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Research article

Effect of translocator protein (18 kDa)-ligand binding on neurotransmitter-induced salivary secretion in rat submandibular glands

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Background information. TSPO (translocator protein), previously known as PBR (peripheral-type benzodiazepine receptor), is a ubiquitous 18 kDa transmembrane protein that participates in diverse cell functions. High-affinity TSPO ligands are best known for their ability to stimulate cholesterol transport in organs synthesizing steroids and bile salts, although they modulate other physiological functions, including cell proliferation, apoptosis and calcium-dependent transepithelial ion secretion. In present study, we investigated the localization and function of TSPO in salivary glands.

Results. Immunohistochemical analysis of TSPO in rat salivary glands revealed that TSPO and its endogenous ligand, DBI (diazepam-binding inhibitor), were present in duct and mucous acinar cells. TSPO was localized to the mitochondria of these cells, whereas DBI was cytosolic. As expected, mitochondrial membrane preparations, which were enriched in TSPO, exhibited a high affinity for the TSPO drug ligand, ^{3}H-labelled PK 11195, as shown by B_{max} and K_d values of 10.0 ± 0.5 pmol/mg and 4.0 ± 1.0 nM respectively. Intravenous perfusion of PK 11195 increased the salivary flow rate that was induced by muscarinic and α-adrenergic agonists, whereas it had no effect when administered alone. Addition of PK 11195 also increased the K^+, Na^+, Cl^- and protein content of saliva, indicating that this ligand modulated secretion by acini and duct cells.

Conclusions. High-affinity ligand binding to mitochondrial TSPO modulates neurotransmitter-induced salivary secretion by duct and mucous acinar cells of rat submandibular glands.

Introduction

TSPO (translocator protein), previously known as PBR (peripheral-type benzodiazepine receptor), was originally identified in the rat kidney as a peripheral site for benzodiazepine binding (Braestrup and Squires, 1977; Papadopoulos et al., 2006). Subsequently, TSPO has been found in all tissues examined, mainly being observed to be localized to mitochondria. This 18 kDa hydrophobic protein has been shown to be involved in various cell functions (Papadopoulos, 1993; Gavish et al., 1999; Beurdeley-Thomas et al., 2000; Casellas et al., 2002; Lacapere and Papadopoulos, 2003; Papadopoulos et al., 2006), including porphyrin transport and haem biosynthesis

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Key words: mitochondria, PK 11195, peripheral benzodiazepine receptor (PBR), salivary secretion, submandibular gland, translocator protein (TSPO).
Abbreviations used: CBR, central-type benzodiazepine receptor; DBI, diazepam-binding inhibitor; PPIX, protoporphyrin IX; TSPO, translocator protein; VDAC, voltage-dependent anion channel.

(Taketani et al., 1995), cholesterol transport, steroidogenesis and bile-salt biosynthesis (Lacapere and Papadopoulos, 2003), cell proliferation (Beinlich et al., 2000), apoptosis (Hirsch et al., 1998) and anion transport (Basile et al., 1988; Gandolfo et al., 2001; Ostuni et al., 2004).

A role for TSPO in transepithelial secretions was proposed (Perez et al., 1985; Ostuni et al., 2004, 2007), and benzodiazepines, which are commonly reported to be involved in symptoms of dry mouth (Scully, 2003), may participate in this TSPO action. The effects of this class of drugs are mediated both by the TSPO and CBRs [central-type benzodiazepine receptors; also known as $GABA_A$ (γ-aminobutyric acid) receptors]. Benzodiazepines act in the central nervous system, as well as in the salivary glands, where both proteins are present (Yamagishi and Kawaguchi, 1998). TSPO has been detected in the parotid and submandibular glands in whole-body sections of rat (Anholt et al., 1985; De Souza et al., 1985). Later, membrane preparations containing TSPO were obtained from the parotid and submandibular glands, and ligand binding was characterized using radiolabelled diazepam (Perez et al., 1985), photoaffinity labelling using PK 14105 (Yamagishi et al., 2000) and radiolabelled isoquinoline carboxamide PK 11195 and benzodiazepine Ro5-4864 (Yamagishi and Kawaguchi, 1998). However, no histological studies have been performed to determine the precise cellular and subcellular localization of TSPO in these tissues.

The inhibitory effect of benzodiazepines on rat salivary secretion has been described previously by Kujirai et al. (2002). These compounds have been shown to modify the ion movement that triggers the process of fluid secretion. In rat parotid acinar cells, diazepam inhibits carbachol-induced phosphoinositide turnover that regulates the release of calcium from intracellular stores (Kujirai et al., 2002). This inhibitory effect of diazepam is prevented by the high-affinity TSPO drug ligand, PK 11195 (Kujirai et al., 2002). Similarly, isoprenaline- or carbachol-induced amylase release by parotid acinar cells is blocked by diazepam and restored in the presence of PK 11195 (Okubo and Kawaguchi, 1998). Information for the submandibular glands is more limited, with one report showing that diazepam does not modify $^{86}Rb^{+}$ release induced by noradrenaline or carbachol (Perez et al., 1985).

In the present study, we investigated TSPO protein expression and pharmacologically characterized TSPO in rat submandibular glands to investigate its function in this tissue. Our results reveal that TSPO is present in the mitochondria of duct cells and mucous acinar cells, and provide evidence that high-affinity ligand binding to mitochondrial TSPO modulates neurotransmitter-induced salivary secretion by these cells.

