Functional characterization and expression of peripheral-type benzodiazepine receptor in rat gastric mucosa: Stimulation of chloride secretion by PBR ligands

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Running Head
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Abstract

Previous studies have demonstrated that gastric mucosa contained high levels of the polypeptide diazepam binding inhibitor, the endogenous ligand of the peripheral-type benzodiazepine receptor (PBR). However, the expression and function of this receptor protein in these tissues have not been investigated. Immunohistochemistry identified an intense PBR immunoreactivity in the mucous and parietal cells of rat gastric fundus and in the mucous cells of antrum. Immunelectron microscopy revealed the mitochondrial localization of PBR in these cells. Binding of isoquinoline PK 11195 and benzodiazepine Ro5-4864 to gastric membranes showed that fundus had more PBR-binding sites than antrum, displaying higher affinity for PK 11195 than Ro5-4864. In Ussing chamber, PK 11195 and Ro5-4864 increased short-circuit current (Isc) in fundic and antral mucosa in a concentration-dependent manner in the presence of GABA_A and CBR blockers. This increase in Isc was abolished after external Cl^- substitution, and was sensitive to chloride channels or transporters inhibitors. PK 11195- induced chloride secretion was also: (i) sensitive to verapamil and extracellular calcium depletion, (ii) blocked by thapsigargin and intracellular calcium depletion and (iii) abolished by the mitochondrial pore transition complex inhibitor cyclosporine A. PK 11195 had not direct effect on H^+ secretion, indicating that it stimulates in fundic mucosa a component of Cl^- secretion independent of acid secretion. These data demonstrate that mucous and parietal cells of the gastric mucosa express mitochondrial PBR functionally coupled to Ca^{2+}-dependent Cl^- secretion, possibly involved in the gastric mucosa protection.

Key words
PBR, Mitochondria, chloride secretion, PK 11195, Ro5-4864
Introduction

PBR is an 18 KDa protein initially identified in the rat kidney (8) as a high affinity binding site for diazepam. It is present in many peripheral tissues such as endocrine and exocrine glands (57), liver (55), as well as in the central nervous system (4). Because of peripheral topography, pharmacological, structural and physiological distinction from the central benzodiazepine receptor (CBR), it was named “peripheral-type benzodiazepine receptor”, or PBR (4, 53). Subcellular fractionation studies found that PBR is primarily localized on the outer mitochondrial membrane (2). It is especially concentrated in the outer/inner mitochondrial membrane contact sites (11) where, as it has been suggested (18, 36, 48), it might form a trimeric complex with the 32 KDa voltage-dependent anion channel (VDAC) and the 30 KDa adenine nucleotide carrier (ANC).

Direct studies of PBR have shown that this protein might be involved in several functions (reviewed in 5, 9, 39). These include porphyrin transport and heme biosynthesis (49), cholesterol transport and steroidogenesis (40) or bile salts biosynthesis (51), apoptosis (21), anion transport (3, 14).

Diazepam binding inhibitor (DBI), also known as endozepine (50), is an 86 amino acid polypeptide, identified for its ability to displace CBR and PBR drug ligands with micromolar affinity. A good correlation between DBI expression and PBR content has been observed in peripheral tissues (7). Proteolytic cleavage of DBI generates a family of several biologically active peptides (13, 44). High concentration of endozepine-like immunoreactivity was observed in the rat gastrointestinal tract, with the highest levels detected in stomach and duodenum (45, 58). Endozepine-like immunoreactivity in those tissues was observed in the secretory and absorptive epithelial cells but not in the myenteric layers or neuronal cells (45). Although these reports establish the presence of endozepines in gastrointestinal tract, there is no direct evidence for PBR localization in these tissues.
Various families of PBR drug ligands have been developed and used for localization and pharmacological characterization studies. Among these, two classes were essential in the identification and/or characterization of PBR: the isoquinolines, such as PK 11195 and benzodiazepines, such as Ro5-4864 (4’-chlorodiazepam). Both PK 11195 and Ro5-4864 have high affinity for PBR (nanomolar range) and low affinity for CBR (high micromolar range: for detailed review of structure and affinity studies of endogenous and exogenous PBR ligands see 5, 39). They, furthermore, have been diagnostic in unveiling the physiological function of this ubiquitous protein in various tissues and cells. The present study is an attempt to examine the presence and distribution of PBR in the rat gastric fundus and antrum by immunohistochemistry and to identify a function of this protein in gastrointestinal biofunction. The results presented demonstrate that PBR is present in mucus and parietal cells of the gastric mucosa and that it is functionally coupled to calcium-dependent chloride secretion, as evidenced by the effect of the high affinity ligands.
Materials and Methods

- **Animals:**

Adult Male Wistar rats weighing 220-260 g (Iffa-Credo, L’Arbresle, France) were housed under controlled conditions (lights on 6 am-6 pm; temperature 24 ± 1 °C) in individual cages with raised mesh floors to prevent coprophagia. Animals were given standard laboratory chow and water *ad libitum* and fasted overnight 14 hours before the experiments. They were sacrificed by cervical dissociation and exsanguination.

