:200), fluorescein anti-mouse antibodies (1:150), TX Red anti-mouse antibodies (1:200), and Texas Red anti-rabbit antibodies (1:200).

For each pancreatic epithelium, all sections (between 50 and 120 per rudiment) were digitized using a Hamamatsu C4742–95 cooled tri-charged coupled device camera (Middlesex, NJ). All images were taken at the same magnification. On each image, the surface areas of the various stains were quantified using Openlab 2.2.5. For each stain, the surface areas per section were summed to obtain the total surface area per rudiment. When a section was missing (<2% of the total section number), we took into account the mean value of the staining in the adjacent sections. To quantify total rudiment volume, we measured and summed the surface areas of all of the sections of that rudiment, and the result was multiplied by the section thickness (4 μm). Statistical analyses were performed with StatView 5.0 (SAS Institute, Cary, NC). Because we could not assume that the data were binomial, we used nonparametric Mann-Whitney *U* tests to compare groups.

Whole-mount in situ hybridization. Tissues were fixed overnight at 4°C in 4% paraformaldehyde in PBS and dehydrated in ethanol. After rehydration in PBS containing 0.1% Tween-20 (PBS-T), tissues were treated with proteinase K (10 μg/ml) (Euromedex, Mundolsheim, France) for 15 min at room temperature. Prehybridization was done at 65°C in hybridization buffer (50% formamide, 5× SSC, 50 μg/ml yeast RNA, 1% SDS, 50 μg/ml heparin). RNA probes were labeled with DIG-UTP by in vitro transcription using the DIG-RNA labeling kit (Boehringer-Mannheim). Hybridization was initiated by the addition of fresh hybridization buffer containing the probe (1 μg/ml) and was continued overnight at 65°C. Then, the tissues were washed for 30 min at 65°C in 50% formamide, 5× SSC, 1% SDS. After treatment with RNase A (100 μg/ml; Sigma) for 1 h at room temperature, the tissues were washed with 50% formamide, 2× SSC for 1 h at 65°C. Nonspecific sites were blocked with 2% blocking reagent (Boehringer-Mannheim) and 10% normal sheep serum (Sigma) in Tris (25 mmol/l, pH 7.5), NaCl (1.4 mol/l), KCl (27 mmol/l), and 0.1% Tween-20. The tissues were incubated overnight at 4°C with peroxidase-conjugated antidigoxigenin antibodies (Boehringer-Mannheim) diluted 1:1,000 in blocking solution. They were rinsed twice with Tris-buffered saline with Tween and washed five times for 1 h each time, at room temperature, in Tris-buffered saline with Tween. The signal was visualized by color development with 5-bromo-4-chloro-3-indol phosphate (Roche, Mannheim, Germany) and nitro blue tetrazolium (Roche) in the presence of 1 mmol/l levamisole (Sigma) to inhibit endogenous alkaline phosphatases. Stained tissues were rinsed in PBS with Tween and photographed in toto using a Sony 3CCD Color Video Camera Exwave HAD. Then, they were postfixed in 4% PFA-PBS, sliced, and used for immunohistochemistry as described above. Each experiment was performed at least three times. The mouse pdx-1 probe was provided by C. Wright (Vanderbilt University Nashville, TN), and the rat VPAC2 probe was provided by L. Journot (CNRS, Montpellier, France).

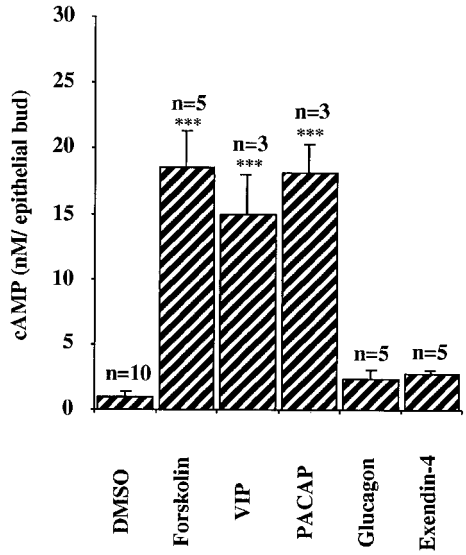

FIG. 1. Effect of various activators of cAMP production in embryonic pancreatic epithelial cells. Batches of four embryonic pancreatic epithelia were treated for 15 min with IBMX supplemented with the factor to be tested. Forskolin was used at 10 μmol/l and all other factors at 100 nmol/l. The cAMP content was measured by radioimmunoassay and presented as nmol per liter per epithelial bud (mean ± SE). *n*, number of experiments; ******P* < 0.005.