Results

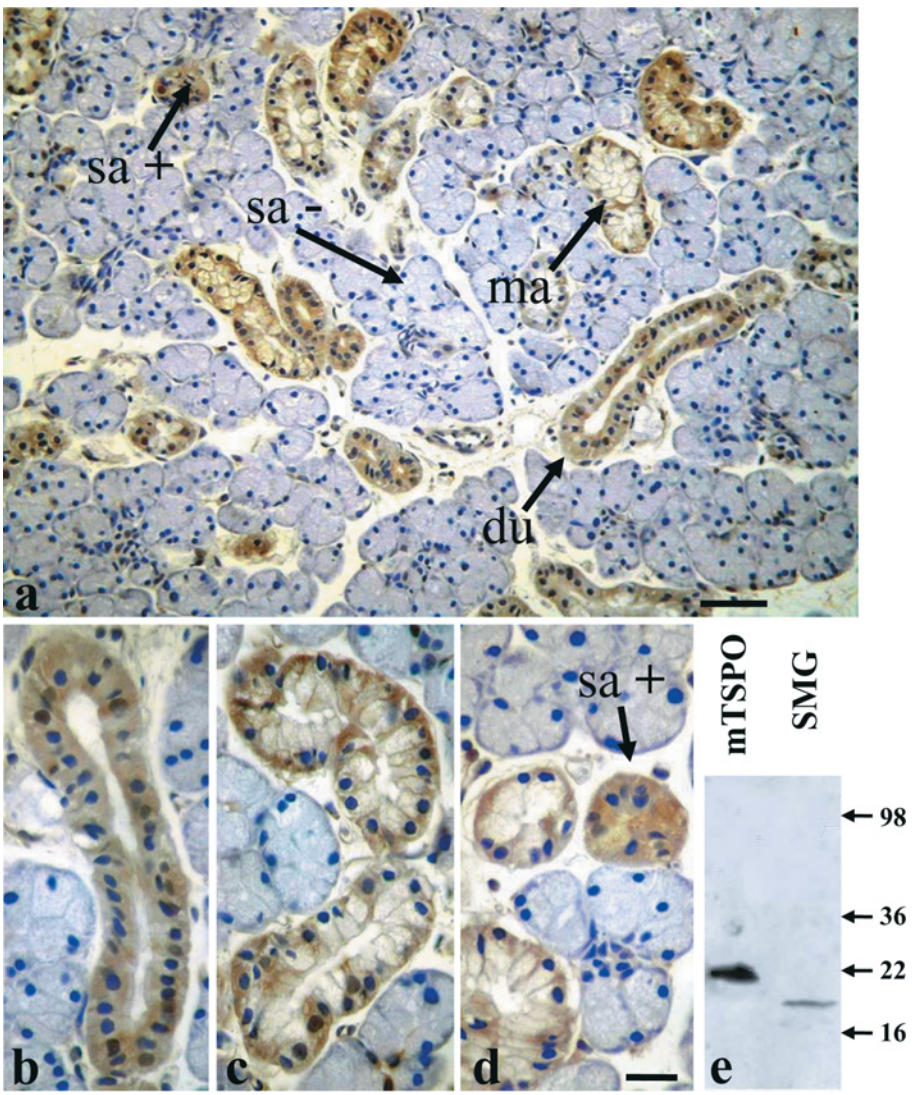
TSPO immunohistochemical staining of submandibular gland tissue revealed a strong immunoreactivity in all duct cells and mucus acini (Figure 1a). In contrast, only a few serous acini were reactive. The highest staining intensity was found in striated duct cells (Figure 1b). Mucous acinar cells, which are easily identified by their large mucous granules, showed strong reactivity (Figure 1c) that was mainly located at their basal pole. Serous acinar cells, which are characterized by their smaller secretory granules, also showed strong reactivity when labelled (Figure 1d). The specificity of the antibody is confirmed by Western blotting with purified recombinant mouse TSPO (Li et al., 2001) compared with membrane homogenate of submandibular glands (Figure 1e).

Confocal microscopy was used to analyse the localization of TSPO (Figures 2a, 2d and 2g) and mitochondria (Figures 2b, 2e and 2h) in ductal cells (Figures 2a–2c), mucus acinar cells (Figures 2d–2f) and serous acinar cells (Figures 2g–2i). A clear overlap between both labels was observed in all cells examined, showing that TSPO co-localizes with mitochondria (Figures 2c, 2f and 2i).

The ultrastructure of the various cells forming the submandibular glands was analysed by electron microscopy. As shown in Figure 3(a), ducts appeared as a lumen surrounded by epithelial cells that had almost perfectly centred nuclei. Mitochondria were situated around the nucleus (Figure 3d), with a high density of mitochondria being present at the basal pole. Mucous (Figure 3b) and serous (Figure 3c) acinar cells had typical ultrastructural appearances, containing many secretory granules of low- and high-electron density respectively (Piludu et al., 2003; Suzuki et al., 2003). In these cells, the secretory granules were present in the apical portion of the cell above the nucleus and were close to the lumen. In contrast, mitochondria

Figure 1 | TSPO expression in the rat submandibular gland

(**a**) Intense TSPO immunoreactivity is present in the duct cells (du), mucus acini (ma) and some of the serous acini (sa). (**b**) In duct cells, TSPO labelling is observed at the basal and apical poles. (**c**) In mucous acini, labelling is present in the basal part of the cell. (**d**) In serous acini positive for TSPO (sa+), labelling is observed throughout the cell. Scale bars: (**a**) 50 μm; (**b–d**), 20 μm. (**e**) Western blotting of recombinant mouse TSPO (mTSPO) and submandibular gland homogenate (SMG) revealed by a specific anti-TSPO antibody (dilution, 1:1000).