- **Morphological Studies**

  **Immunolocalization:** For detection of PBR in embedded tissue samples a rabbit anti-mouse polyclonal antibody raised against, an amino acid sequence (amino acid 9 to 27) conserved across species (ab-PBR-9-27) (33). To examine immunoreactivity for PBR by light and/or electron microscopy, mucosal samples from fundus or antrum were fixed in 4% paraformaldehyde during 2 hours at room temperature and embedded in paraffin, or fixed in a mixture of 4 % paraformaldehyde with 0.3 % glutaraldehyde and embedded in LX-112 resin. DBI immunolocalization was examined using an anti-bovine DBI antibody as previously described (16).

  **Light Microscopy:** Paraffin sections were re-hydrated and endogenous peroxydase activity was inactivated followed by the peroxidase-antiperoxidase (PAP) technique. Sections were incubated with the PBR antibody (1:120 dilution) or DBI antibody overnight at 4°C. Peroxidase complexed secondary antibody was purchased from Dakopatts Ltd. (Trappes, France). Sections were counterstained with Mayer’s hematoxilin (E.M.S.) (Euromedex, Souffelweyersheim, France). To test the specificity of the immunohistochemical signals, control experiments were performed in the absence of the primary antibody and immunoabsorption (8 µg of recombinant 18 kDa PBR protein/ ml of diluted antibody).
**Electron Microscopy:** LX-112 resin sections were treated by 10 % H₂O₂ during 5 minutes to expose antigenic sites. An indirect immunocytochemical technique was applied (15). Incubation with increasing primary antibody concentrations (from 1/600 to 1/200) was performed overnight at 4°C. Secondary antibody was coupled to 15 nm colloidal gold (Biovalley, Marne la Vallée, France). Grids were contrasted with 2% uranyl acetate and observed in a JEOL 1200 EX electron microscope operated at 80 kV accelerated voltage.

**Morphometric studies:** Grids were double stained with uranyl acetate and lead citrate. Twenty centrally sectioned cells with clear nuclear profile (cell:nucleus ratio < 2:1) from each experimental protocol were analyzed to determine the surface density of mitochondria at ultrastructural level as previously described (54).

- **Ussing Chamber Experiments**

  **Tissue preparation:** Stomach was rapidly removed after sacrifice and the rumen and pylorus were discarded. The antrum area was separated from corpus and opened along the small curvature. Tissue was gently washed with Ringer solution, and serosa was stripped off by dissection with clamps and scissors. The remaining piece of fundus was cut along the large curvature and the muscular layer was stripped off using forceps.

  **Solutions:** The Krebs Ringer (KRB) bathing solution contained: 115.4 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 1.5 mM Na H₂PO₄, 1.2 mM MgCl₂, 1.2 mM CaCl₂, 10 mM glucose, 25 mM NaHCO₃. The calcium free solution was prepared as the KRB but without calcium added and with 0.2 mM EGTA. The Cl⁻-free solution contained: 115 mM Na-Gluconate, 5 mM KHPO₄, 1.2 mM MgSO₄, 0.6 mM NaH₂PO₄, 1.2 mM Ca-Gluconate, 25 mM NaHCO₃ and 10 mM glucose.

  **Isc measurements:** Isc was measured in vitro in Ussing chambers (AD Instruments, Colorado Springs, CO, USA). Stripped antrum or fundus mucosa were mounted vertically between the
two compartments of the chamber with an exposed surface area of 0.5 cm². Each compartment contained 4 mL of KRB solution gassed with 95%O₂:5%CO₂ (pH 7.4) and kept at 37°C. The tissue was continuously short-circuited using a voltage-clamping amplifier (DVC-1000 WPI, Aston, England) as previously described (19). The transepithelial potential was measured through 3 M KCl-agar bridges connected to a pair of calomel electrodes. The transepithelial current was applied across the tissue through a pair of Ag-AgCl electrodes kept in contact with the mucosal and serosal bathing solutions through a pair of 3M KCl bridges. Isc was recorded using the MacLab/8 connected with the V3.3 software (AD Instruments, Colorado Springs, CO, USA). Results were expressed as the intensity of the Isc (µA/cm²) or as the difference (ΔIsc) between the Isc measured within 10 minutes after addition of the compounds and the basal Isc (measured just before addition of the compound). Each drug was applied from a concentrated stock solution dissolved in water or ethanol. The final volume of ethanol was always lower than 0.1% v/v. It had been established in preliminary experiments that ethanol added either in mucosal or serosal reservoir up to 1 % v/v did not produce any effect on Isc, neither in fundus nor in antrum.