BrdU and Tdl-mediated dUTP nick-end labeling assays. For labeling cells in the S phase, pancreatic epithelia were incubated with BrdU (10 μmol/l; Sigma) for 1 h. Explants were fixed, sectioned, and stained using anti-BrdU antibodies (dilution 1:2; Amersham Pharmacia Biotech).

To label apoptotic cells, we used the In Situ Cell Death Detection Kit (Roche). Sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min at room temperature, washed three times for 5 min in PBS, and incubated with 50 μl of Tdl-mediated dUTP nick-end labeling (TUNEL) reaction mixture for 60 min at 37°C. The sections were visualized using a fluorescence microscope (Leica, Leitz DMRB). Results are presented as the number of positive cells per mm^2.

RESULTS

Screening for ligands of protein G–coupled receptors activating cAMP production in embryonic pancreatic epithelial cells. We searched for ligands of protein G–coupled receptors that increase cAMP accumulation in embryonic pancreatic epithelial cells. Forskolin, an activator of adenyl cyclase, was used as a positive control. As shown in Fig. 1, both VIP and PACAP at 100 nmol/l activated cAMP accumulation in E13 pancreatic epithelial cells. No increase in cAMP accumulation was detected when the epithelial rudiments were treated with glucagon, exendin-4 (an analog of glucagon-like peptide-1), glucagon-like peptide-2, parathyroid hormone, gastrin, cholecystokinin, or isoproterenol (a β-adrenergic receptor agonist; data not shown).