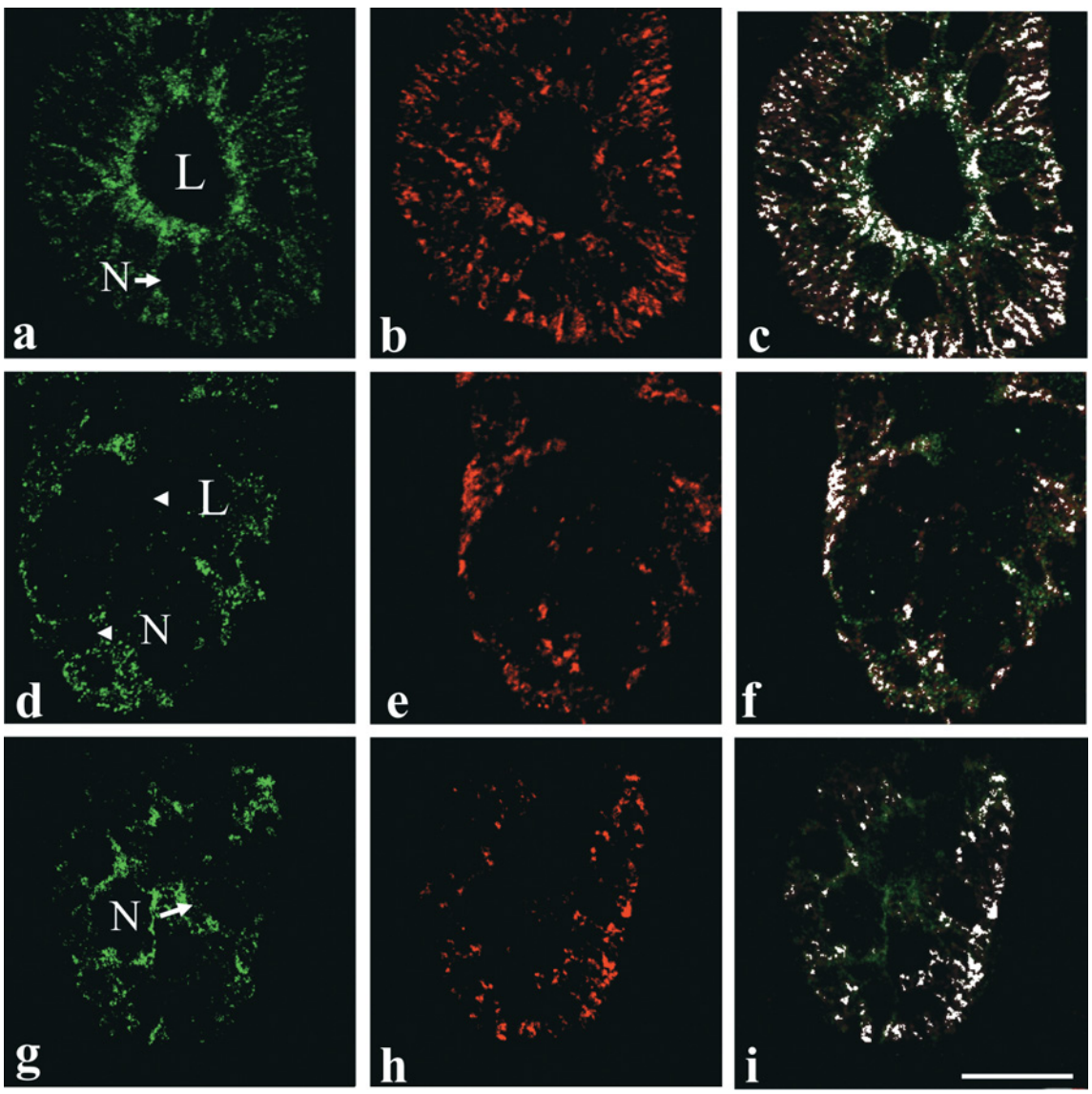


were primarily located at the basal part of the cell below the nucleus (Figures 3e and 3f). Characterization of the subcellular localization of TSPO was further performed by immunogold labelling. It was strictly localized to the mitochondria of the duct (Figure 3g) and mucus acinar cells (Figure 3h). It was observed only in the mitochondria of some serous acinar cells (Figures 3i and 3j), as observed by immunohistochemistry (Figure 1). It was not observed in other membranes, such as the plasma or nuclear membranes, as was reported previously for some other tissue types (O'Beirne et al., 1990; Kuhlmann and Guilarte, 2000).

The subcellular distribution of the endogenous TSPO ligand, DBI (diazepam-binding inhibitor), in rat submandibular glands was also investigated (Figure 4). DBI immunoreactivity was present in the same cells where TSPO was observed, specifically

Figure 2 | Subcellular localization of TSPO in the rat submandibular gland

Confocal microscopy images of duct cells (**a–c**), mucous acini (**d–f**) and serous acini (**g–i**). Cells are co-labelled for TSPO (green; **a**, **d** and **g**) and the mitochondrial marker VDAC (red; **b**, **e**, and **h**). Merged images reveal a strong co-localization of both labels (white; **c**, **f** and **i**). Images are representative of at least 10 immunolocalization experiments. N, nucleus; L, lumen. Scale bar, 10 μm.



the ductal (Figure 4a), mucous acinar (Figure 4b) and serous acinar (Figure 4c) cells. However, the intracellular localization of DBI differed from that of TSPO. In duct cells, DBI immunoreactivity was mainly observed at the apical pole, whereas it was dispersed throughout acinar cells. DBI immunoreactivity did not co-localize with mitochondrial markers (data not shown), confirming its cytosolic location.