• Radioligand Binding Assays

Binding of [³H]PK 11195 and [³H]Ro5-4864 to membranes prepared from the rat fundus and antrum was carried out as described previously (17). In brief, each tissue was first minced and homogenized in 5 ml of phosphate buffered saline solution (PBS), pH 7.3 at 4°C with the use of an Ultra-Turrax polytron 15 sec at half maximal setting (13500 rpm). Then, the homogenate was processed in a glass-teflon homogenizer to obtain homogeneous protein extract, and centrifuged at 500 x g for 10 minutes to dispose of connective tissues. Subcellular membrane fractions were obtained as follow: whole homogenates from fundus and antrum were obtained as previously described using 10 mM Tris-HCl, 250 mM sucrose, 1 mM EDTA buffer, pH 7.3, supplemented with 1 mM PMSF. They were centrifuged at 750
The pellet containing nucleus and the supernatant are referred as “nuclear fraction” and “post-nuclear fraction”, respectively. Supernatant was further centrifuged at 10,000 x g for 20 minutes to obtain “mitochondrial” (pellet) and “post-mitochondrial” (supernatant) fractions. Protein levels were quantified using the dye-binding BioRad assay with BSA as standard. PBR-ligands binding studies were performed at room temperature, in a final incubation volume of 0.3 ml of PBS, containing various concentrations of the radioligand with and without 1000-fold excess of unlabelled ligand. After 25 minutes incubation, assays were stopped by filtration through Whatman GF/F or GF/B for [3H]PK 11195 and [3H]Ro5-4864 binding studies, respectively. Radioactivity trapped on the filters was determined by liquid scintillation counting. Displacement experiments in identical conditions were performed with membranes incubated in the presence of constant radioligand concentration (0.3, 1 or 10 nM) and no labeled ligand concentrations up to 30 μM. The dissociation constants (Kd) and the number of binding sites (Bmax) for PK 11195 and for Ro5-4864 were determined from the saturation isotherms by Scatchard plot analysis.

Gastric acid secretion test

Experiments were performed on male Wistar rats weighing 225-250 g fasted for 18 h with free access to water. Animals were anesthetized by intramuscularly injection of urethane (0.6-0.7 ml of 25 % solution per 100 g of body weight). A polyethylene catheter was inserted through the esophagus in the cardia, and connected to a peristaltic pump (Minipuls 2, Gilson Medical Electronics) to deliver a solution of pre-warmed 0.9 % NaCl at a constant rate of 1 ml/min. This perfusate was collected through another catheter placed through the pylorus and secured with a ligature. The test started after stabilization of the gastric perfusion, which was achieved between 30-60 minutes after completion of the surgical preparation. During this period, an intravenous of physiological saline was infused at 2.4 ml/h (perfusor Braun, Roucaire, France) into the dorsal vein of the penis. Stimulation by pentagastrin or PK 11195
was obtained by continuous infusion through the same route. Gastric secretions were collected every 20 min and the amount of H\(^+\) measured by titration with 0.01 N NaOH to pH 7.0. Results are expressed as µEq of H\(^+\)/min.

- **Statistical analyses:**
  Data are presented as the mean ± standard error of mean (SEM). Experimental data were studied using one-way ANOVA. Scheffe homogeneity test was performed in order to determinate differences between means of each experimental group and control counterpart. Differences were considered significant for P value < 0.05.

- **Reagents:**
  PK 11195, Ro5-4864 and all chemical reagents used were purchased from Sigma (St. Louis, MO, USA). Flumazenil was kindly provided by Dr. Pierre Weber (S. Hoffman-Laroche SA, Basel, Switzerland). \([^3]H\)PK 11195 (83.5 Ci/mmol) and \([^3]H\)Ro5-4864 (86 Ci/mmol) were purchased from Dupont-New England Nuclear (Boston, MA, USA).
Results

**Immunohistochemistry:** PBR immunoreactivity was detected in the gastric fundus and antrum. The immunoreactivity in the fundus (Fig. 1A) was located all along the glands. Mucous cells, localized at the tissue surface, showed a strong PBR immunoreactivity (Fig. 1B). Large-sized parietal cells contained round nuclei and also exhibited a strong immunoreactivity to PBR (Fig. 1C). PBR immunoreactivity in the antrum was located mainly at the surface of glands, where mucous cells appeared strongly labeled (Figs 2A, 2B).

Figure 3 show the localization of the endogenous PBR ligand DBI in fundus (A, B, C) and antrum (D,E). The immunoreactivity in the fundus was located all along the glands (Fig. 3A) with both, mucous (Fig. 3B) and parietal (Fig. 3C) strongly labeled. In the antrum, immunoreactivity anti-DBI was located mainly at the surface of glands (Fig. 3E,D).

**Immunoelectron microscopy:** These studies were further performed to characterize the subcellular localization of PBR. Immunolabelling was strictly localized to the mitochondria of the gastric fundus and antrum (Figs. 1D, 1E and 2C). The labeling was not observed in membranes, such as the plasma or nuclear membranes, as it was previously reported for some other tissues (28, 37). Mitochondria were numerous in parietal cells, round shaped and spread all over the cytoplasm (Fig. 1E). In the surface mucous cells, as well as in the fundus and antrum, however, they were located beneath the secretory mucous granules (Figs. 1D and 2C). PBR immunoreactivity was markedly different in superficially located mucous cells and those situated towards the base of the antral glands. In order to verify whether or not these differences were due to the mitochondrial content of the two cell groups, morphometric analyses were performed using transmission electron micrographs. The results of morphometric analyses indicated that mitochondrial surface density was similar in both surface and basally located cells of the antral glands. Consequently, this finding alone could not account for the observed differences in labeling characteristics of the two cell groups.
Radioligand binding assays: Pharmacological studies have shown that PK 11195 and Ro5-4864 were high affinity specific PBR ligands (30, 31). PBR drug ligands binding was measured on fundus and antral membrane preparations at room temperature. Values of Kd and Bmax for the isoquinoline PK 11195 and the benzodiazepine Ro5-4864 are summarized in Table I. There were approximately 50 % more binding sites for the PBR ligands in the fundus than in the antrum. The affinities for both PBR ligands were different (3 and 230 nM for PK 11195 and Ro5-4864, respectively), and no affinity differences were observed between fundus and antrum. Displacement studies between [³H]PK 11195 and PK 11195 (squares), Ro5-4864 (triangles) or diazepam (circles) are shown in figure 4A. Apparent IC₅₀ of 3.5 nM, 200 nM and 1.4 µM were calculated for PK 11195, Ro5-4864 and diazepam, respectively. It was clear that there were no affinity differences between the fundus (open symbols) and antrum (closed symbols). [³H]PK 11195 binding in subcellular fractions are shown in figure 4B. Mitochondrial enriched fractions from fundus and antrum showed about 2.5 fold more [³H]PK 11195 binding than the respective whole homogenates. Nuclear and post-nuclear fractions [³H]PK 11195 binding were not significantly different, and the binding to the post-mitochondrial fraction was significantly lower than those to the whole homogenate (Fig. 4B).