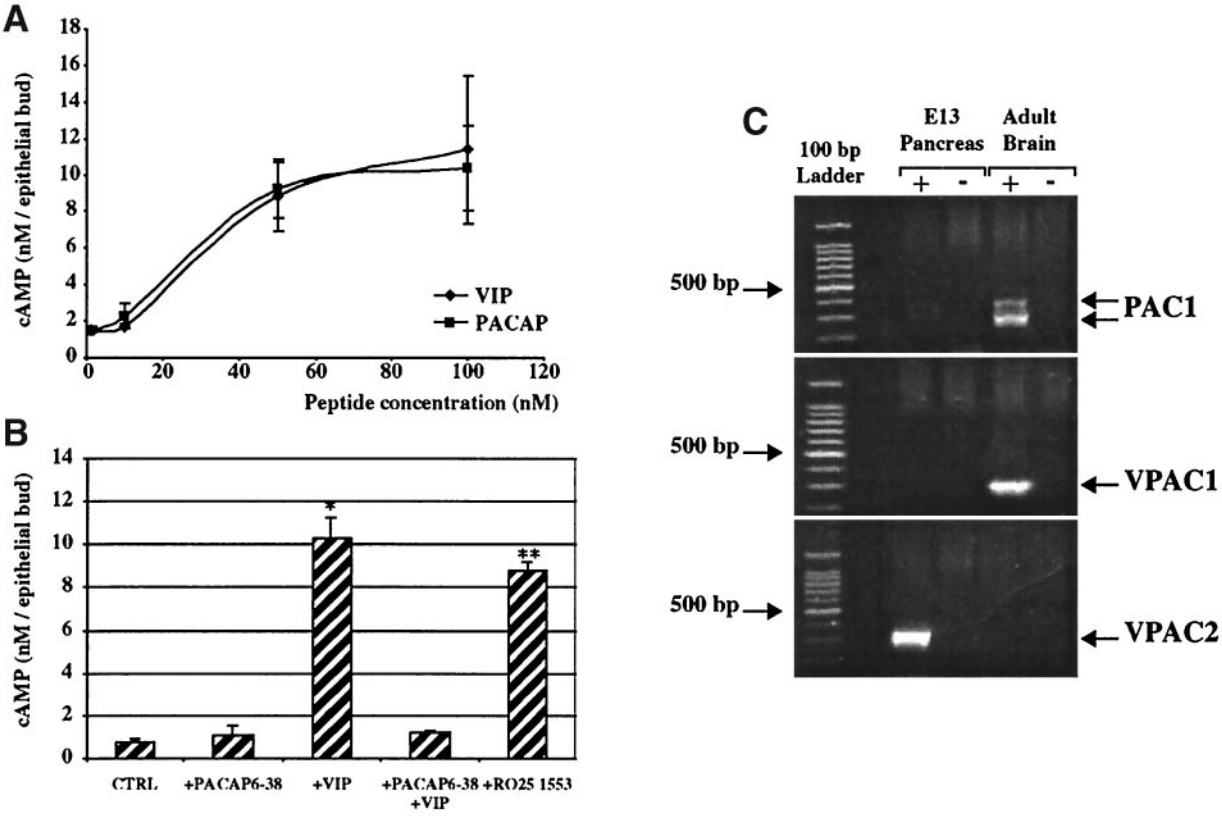

FIG. 2. VIP and PACAP signal through VPAC2 in embryonic pancreatic epithelium. *A*: Dose-dependent effect of VIP and PACAP on cAMP production by embryonic pancreatic epithelial cells. *B*: Embryonic pancreatic epithelia were treated for 15 min with IBMX supplemented with one of the following: $PACAP_{6-38}$, a PAC1/VPAC2 antagonist (10 μmol/l); VIP (100 nmol/l); $PACAP_{6-38}$ (10 μmol/l) plus VIP (100 nmol/l); or RO 25-1553, a VPAC2 agonist (100 nmol/l). The cAMP content measured by radioimmunoassay is reported as nanomoles per liter per epithelial bud (mean ± SE). Three samples were analyzed for each group. *C*: RT-PCR analysis for PAC1, VPAC1, and VPAC2 using RNA from E13 pancreas or adult brain. RT+ and RT−, reverse transcription with or without reverse transcriptase.

VIP and PACAP generate signals via VPAC2 in embryonic pancreatic epithelium. Three receptors for VIP and PACAP (PAC1, VPAC1, and VPAC2) have been cloned. Whereas PAC1 binds only PACAP with high affinity, VPAC1 and VPAC2 bind both VIP and PACAP (26). First, to define the functional signaling receptor for VIP and PACAP in E13 pancreatic epithelium, we evaluated the dose-dependent accumulation of cAMP in response to increasing concentrations of VIP and PACAP. As shown in Fig. 2*A*, epithelial cells respond in a similar manner to the addition of various concentrations of VIP and PACAP. As shown in Fig. 2*B*, the stimulatory effect of VIP on cAMP accumulation was inhibited by $PACAP_{6-38}$, an antagonist of both PAC1 and VPAC2 (27), whereas the effect of VIP was mimicked by Ro 25-1553, a specific VPAC2 agonist (28). Finally, as shown in Fig. 2*C*, RT-PCR analysis revealed that embryonic pancreatic epithelial cells expressed VPAC2, whereas no expression of VPAC1 and PAC1 was detected. Taken together, these results indicated that VIP generated signals via VPAC2 in embryonic pancreatic epithelial cells.

In vitro effect of VIP on the development of embryonic pancreatic epithelium. To determine whether VIP influenced pancreatic development, we cultured E13 pancreatic epithelia for 3 days with and without VIP. As shown in Fig. 3*A*, during culturing, the total tissue size did not increase in the absence of VIP but increased 2.2-fold in the presence of VIP. VIP treatment had no effect on the few fibroblasts that remained attached to the epithelium. Then, we quantified cell differentiation. The absolute volume occupied by acinar cells decreased under control conditions but not in the presence of VIP. The volume occupied by endocrine cells showed a far greater increase with VIP (4.8-fold) than without VIP (2.8-fold; Fig. 3). This difference was due to an increase in absolute cell numbers, the cell sizes remaining unchanged under the various conditions (data not shown). In addition, as shown in Fig. 4, the increase in endocrine development was mainly due to an increase in the development of insulin-expressing cells. The volume occupied by glucagon-expressing cells was similar with and without VIP (Fig. 4). Finally, embryonic pancreatic epithelium cultured with the VIP analog Ro 25-1553, specific for VPAC2, showed similar development to that obtained with VIP (data not shown).

VIP activated both the proliferation and the survival of embryonic pancreatic epithelial cells. We investigated a number of possibilities by which VIP might increase the final mass of insulin-expressing cells. First, we used the TUNEL assay to examine the effect of VIP on E13 epithelial cell apoptosis. As shown in Fig. 5, when embryonic pancreatic epithelial rudiments were cultured for 8 h with VIP, a twofold decrease in the number of apoptotic cells was observed as compared with rudiments grown without VIP. This effect of VIP on cell survival was confirmed by counts of the number of pyknotic nuclei after Hoechst 33342 staining (data not shown). Next, we examined the effect of VIP on E13 epithelial cell prolifer-