Radioligand-binding analyses performed with mitochondrial membrane preparations from submandibular glands revealed that these preparations bound the TSPO drug ligand, ^{3}H-labelled PK 11195, with high affinity (K_d, 4.0 ± 1.0 nM) and capacity (B_{max}, 10.0 ± 0.50 pmol/mg) (Figure 5a). Enrichment of TSPO in isolated mitochondria was confirmed by ^{3}H-labelled PK 11195-binding studies

Figure 3 | Electron microscopy of duct cells (a, d and g), mucus acini (b, e and h), and serous acini (c, f, i and j) of the rat submandibular gland

Mitochondria (arrows) are primarily localized to the basal poles of duct cells (**d**), mucus acini (**e**) and serous acini (**f**) cells. TSPO is revealed by immunocytolocalization in the mitochondria of duct cells (**g**), mucus acini (**h**) and serous acini (**i**) cells with gold labelling (15 nm beads). Mitochondria of serous acini cells negative for TSPO are shown in (**j**). L, lumen; N, nucleus; SG, secretory granules. Scale bars: (**a**–**c**) 5 μm; (**d**–**f**) 2 μm; (**g**–**j**) 0.5 μm.

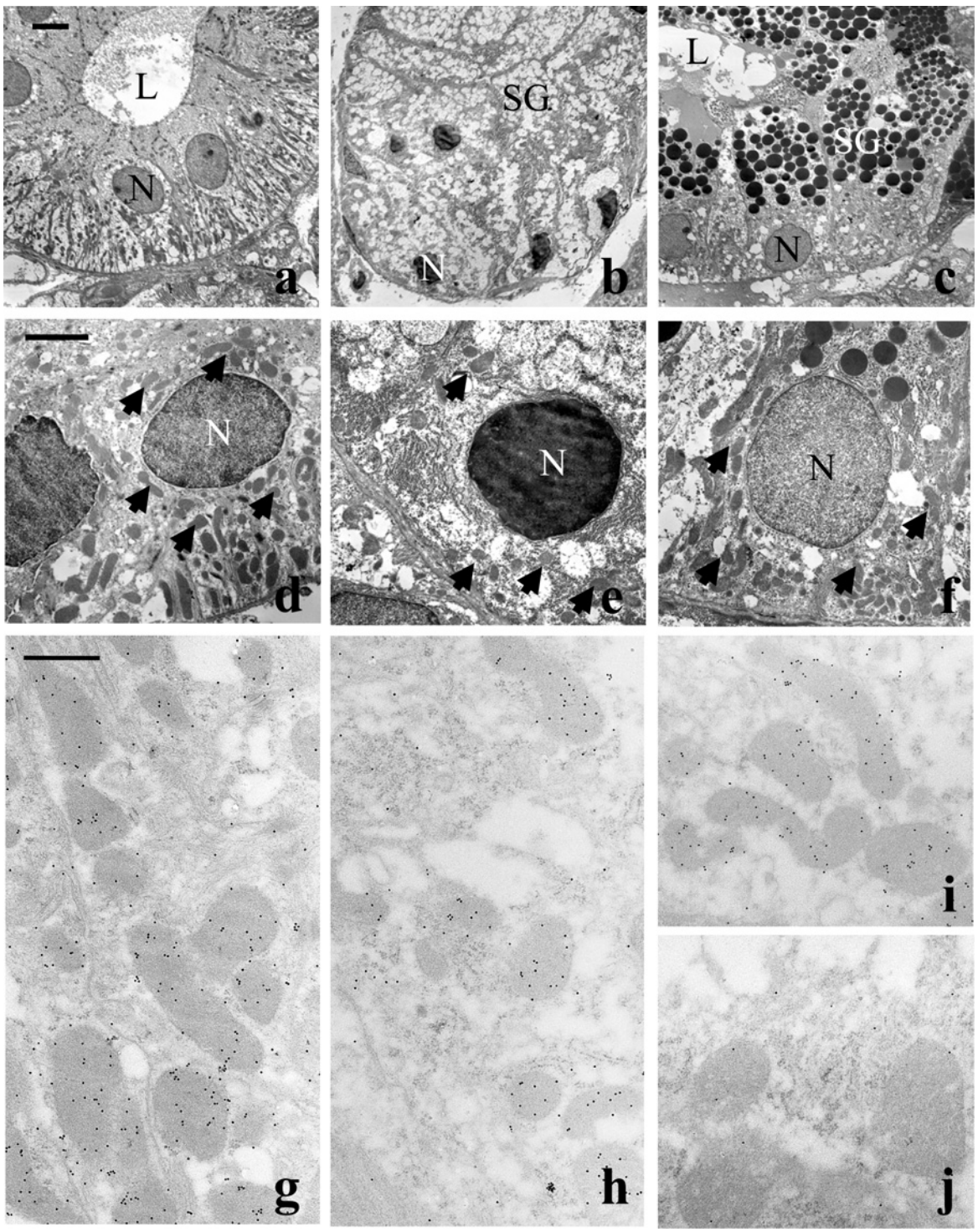
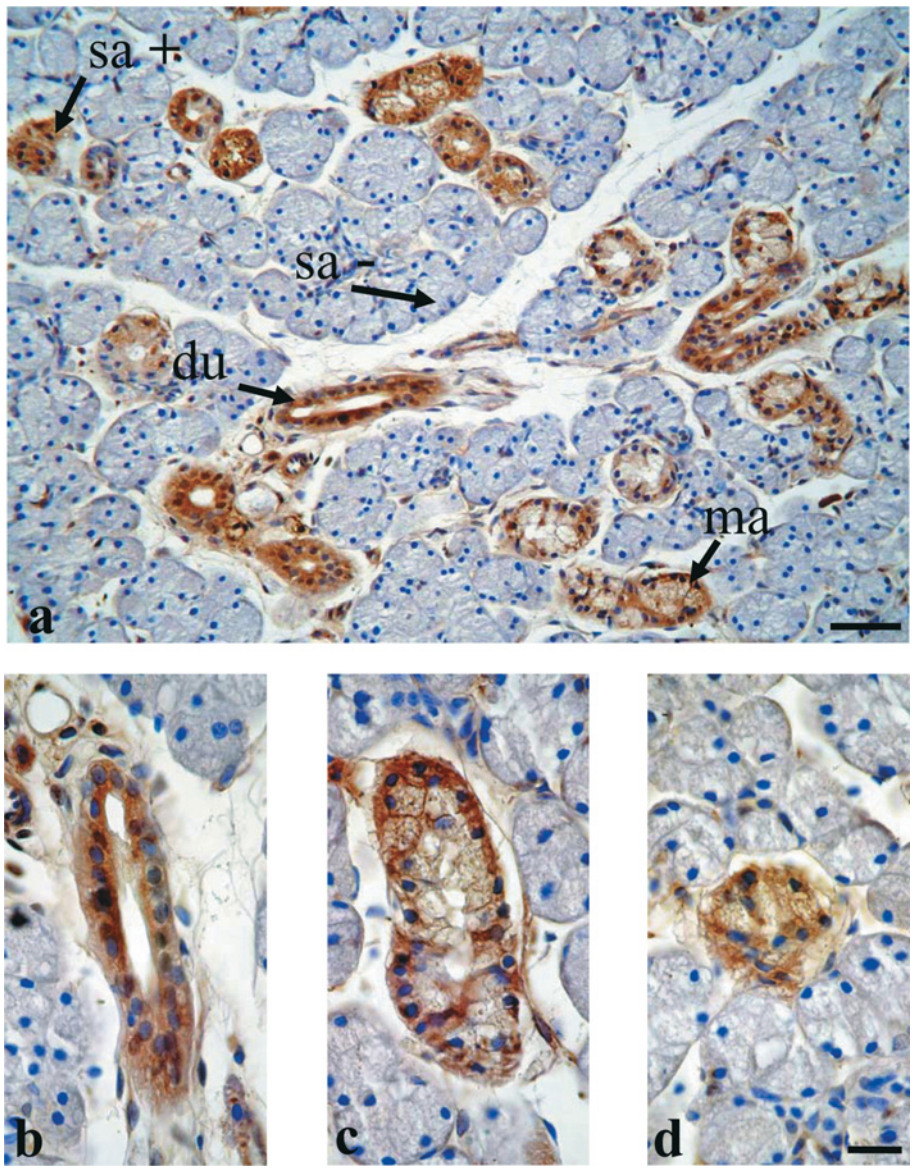