Effect of PBR ligands on the Isc: The effect of diazepam, a widely used benzodiazepine molecule known to bind to both CBR and PBR with mid-micromolar affinity, was first examined. Diazepam stimulated Isc in the fundus and antrum but with some differences (Fig. 5). It produced in the antrum (Fig. 5B) a concentration-dependent increase in Isc reaching a plateau at 1 µM. In the fundus diazepam also induced a concentration-dependent increase in Isc also (Fig. 5A), which was significant at concentrations higher than 0.1 µM. No saturation of the response was observed at concentrations up to 100 µM, suggesting a higher EC₅₀ as it was previously indicated for the native and recombinant PBR (2, 17, 32) and in line with the
results of displacement binding assays (Fig. 4). The effect of diazepam in the fundus and/or antrum was completely blocked (open circles) by the addition of either 50 µM bicuculline methiodide and 50 µM flumazenil, that are GABA$_A$ and central benzodiazepine receptor (CBR) antagonists, respectively. In order to eliminate the CBR-mediated effects in all subsequent experiments: (i) bicuculline and flumazenil were used to block events mediated by GABA$_A$ and/or CBR; and (ii) two distinct high affinity PBR exclusive drug ligands were used.

The respective effects of increasing concentrations of PK 11195 and Ro5-4864 on the Isc across the gastric mucosa were further examined (Figs 6, 7). PK 11195, when added to the serosal side of fundic preparations, induced a concentration-dependent increase in Isc reaching a maximum at 1 µM ($\Delta$Isc: 15.1 ± 3.2 µA/cm$^2$), with an apparent EC$_{50}$ of 3 nM (Fig. 6A). It is noteworthy that the response to PK 11195 started after a delay of a few minutes, which might account for the entry of the drug into the cells (data not shown). PK 11195 also induced a significant increase in Isc in the antral mucosa, although this increase was smaller and reached a maximum of 6.0 ± 1.1 µA/cm$^2$, with an apparent EC$_{50}$ of 3 nM (Fig. 6B).

Although the serous layer was stripped off, the eventual presence of neuronal cells in the preparation of gastric mucosa cannot be excluded. Experiments performed in the presence of the neurogenic blocker tetrodotoxin (TTX) clearly showed that pre-treatment with 1 µM TTX had no effect on PK 11195-induced Isc either in fundus or in antrum (Fig. 6, open triangles). According to the methodology used herein, the increase in the short-circuit current could be the consequence of either an anion secretion, or cation absorption, or a combination of both events. In order to identify the nature of the PK 11195-increased Isc, the effect of extracellular chloride replacement on this process was examined (Figure 6 open squares). The absence of Cl$^-$ in KRB solutions, bathing both sides of the tissue, prevented the increase of Isc in response to PK 11195 in the fundus (maximum $\Delta$Isc: 1.00 ± 0.30 µA/cm$^2$, Fig. 6A) and
also in the antrum (maximum ΔIsc: 1.25 ± 0.40 µA/cm², Fig. 6B). These findings indicated that Cl⁻ is the main ionic species contributing to the Isc induced by PK 11195 in the gastric mucosa.

In the fundus, Ro5-4864 was also observed to induce an increase in Isc. This increase was concentration-dependent, reaching an extrapolated maximal response of 4.50 ± 1.0 µA/cm² giving an apparent EC₅₀ of 200 nM. Cl⁻ movements could likely account for this effect since extracellular Cl⁻ suppression drastically decreased the Isc (Fig. 7, open squares). In the antrum, Ro5-4864 had no significant effect on Isc.

Chloride secretion from epithelial cells is a complex process involving several Cl⁻ input and output mechanisms (26,27). We further examined the effect of different chloride channels or chloride transporters inhibitors to PK 11195-induced Isc. As shown in Fig.8, chloride channel blocker NPPB, basolateral Cl⁻/HCO₃⁻ exchanger inhibitor DIDS or basolateral Na⁺/K⁺/2Cl⁻ cotransporter inhibitor bumetanide all dramatically reduced Isc in both fundus and antrum in agreement with the involvement of PK 11195-induced chloride movements.