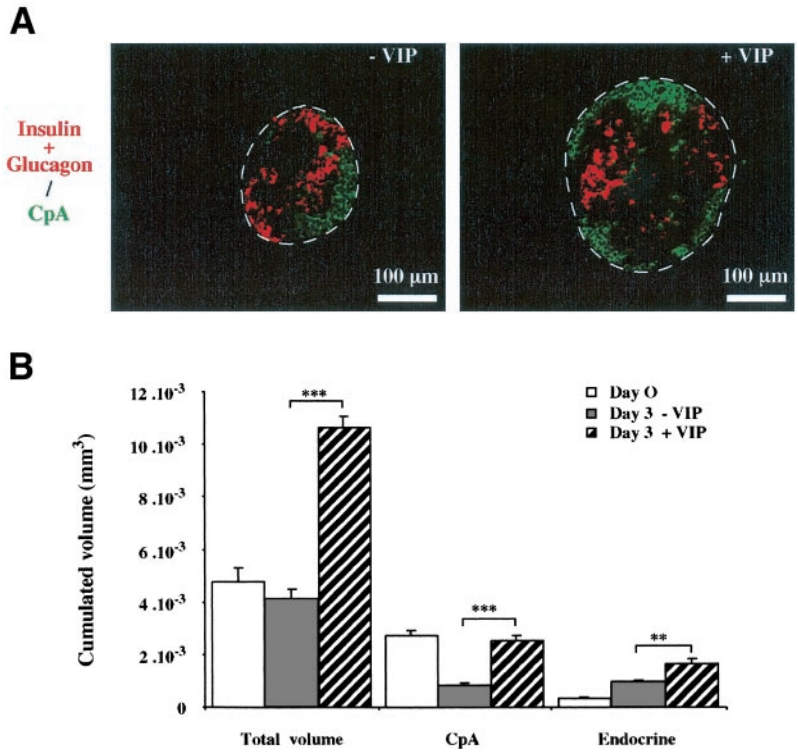

FIG. 3. Immunohistological analysis of pancreatic epithelium grown in vitro for 3 days with or without VIP. *A*: Pancreatic epithelia were grown for 3 days with or without VIP, sectioned, and stained for insulin plus glucagon (red) and carboxypeptidase-A (green). *B*: Absolute volume (mm^3) of epithelia occupied by carboxypeptidase-A–positive cells and by endocrine cells (insulin- plus glucagon-expressing cells) in rudiments before culturing or after 3 days of culturing with or without VIP. Eight rudiments were analyzed for each condition. $^{**}P < 0.01$; $^{***}P < 0.005$.

ation. When embryonic pancreatic epithelial rudiments were cultured for 8 h and pulsed with BrdU during the last hour, the number of cells that incorporated BrdU was increased 1.8 times in epithelia cultured with VIP as compared with without VIP (Fig. 5). Finally, we investigated whether the increase in the final β-cell mass might be related to VIP-induced activation of the proliferation of mature β-cells during culturing. No increase in the number of insulin-expressing cells incorporating BrdU was detected in epithelial buds cultured with VIP as compared with without VIP (data not shown).

Pancreatic expression of VPAC2. In situ hybridization

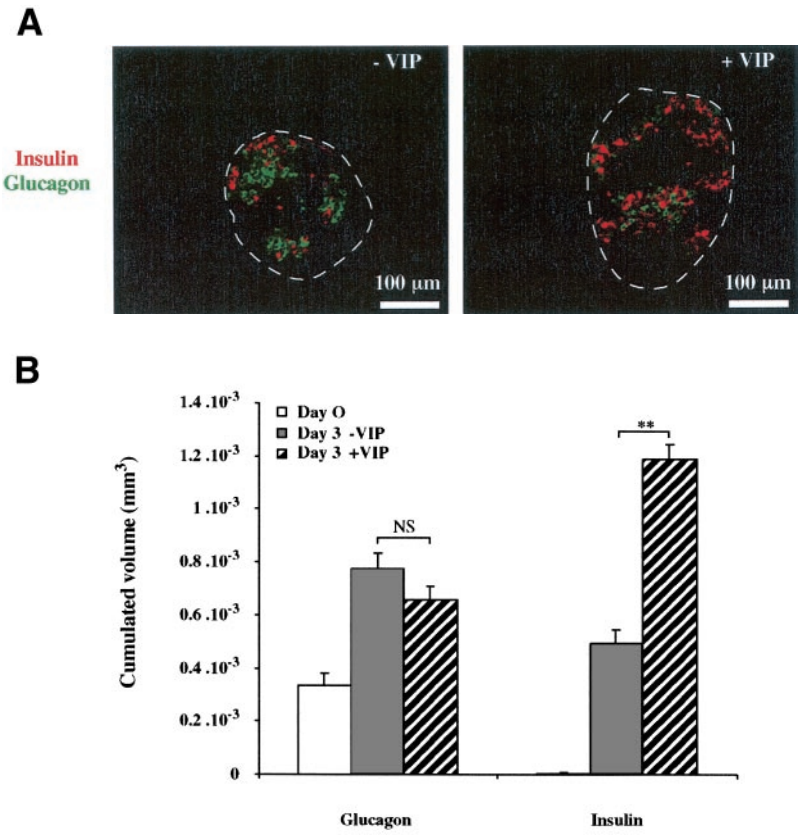

FIG. 4. VIP treatment increased the absolute number of insulin-expressing cells in pancreatic epithelium grown in vitro. *A*: Pancreatic epithelia were grown for 3 days with or without VIP, sectioned, and stained for insulin (red) or glucagon (green). *B*: Absolute volume (mm^3) of epithelia occupied by insulin- or glucagon-expressing cells in rudiments before culturing or after 3 days of culturing with or without VIP. Eight rudiments were analyzed for each condition. $^{**}P < 0.01$.