Figure 4 | DBI expression in the rat submandibular gland

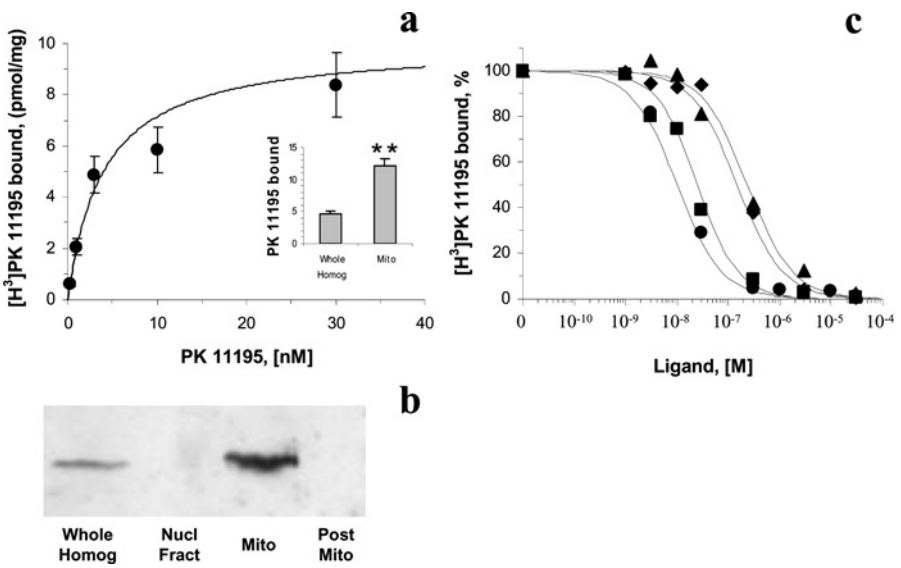
(**a**) Intense DBI immunoreactivity is present in the duct cells (du), mucus acini (ma) and some of the serous acini (sa). (**b**) In duct cells, labelling is observed close to the apical pole. (**c**) In mucous and (**d**) serous acini, labelling is dispersed throughout the cells. Scale bars: (**a**) 50 μm; (**b–d**) 20 μm.