Movements of water and electrolytes resulting from activities of secretory epithelial cells are an essential mechanism for maintaining the function of the gastrointestinal tract. Cl⁻ secretion is the main active process responsible for secretion of water. Cytosolic calcium concentration fluctuations are involved in regulation of chloride movements in epithelial secretory cells (for detailed review see 26, 27). Indeed, Ca²⁺ released from the endoplasmic reticulum and repumped by Ca-ATPase as well as Ca²⁺ influx through the plasma membrane (for instance by L-type calcium channels) are the principal source generating fluctuation of free intracellular Ca²⁺. To examine the role of such a mechanism in PBR-mediated Cl⁻ movement the effects of thapsigargin, an endoplasmic reticulum Ca²⁺-ATPase inhibitor, and of verapamil, a L-type Ca²⁺ channel blocker, on the increase of Isc induced by PK 11195 were investigated. In the fundus, 2 µM thapsigargin reduced the PK 11195-induced increase in Isc by about 75 %
(Fig. 9A). In the antrum thapsigargine abolished the PK 11195-induced Isc increase (Fig. 9B). The addition of 10 µM verapamil also produced a marked drop of the Isc induced by PK 11195, an event which was more pronounced in the fundus than in the antral mucosa (Fig. 9). The addition of 25 µM intracellular calcium chelator BAPTA-AM, produced a significant inhibition of PK 11195-induced Isc in the fundus and antrum (65 % and 77 % respectively; Fig. 9). In both tissues, extracellular calcium depletion produced a significant inversion of current polarity (Fig. 9).

We found that PBR was located in the mitochondria of cells in the gastric fundus and antrum (Figs 1, 2). The effect of the luminal versus basolateral addition of the PBR drug ligands was next verified. PK 11195 had no significant effect when added apically either in the fundus or in the antrum (data not shown). The latter finding suggested that physiologic activation of PBR was occurring from the serosal side of the mucosa, while the luminal access to the receptor would have little, if any functional significance.

PBR has been reported to be closely associated with the permeability transition pore (PTP), a mitochondrial channel that appears to be involved in the mitochondrial Ca\(^{2+}\) signal (9). In order to estimate the importance of this channel in the process induced by PBR ligands, the effect of the PTP complex inhibitor cyclosporine A on PK 11195-induced Isc was examined. Cyclosporine A (100 nM) reduced the Isc by 80% in the fundus (Fig. 9A), while in the antrum the PK 11195-induced Isc was completely abolished (Fig. 9B). These results strongly suggested that PTP is involved in the PBR-induced chloride secretion in the gastric mucosa.

Since in the gastric mucosa chloride secretion is an important component of acid secretion, we examined the effect of proton pump inhibitor omeprazole on PK 11195-induced chloride secretion (Fig. 10A). Omeprazole (10 µM) significantly reduced (-40%) PK-induced Isc suggesting that PK might stimulate gastric acid secretion. To verify this possibility, we measured the effect of PK 11195 on H\(^+\) output in vivo in anaesthetized rats. These
experiments showed that PK 11195 (alone or with bicuculine) does not stimulate H\(^+\) secretion when administered intravenously from 3 to 300 µg/kg/h (i.e., ~1 to 100 µM) Fig. 10B).
Discussion

The results presented herein show that the high affinity PBR drug ligands, the isoquinoline PK 11195, and to a lesser extent the benzodiazepine Ro5-4864, exert a direct stimulatory effect on gastric epithelial cells resulting in a marked Ca$^{2+}$-dependent chloride secretion.

Mucous secreting cells of the gastric epithelium, and parietal cells of the gastric fundus exhibit marked immunoreactivity for PBR and its endogenous ligand endozepine DBI (compare Figs. 1A to 3A and 2A to 3D). Although the presence of DBI in gastric epithelium was previously reported (45, 58), our findings that DBI is present throughout the epithelium is in contrast to its previously reported exclusive localization in the deep layer of the mucosa (45). Intense PBR immunoreactivity was observed in mucous cells localized only on the surface of the epithelium. This finding may be related to the coexistence of different type of mucous cells that was previously shown on the surface and in the more basal location of the rat antral mucosa (24, 25). Immunelectron microscopy further revealed that PBR was mainly localized in the mitochondrial compartment of the parietal cells in the fundus and in the mucous secreting cells of the antrum, results that were confirmed by PBR ligand binding experiments (Fig. 4B). This finding is in agreement with the subcellular localization of PBR that has been previously reported in several other tissues (2, 11). The abundant expression and colocalization of PBR and its ligand, DBI, in the rat and mouse gastric mucosa (Figs.1, 2 and 3 and references 45 and 58) suggest that this receptor, upon activation by its endogenous ligand, could play an important role in gastric function. Indeed, the mucosa of antrum and fundus was shown to express the highest level of DBI/ODN immunoreactivity in the gastrointestinal tract (45). The localization of the receptor in the epithelial cell, along with previously reported DBI production by the deep layer of the gastric mucosa (45), indicated that PBR may be involved in the regulation of electrolyte, water and/or mucous secretion.
Based on these observations, PBR function on transepithelial ionic secretion was examined using structurally distinct high affinity PBR drug ligands.