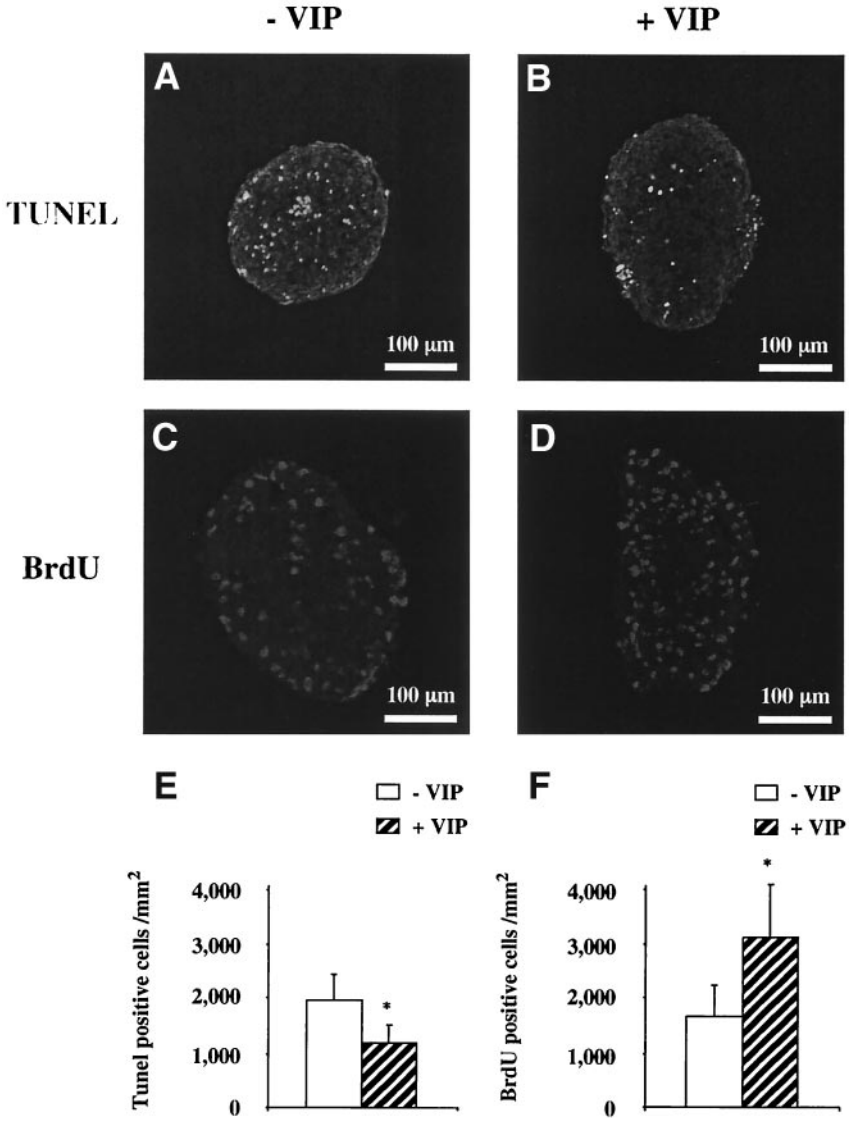

FIG. 5. VIP treatment decreased the number of apoptotic cells and increased cell proliferation. Pancreatic epithelia were grown for 8 h with or without VIP and were pulsed with BrdU during the last hour. TUNEL-positive cells were revealed in green (*A* and *B*) and counted (*E*). BrdU-expressing cells were revealed in red (*C* and *D*) and counted (*F*). Three rudiments were analyzed for each condition. *$P < 0.05$.

was performed on digestive tracts of E13 rats to define the expression profile of VPAC2. As shown in Fig. 6*A*, *a* and *b*, the expression profile of VPAC2 resembled that of Pdx-1, a transcription factor expressed by pancreatic epithelial cells. To define further the cell types expressing VPAC2, we sectioned and stained digestive tracts hybridized with a VPAC2 probe with an anti–Pdx-1 antibody. VPAC2-expressing cells present in pancreatic buds stained positively for Pdx-1 (Fig. 6*B*). As shown in Fig. 7, VPAC2-expressing cells were negative for insulin and glucagon. Few VPAC2-expressing cells stained positively for the acinar marker carboxypeptidase.