of whole homogenate and mitochondrial fractions (Figure 5a, inset). The 2- to 3-fold enrichment in TSPO-binding sites that we observed in mitochondrial membrane preparations was close to that seen for other tissue or cell types (Nayyar et al., 1996; Ostuni et al., 2004, 2007; Costa et al., 2006). This enrichment was further confirmed using immunoblotting of the various cellular fractions (Figure 5b). Pharmacological characterization of PK 11195 binding revealed a classical TSPO pharmacological profile (Figure 5c) in which the benzodiazepine, Ro5-4864, binds with high affinity (IC_{50}, 20 ± 0.29 nM), and both diazepam and PPIX (protoporphyrin IX) bind with lower affinity (IC_{50}, 200 ± 15 and 250 nM ± 27 respectively). The IC_{50} at which Ro5-4864 and diazepam displaced PK 11195 were of

Figure 5 | Binding and pharmacological profiles of mitochondrial membrane preparations from the rat submandibular glands

(**a**) Saturation curve for ^{3}H-labelled PK 11195 binding. Line indicates the best-fit regression curve. Inset: binding experiments performed with membranes from whole homogenates and from purified mitochondria. (**b**) Western blot of the various membrane-enriched fractions isolated from rat submandibular glands revealed by the specific anti-TSPO antibody. Whole Homog, whole homogenate; Nucl Fract, nuclear fraction; Mito, mitochondria; Post Mito, post-mitochondria. (**c**) Pharmacological profiles of the PK 11195-binding sites in mitochondrial membrane preparations. Ligand displacement with PK 11195 (●), Ro5-4864 (■), diazepam (▲) and PPIX (◆). Results are representative of three independent experiments performed in triplicate for each point. The S.E..M. values for each point were consistently less than 15 % of the mean. Lines indicate the best-fit curves. $^{**}P < 0.01$.



the same order of magnitude as that reported previously for whole-membrane homogenates from salivary glands (Yamagishi and Kawaguchi, 1998).

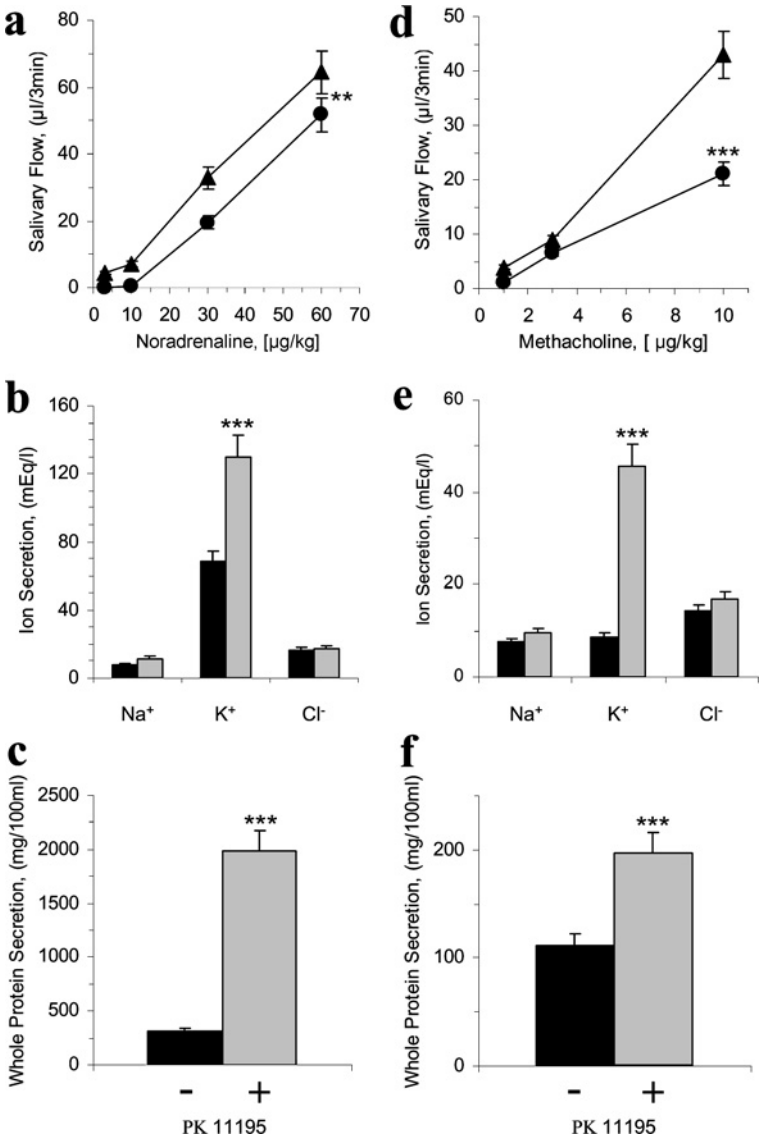
In vivo, rat salivary secretion is enhanced following stimulation of muscarinic, cholinergic and α-adrenergic receptors (Baum, 1987). Stimulation of salivary secretion was dose-dependent and more efficient with methacholine (Figure 6a) than with noradrenaline (Figure 6d). A 20-fold increase in the rate of salivary flow was observed with 10 μg of methacholine/kg of body mass, whereas 30 μg of noradrenaline/kg of body mass was required to simply elicit saliva secretion. In both cases, simultaneous perfusion of 25 nM PK11195 increased the flow rate. PK 11195 alone did not affect salivary secretion. Analysis of the electrolyte composition (i.e. K^+, Na^+ and Cl^-) of the salivary secretion revealed that the K^+ content of the secretion elicited by either 60 μg of noradrenaline/kg of body mass (Figure 6b) or 10 μg of methacholine/kg of body mass (Figure 6e) was increased by PK 11195. PK11195 also increased the protein content of the secretion elicited by noradrenaline (Figure 6c) and methacholine (Figure 6f).