Diazepam, a benzodiazepine with affinity for CBR and PBR, was initially used to identify PBR in peripheral tissues (8). Present findings showed that it induced an increase in Isc in fundus as well as in antrum (Fig. 5). However, this effect was completely inhibited by the CBR and GABA\textsubscript{A} antagonists’ flumazenil and bicucullin, respectively. This is in agreement with previous observations that diazepam acts only on these receptors in the gastric mucosa (34). In an effort to distinguish between CBR and PBR, the PBR specific high affinity drug ligand PK 11195 was used. The latter showed indeed a strong stimulatory effect on electrolyte movement that was not affected by the presence of bicuculline and flumazenil (Fig. 6), thus indicating a PBR-mediated function. This effect was further tested while using varying PK 11195 concentrations, with an apparent EC\textsubscript{50} of 3 nM in both fundus and antrum, a concentration similar to the affinity for PBR calculated from Scatchard analysis of the saturation isotherms (Table I). These values of Kd and Bmax for PK 11195 were consistent with those previously reported for whole rat stomach membranes (29). The stimulatory effect on electrolyte transport was also demonstrated using Ro5-4864, a high affinity PBR drug ligand that belongs to the benzodiazepine family. However, the effects observed with this ligand were less pronounced in the fundus and absent in the antrum. These differences between PK 11195 and Ro5-4864 effects might account for the 70-fold difference in ligand affinities and for the presence of a higher number of binding sites in the fundus, as previously reported for some other tissues (56). It is noteworthy that the differences in PBR affinity for Ro5-4864 between herein and previous work (56) might arise from different incubation temperature of the ligand binding assays. This ligand-induced PBR activation was generated in a polarized manner with a more pronounced effect at the basolateral than the apical cell membrane. The polarity of the effect may be of physiological relevance because DBI could
stimulate the gastric mucosa by an endocrine and/or paracrine manner, as previously reported for Leydig cells (16). In line with this hypothesis, the presence of DBI has been described throughout the gastric epithelium (58 and present data) including the deep layer of the rat mucosa (45) as well as in the human plasma (52). Finally, the absence of effect of the neurogenic blocker TTX on the PBR ligand-stimulated electrolyte transport suggests a direct action of PBR ligands in the gastric epithelium, but not through terminal nervous connections.

The present work contributes in our understanding of how PBR drug ligands induced a calcium-dependent chloride current in the gastric mucosa. In the absence of chloride from the bathing solution, the Isc was indeed virtually abolished, indicating that the effect of PBR ligands on Isc was entirely due to transepithelial Cl− secretion. This was also supported by the action of different inhibitors of proteins involved in chloride traffic as NPPB, DIDS or bumetanide, which all blocked PK 11195-induced Isc. The stimulation of Isc by PBR ligands was further shown to be related to cytosolic Ca2+ regulation since thapsigargin (endoplasmatic Ca-ATPase inhibitor), verapamil (blocker of L-type voltage dependent Ca2+ channels), and cytosolic calcium depletion by BAPTA-AM markedly reduced this stimulation (Fig. 9). Furthermore, when calcium was removed from extracellular medium, PK 11195 induced an inversed current, possibly due to the calcium efflux from the cytosol. The effect of exogenous and endogenous PBR ligands upon activation of L-type Ca2+ channels has been previously reported (14, 59). It was also shown that PBR ligands provoke chloride efflux related to calcium influx via L-type Ca2+ channels in the rat astrocytes (14). On the other hand, it has been indicated that DBI elicits intracellular Ca2+ oscillations in STC-1 cells inhibited by L-type Ca2+ channel blockers (59). The latter authors suggested that these Ca2+ oscillations might involve the ryanodine receptor from the endoplasmic reticulum through a calcium-induced calcium release mechanism. In line with these hypotheses, the
present findings ascertained a very strong inhibition of PK 11195-induced Isc by verapamil in the mucosa of gastric fundus, and by thapsigargin in antral mucosa. Consequently, it appears that L-type Ca\(^{2+}\) channel and endoplasmic reticulum calcium stores are involved in the cascade of events following ligand-induced PBR activation.

Because PBR has been suggested to be among the proteins forming the permeability transition pore (PTP) complex (18, 36, 48), the effect of PTP complex inhibitor cyclosporine A on PK 11195-induced Cl\(^{-}\) current was further investigated in the mucosa of fundus and antrum (Fig. 9). It was found that cyclosporine A inhibited the chloride secretion induced by nanomolar concentration of PK 11195 in both tissues, suggesting that the first step in the PBR ligand-induced Cl\(^{-}\) current could be the change in the PTP state. PTP is a mitochondrial megachannel that appears to operate at the crossroad of two distinct physiological pathways, i.e., the Ca\(^{2+}\) signaling network during the life of the cell and the effector’s phase of the apoptotic cascade during Ca\(^{2+}\)-dependent cell death (23). Transient opening of the PTP could induce mitochondrial Ca\(^{2+}\)-induced Ca\(^{2+}\)-release. This, in turn, leads to depolarization and/or Ca\(^{2+}\) rise that could rapidly travel inside the cell via the mitochondrial network to stimulate these organelles in determining the overall Ca\(^{2+}\) signal of a cell (22). Several studies in malignant cells recently demonstrated that PBR ligands, at micromolar concentrations, induce apoptosis (12, 35). These ligands, however, exerted an anti-apoptotic effect at nanomolar concentrations (6). Present results suggest the possibility that the opening of mitochondrial PTP, upon PBR activation, could have other important functional implications unrelated to cell death. Further studies would be required to examine whether nanomolar concentration of PK 11195 could also exert anti-apoptotic effects on gastric epithelium.