Next, we used in situ hybridization to investigate the ontogeny of VPAC2 expression in the pancreas. VPAC2 expression was not detected at E11, the earliest age examined (data not shown). As shown in Fig. 8, at E12, VPAC2 transcripts were detected in the two pancreatic buds (dorsal and ventral). Receptor transcripts were maintained in the pancreas, until E16. At later stages, the expression of VPAC2 decreased, being detected neither at E18 nor in adult pancreas.

DISCUSSION

In the present work, we demonstrated that embryonic pancreatic epithelial cells are sensitive to VIP. In such cells, VIP generates signals via VPAC2 and induces cAMP accumulation. VIP affects epithelial cell survival and proliferation, inducing an increase in the number of insulin-expressing cells available for development.

Whereas data have been reported on the effect of ligands of receptor tyrosine kinases on pancreatic development during embryonic life (8,10,12,13,29–31), little information is available about the implication of factors that generate signals via other pathways. Thirty years ago, it was suggested that cAMP may be a regulatory intermediary in pancreas development (32). Glucagon, a factor that is expressed at the early stages of pancreas development and that increases cAMP levels, has been suggested as one of the endogenous pancreatic factors acting via these other pathways (33). In the present work, we screened for a number of factors known to increase cAMP accumulation in target cells. Our finding that cAMP accumulation did not occur in E13 epithelia exposed to glucagon suggests that,