Discussion

The immunohistochemical and radioligand-binding results in the present study demonstrate that the submandibular gland contains high levels of TSPO. In agreement with these results, TSPO ligand-binding sites have been detected in the parotid and submandibular glands of various species, including rat, rabbit, mouse and human (Yamagishi et al., 2000). Furthermore, whole-body autoradiography of neonatal rats using the radiolabelled benzodiazepine Ro5-4864 demonstrated substantial levels of TSPO (Anholt et al., 1985). Nevertheless, these studies did not provide any indications as to the cellular and subcellular localization of TSPO. Our present results clearly demonstrate that TSPO is localized primarily in striated ducts and mucous acini of the submandibular gland, as well as in some of the serous

Figure 6 | Effect of PK 11195 on rat salivary secretion stimulated by noradrenaline or methacholine

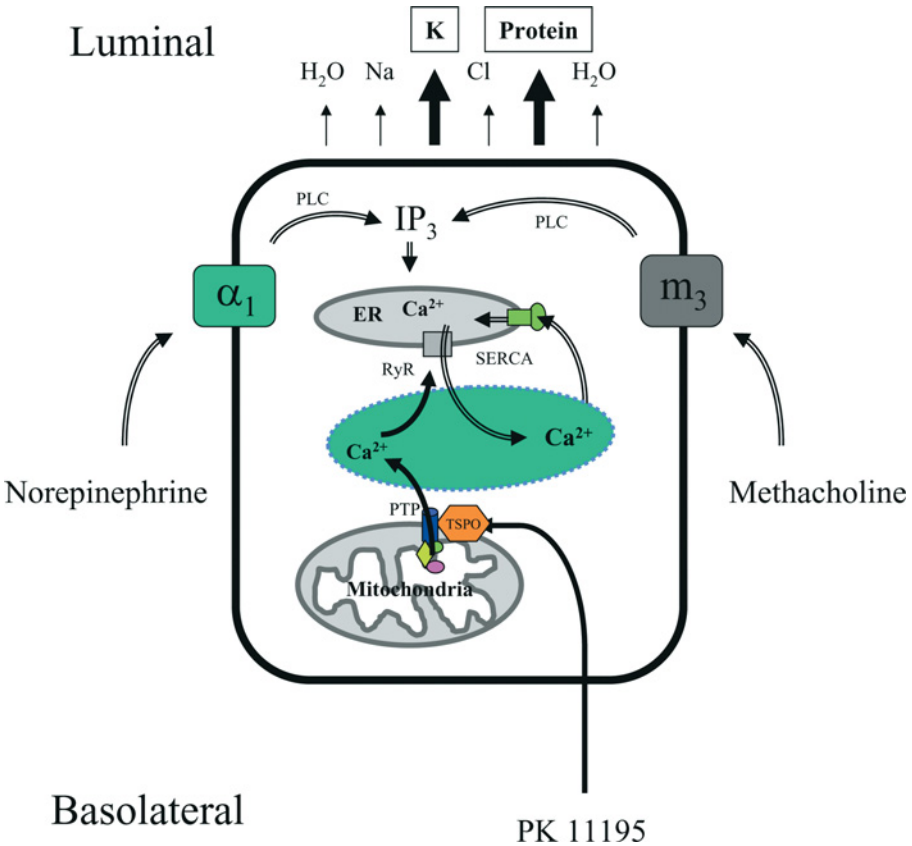
Salivary flow rate as a function of noradrenaline ('Norepinephrine') (**a**) or methacholine (**d**) concentration in the absence (●) or presence (▲) of 25 nM PK 11195. Ion and protein content of salivary secretions elicited by 60 μg of noradrenaline/kg of body mass (**b** and **c**) or 10 μg of methacholine/kg of body mass (**e** and **f**) in the presence (grey bars) or absence (black bars) of PK 11195. $^{**}P < 0.01$; $^{***}P < 0.001$.



acini. These results are in disagreement with a previous report (Perez et al., 1985) that TSPO is present in acinar, but not duct, cells. This discrepancy may be due, in part, to the fact that these results were based on binding experiments with diazepam, which is a mid-affinity TSPO drug ligand that binds to both CBRs and TSPOs. Interestingly, TSPO distribution in the submandibular gland was closely overlapped with the expression of its endogenous polypeptide ligand, DBI. This finding is consistent with previous results showing the importance of DBI and the naturally occurring DBI fragment, ODN (octadecaneuropeptide), in cells expressing TSPO (Ostuni et al., 2004, 2007).

In all of the three types of salivary cells, TSPO co-localized with mitochondria, as revealed by confocal

Figure 7 | Proposed mechanism underlying the synergetic effect of TSPO ligand on saliva secretion
α1, adrenergic receptor; ER, endoplasmic reticulum; IP_3, inositol 1,4,5-triphosphate; Mi, mitochondria; m3, muscarinic receptor type 3; Norepinephrine, noradrenaline; PTP, permeability transition pore; PLC phospholipase C; RyR, ryanodine-sensitive calcium channel; SERCA, sarco-endoplasmic reticulum calcium ATPase.



or electron microscopy and radioligand-binding studies. This finding is in accordance with mitochondrial localization of TSPO in a large majority of other tissues and cells (Papadopoulos et al., 2006), excluding astrocytes and breast cancer cells in which TSPO is localized to the plasma membrane (Itzhak et al., 1993; Ahlo et al., 1994; Hardwick et al., 1999). In a previous study, nuclear and mitochondrial fractions from the submandibular gland displayed diazepam-binding characteristics similar to those observed in the present study and contained 44 % and 51 % of the total binding sites respectively (Perez et al., 1985). These results could be explained by the fact that in salivary cells, especially ducts cells, mitochondria are located mainly in the perinuclear region (Figure 3), and it is usually difficult to obtain nuclear membrane fractions devoid of mitochondrial contamination.