As schematized in Fig 11, drug ligand binding to PBR might result in PTP opening and, consequently, lead to an initial calcium release. This signal can be further amplified by calcium-induced calcium release mechanism activating L-type Ca channels and endoplasmic
reticulum calcium stores. This intracellular calcium rise would, in turn, activate a chloride channel responsible for the transepithelial Cl− secretion (27).

Elevation of intracellular Ca^{2+} concentration increases mucous secretion (43). Since PBR was found in some mucous secreting cells, it is likely that activation of the receptor could also result in an increase of mucous secretion. On the other hand it has been established that Cl− secretion across mucosa of the gastric fundus is the result of at least two components, an acidic chloride transport (coupled to gastric acid secretion) and a non-acidic chloride transport (20). Although our results showed that PK 11195 does not stimulate gastric acid secretion (Fig. 10B), the partial inhibition of PK 11195-induced Isc observed with omeprazole (Fig. 10A) could then not be attributed to the acidic component of Cl− secretion. However this effect of omeprazole appears related to acid-producing mucosa since it was found in fundic but not antral mucosa. At present, we have no explanation for this effect of omeprazole. Inhibition of Cl−/HCO_{3}− transport by omeprazole has been previously reported (42). Here an inhibition of the apical Cl−/HCO_{3}− exchanger of resting fundic mucosa by omeprazole would decrease PK 11195-induced chloride secretion by diminishing the available intracellular chloride concentration in parietal cells. PAT1, a putative anion transporter for Cl−/HCO_{3}− was recently found co-expressed with H-K-ATPase in apical tubulovesicle membranes of parietal cells (41). We speculate that omeprazole could interfere with the activity of this exchanger, possibly as a secondary effect of H-K-ATPase inhibition. However, further experiments are needed to verify this possibility.

In summary, the presence of the 18 kDa PBR protein in the mitochondria of mucous and parietal cells in the rat gastric mucosa was demonstrated. It was also shown that PBR ligands induce a Ca^{2+}-dependent Cl− current, the first step of which could be the opening of the PTP mitochondrial channel. The fact that this chloride secretion is not related to acid secretion examined in vivo but possibly to HCO_{3}− secretion, suggest that activation of PBR
could participate at the protective functions of the gastric mucosa. Taken together our data indicate a new role for PBR in the regulation of electrolyte, water and mucous secretion in the gastric mucosa.
Acknowledgements

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References


Legends to Figures

Figure 1: Illustration of PBR immunoreactivity in the rat mucosa of gastric fundus. Intense and homogeneous immunoreactivity for PBR are localized all along the epithelium (A). Only mucous (B) and parietal (C) cells are strongly labeled by peroxydase conjugated anti-PBR antibody. Electron microscopy reveals immunoreactivity for PBR localized in the mitochondria of mucous (D) and parietal (E) cells. Bars: A, 100 µm; B and C, 50 µm; D and E, 1 µm. MC: mucous cell; PC: parietal cell; CC: chief cell; MG: mucous granules; Mi: mitochondria; TV: tubule-vesicles; Cc: canaliculus.

Figure 2: Illustration of PBR immunoreactivity in the rat mucosa of gastric antrum. Intense and specifically localized immunoreactivity for PBR is observed in the epithelium (A). Only surface mucous cells are strongly labeled by peroxydase conjugated anti-PBR antibody; labeling in the bottom of the gland is due to a few parietal cells still present in this region (B). Electron microscopy reveals immunoreactivity for PBR localized in the mitochondria (C). Bars: A, 100 µm; B, 50 µm, and C, 1 µm. MC: mucous cell; MG: mucous granules; Mi: mitochondria.

Figure 3: Illustration of immunoreactivity for DBI in the rat gastric mucosa. Intense and homogeneous immunoreactivity for DBI are localized all along the fundus epithelium (A). Only mucous (B) and parietal (C) cells are strongly labeled by peroxydase conjugated anti-DBI antibody. In the antrum epithelium, intense and specifically localized immunoreactivity for DBI is observed (D). Only surface mucous cells are strongly labeled by peroxydase
conjugated anti-DBI antibody (E). Bars A and D: 100 µm; bars B, C and E: 50 µm. MC: mucous cell; PC: parietal cell; CC: chief cell.

**Figure 4.** PBR drug ligand and displacement studies. A) Specific binding of $[^3\text{H}]$PK 11195 (0.3 nM) to fundus (open symbols) or antrum (filled symbols) membranes was measured in the presence and absence of the indicated concentration of PK 11195 (square), Ro5-4864 (triangles), or diazepam (circles). B) Specific binding of $[^3\text{H}]$PK 11195 (3 nM) to fundus (open bars) or antrum (filled bars) subcellular fractions. Binding experiments were performed as described in materials and methods. 100 µg of fundus or antrum membrane protein were used. Results shown are representative of three independent experiments performed by triplicate. The SE values for each point were consistently less than 15% of the mean.