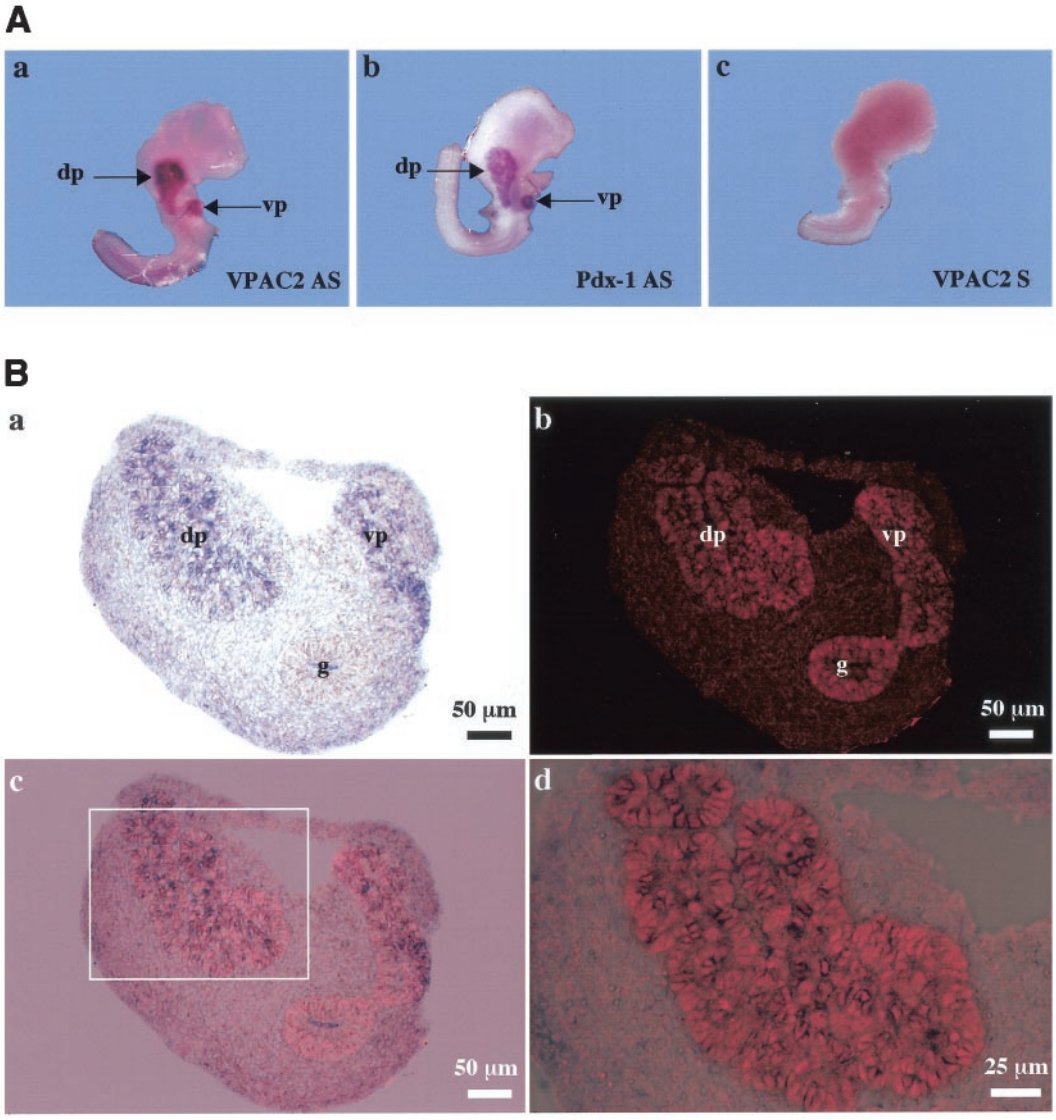

FIG. 6. Expression of VPAC2 in the pancreas at E13. *A*: Whole-mount in situ hybridization of dissected gastrointestinal tracts hybridized with a VPAC2 antisense (AS) probe (*a*), a Pdx1 AS probe (*b*), or a VPAC2 sense probe (*c*). *B*: Gastrointestinal tracts hybridized with a VPAC2 AS probe were sectioned and stained with a Pdx-1 antibody (red). *a*: VPAC2 expression in the dorsal and ventral pancreas. *b*: Pdx-1 expression. *c*: *a* and *b* are fused. *d*: *c* is enlarged. dp, dorsal pancreas; vp, ventral pancreas; g, gut.

at this stage of development, pancreatic epithelial cells are not sensitive to glucagon.

The effect of Glp-1 on cAMP accumulation in embryonic epithelial cells was also tested. Glp1 has been shown to play important roles in β-cell formation during postnatal life. It activates β-cell neogenesis in adult rats after pancreatectomy (34) and in adult diabetic mice (35) and stimulates β-cell neogenesis in newborn diabetic rats (35). Finally, it has recently been shown that Glp-1 can induce differentiation of pancreatic epithelial ductal cell lines into insulin-secreting cells (36). To the best of our knowledge, data on the effect of Glp-1 on the embryonic pancreas are scarce. Our findings indicate that Glp-1 and its analog exendin-4 are poor activators of cAMP accumulation in embryonic pancreatic epithelial cells, suggesting that these cells are not sensitive to Glp-1. However, we found here that both VIP and PACAP were strong activators of cAMP accumulation in embryonic pancreatic epithelial cells. VIP and PACAP are known to generate signals via three different receptors: VPAC1, VPAC2, and PAC1 (26). The pharmacological and RNA analysis results presented here indicate that VIP and PACAP generate signals via VPAC2 in embryonic pancreatic epithelial cells. VPAC2 is the only receptor of this family expressed at this stage by embryonic pancreatic epithelial cells. In contrast, mRNA encoding all three receptors has been detected in adult pancreas. Pancreatic islets express mRNA encoding PAC1, VPAC1, and VPAC2, and both VIP and PACAP increase glucose-induced insulin secretion. This effect on insulin secretion seems to be mediated by all three receptors (19,37,38).

Our analysis of VPAC2 expression during pancreatic development revealed that VPAC2 was expressed at E13 by Pdx-1–positive cells present in both the dorsal and the ventral pancreas. At this stage, VPAC2 expression was detected neither in the stomach nor in the duodenum. Later, VPAC2 expression decreased to low levels (39) that were under the limit of detection in our conditions.

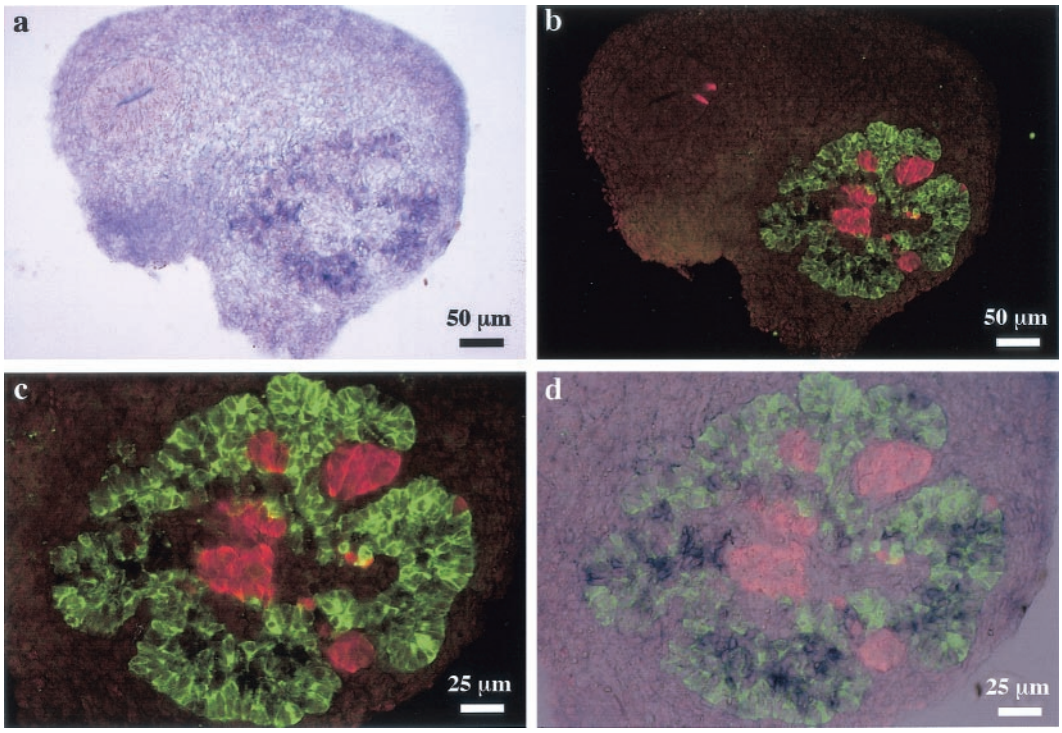

FIG. 7. The vast majority of VPAC2-expressing pancreatic cells stained negative for endocrine and acinar markers at E13. Gastrointestinal tracts hybridized with a VPAC2 AS probe were sectioned and stained for insulin plus glucagon (red) and carboxypeptidase-A (green). *a*: VPAC2 expression in the dorsal pancreas. *b*: Expression of insulin plus glucagon (red) and carboxypeptidase-A (green). *c*: *b* is enlarged. *d*: Fusion, allowing the visualization of insulin plus glucagon (red), carboxypeptidase-A (green), and VPAC2 (blue).

We found that VIP increased the final number of insulin-expressing cells developing in vitro from E13 pancreatic epithelium. Our data strongly suggest that this effect was due to an increase in the pool of endocrine progenitors available for differentiation into insulin-expressing cells. This hypothesis is based on the following arguments. First, in our experimental system, β-cells develop from progenitor cells (22). Second, we found that at E13, the proliferation and survival of immature pancreatic epithelial cells was increased in the presence of VIP, whereas VIP had no effect on the proliferation of mature β-cells. Third, in various other models, VIP has been shown to regulate both cell survival and cell proliferation. For example, VIP has been reported to regulate the survival of neuronal cells and overall cell proliferation during embryonic life (20,40).

An important point is the source of VPAC2 ligands during embryonic pancreas development. During postnatal life, PACAP and VIP may be present in nerves throughout the pancreatic gland (19). Theoretically, during embryonic life, VPAC2 ligands could derive from various sources, including the nerves, pancreatic tissue, other embryonic tissues, or maternal tissues. By immunohistochemistry, we were unable to detect VIP in the pancreatic nerves or epithelial or mesenchymal cells at E13, whereas in adult pancreas, we found VIP in nerves throughout the gland (data not shown). The source of VIP may be extrapancreatic embryonic tissues that express VIP mRNA during embryonic life (41). Maternal tissue may be another source of VIP acting on VIP binding sites in the embryo: VIP levels are high in maternal blood, and VIP can be transported from the maternal blood to the embryo (42).

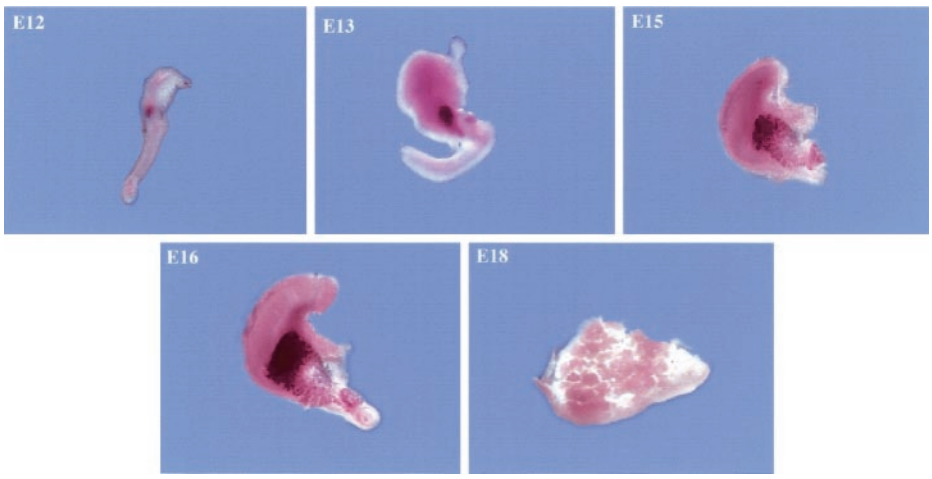

FIG. 8. Expression of VPAC2 during pancreas development. Whole-mount in situ hybridization of dissected gastrointestinal tracts hybridized with a VPAC2 antisense (AS) probe at E12, E13, E15, E16, and E18.

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