**Figure 5:** Effect of diazepam on Isc in gastric mucosa. Isc, expressed as ∆Isc, was measured in response to increasing concentration of diazepam in the rat mucosa of gastric fundus (A) and antrum (B). Experiments were conducted in the absence (●) or in the presence (○) of 50 µM bicuculline plus 50 µM flumazenil. Reagents were added at the serosal compartment. Each point represents the means ± SE (n = 4-6).

**Figure 6:** Dose-response curves for PK 11195-induced Isc in the rat mucosa of gastric fundus (A) and antrum (B) and effect of tetrodotoxin (TTX) and of Cl’ replacement. All the experiments were performed with 50 µM bicuculline and 50 µM flumazenil in the incubation medium. Reagents were added at the serosal compartment. (○) Control group; (▽) + 1µM TTX; (□) Cl’ free medium. Each point represents the means ± SE (n = 4-6).
Figure 7: Concentration-response curves for PBR ligand Ro5-4864 performed in the mucosa of gastric fundus in the presence of 50 µM bicuculline and 50 µM flumazenil and effect of Cl⁻ free medium. Reagents were added at the serosal compartment. (○) Control; (□) Cl⁻ free medium. Each point represents a mean ± SE (n = 4-6).

Figure 8: Illustration of the effect of inhibitor of Cl⁻ cellular traffic on Iˢc induced by PK 11195 in the rat mucosa of gastric fundus (A) and antrum (B). 100 µM bumetanide, NPPB and or DIDS were added 10 min before the addition of 10 nM PK 11195. Bumetanide was added in the serosal side. NPPB and/or DIDS were added in both compartments. All experiments were performed in the presence of 50 µM bicuculline and 50 µM flumazenil in the incubation medium. Each bar represents the means ± SE (n = 4-6). * P< 0.05 and ** P< 0.01 significantly different from the control group.

Figure 9: Illustration of the effect of inhibitor of Ca²⁺ activation pathway on Iˢc induced by PK 11195 in the rat mucosa of gastric fundus (A) and antrum (B). 2.5 µM thapsigargine (+ TG), 10 µM verapamil (+ VP) or 0.1 µM cyclosporine A (+ CsA) were added in the serosal reservoir 5 minutes before the addition of 10 nM PK 11195. Experiments without calcium were performed by adding calcium free buffer on both compartments of Ussing chambers. BAPTA experiments were performed adding 25 µM BAPTA-AM to both compartments of Ussing chambers 30 min before the stimulation. All experiments were performed in the presence of 50 µM bicuculline and 50 µM flumazenil in the incubation medium. Each bar represents the means ± SE (n = 4-6). * P< 0.05 and ** P< 0.01 significantly different from the control group.
**Figure 10:** A) Effect of omeprazole on PK 11195-induced Isc in the mucosa of gastric fundus: samples were incubated with 1 µM PK 11195 with or without 10 µM omeprazole. All experiments were performed with 50 µM bicuculline and 50 µM flumazenil in the incubation medium. Each bar represents the means ± SE (n = 4). ** P< 0.02 significantly different from the control group. B) Time-course effects of increasing doses of pentagastrine (open triangles) and PK 11195 (open circles) on gastric acid output in anesthetized perfused rat stomach. Arrows indicates time of drug addition, (1) 16 or 3 µg/kg/h, (2) 32 or 30 µg/kg/h and (3) 48 or 300 µg/kg/h of pentagastrine or PK 11195, respectively. Results represent the mean ± SE of 7 independent experiments.

**Figure 11:** Schematic representation pf the proposed mechanisms underlying the chloride secretion induced by PBR ligand in gastric epithelium. Abbreviations used are: Mitochondria (Mi), endoplasmic reticulum (ER), cyclosporine A (CsA), thapsigargine (TG), verapamil (VP), peripheral benzodiazepine receptor (PBR), permeability transition pore (PTP), ryanodyne sensitive calcium channel (RyR), sarcoendoplasmic reticulum calcium ATPase (SERCA), calcium-sensitive chloride channel (Cl-C), L-type calcium channel (L-typeCaC), anion exchanger (AE), Na⁺/K⁺/2Cl⁻ cotransporter (NKCC), intracellular calcium chelator (BAPTA-AM).
**Table I.** Binding properties of PBR ligands PK 11195 and Ro5-4864 to rat gastric membranes.

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<tr>
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<td>Kd</td>
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<tr>
<td></td>
<td>(nM)</td>
<td>(pmol/mg)</td>
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<tr>
<td>PK 11195</td>
<td>3.09 ± 0.41</td>
<td>7.32 ± 1.02</td>
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<tr>
<td>Ro5-4864</td>
<td>230.2 ± 25.1</td>
<td>7.45 ± 1.09</td>
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Kd and Bmax were calculated from Scatchard plot analysis from data of three to five independent experiments performed in triplicates. Values are expressed as the mean ± SE.
Figure 4
Figure 5

**A**

**B**
Figure 6
Figure 7
Figure 8
Figure 9

**A**

Δ I sc (µA/cm²)

PK 11195 + VP + CsA + BAPTA

[Ca²⁺]out 0

**B**

Δ I sc (µA/cm²)

PK 11195 + VP + CsA + BAPTA

[Ca²⁺]out 0
Figure 10