The density of high-affinity TSPO-binding sites in mitochondrial membrane preparations from the submandibular gland is relatively high compared with that in preparations from other tissues, but still lower than in adrenal or testis preparations (Krueger and Papadopoulos, 1990; Oke and Suarez-Quian, 1993; Culty et al., 1999). In the present study, we characterized the pharmacological profile of TSPO in mitochondrial membrane preparations from the submandibular glands and found that these preparations bind PK 11195 and Ro5-4864 with similar affinity, but bind diazepam and PPIX with almost 50-fold lower affinity than PK 11195. Similar findings have been obtained with whole-membrane preparations from both the submandibular and parotid gland (Yamagishi and Kawaguchi, 1998; Yamagishi et al., 2000). In these studies (Yamagishi and Kawaguchi,

1998; Yamagishi et al., 2000), the membrane preparations contained both CBR and TSPO proteins. However, the submandibular glands contain very low amounts of CBR (0.2%, Yamagishi and Kawaguchi, 1998), and thus enrichment in the mitochondrial membrane would not be expected to affect the pharmacological profile of TSPO.

To investigate the function of TSPO in submandibular glands, we examined the effect of PK 11195 on salivary secretion *in vivo*. Saliva is formed by a two-stage process, in which a 'primary fluid' is secreted by the salivary acini and then modified by duct cells to produce saliva (Thaysen et al., 1954; Martinez, 1987; Castle and Castle, 1998). Salivary secretion is controlled by a number of factors, but it occurs mainly in response to parasympathetic and sympathetic stimulation (Schneyer et al., 1972). We observed that PK 11195 alone did not affect salivary secretion. However, PK 11195 enhanced salivary secretion when it was added simultaneously along with noradrenaline or methacholine, both calcium-mobilizing agents. The synergetic action of PK 11195 involved the flow rate and protein secretion, as well as the ionic composition of saliva, indicating that both acini and duct cells are affected by PK 11195. This finding is in agreement with TSPO immunolocalization to both these cells.

The benzodiazepine diazepam has been shown to suppress parotid secretion in dose-dependent and non-competitive manner when injected into rats (Kawaguchi and Yamagishi, 1996). The inhibitory effect of diazepam is thought to be mediated by both CBRs and TSPOs (Kujirai et al., 2002). Various benzodiazepines, such as diazepam, clonazepam, and Ro5-4864, were also able to inhibit amylase secretion induced by isoprenaline or carbachol in a dose-dependent manner (Okubo and Kawaguchi, 1998). In these studies, PK 11195 was used as a TSPO ligand and antagonist, and was able to diminish the inhibitory action of diazepam (Okubo and Kawaguchi, 1998; Kujirai et al., 2002). In parotid acinar cells, diazepam acts through a calcium signalling pathway, as seen by its ability to inhibit IP_3 (inositol 1,4,5-trisphosphate) production and the increases in intracellular calcium induced by carbachol and phenylephrine (Kujirai et al., 2002). More recently, PK 11195 has been shown to induce a transient calcium release in mitochondria in a human colon cell line (Ostuni et al., 2007). These findings support the hypothesis that the high-affinity TSPO ligand PK 11195 activates pathways which induce a rise in intracellular calcium concentrations (Figure 7).

In conclusion, binding of PK 11195 to mitochondrial TSPO may modulate neurotransmitter-stimulated salivary secretion by sensitizing an intracellular calcium pathway which is driven by calcium release in mitochondria (Ostuni et al., 2007) or by increasing the time needed to restore calcium to baseline levels, as demonstrated previously for somatostatin (Ostuni et al., 2003). Regardless, the use of PK 11195 may be an interesting approach for the treatment of xerostomy, especially benzodiazepine-induced dry mouth syndrome.

Materials and methods

Animals

Adult male Wistar rats weighing 250–300 g (Iffa-Credo, L'Arbresle, France) were housed under standard laboratory conditions (12 h light/12 h dark cycle at 21–23°C) in individual mesh-wire cages to prevent coprophagia. Animals were given free access to standard laboratory chow and water. They were killed by cervical dislocation for immunohistochemistry experiments or by anaesthesia overdose after *in vivo* experiments. All animals were treated in accordance with European Committee guidelines concerning the care and use of laboratory animals.

Electrophoresis and Western blotting

Proteins were separated by SDS/PAGE under reducing condition. They were transferred on to nitrocellulose membranes (0.45 μm pore size; Sartorius AG, Göttingen, Germany), which were then incubated in with a rabbit anti-TSPO polyclonal antibody (1:1000), raised against amino acids 9–27 of TSPO (Li et al., 2001). The protein bands were revealed using an anti-rabbit peroxidase-coupled secondary antibody (Sigma–Aldrich, Saint Quentin, France) and a chemiluminescence kit (PerkinElmer, Courtaboeuf, France).

Immunohistochemistry

Submandibular glands from male Wistar rats were removed and stripped of all connective tissue. They were cut in small pieces and immediately fixed in 4% paraformaldehyde for 4 h at room temperature (25°C), and then embedded in paraffin. Paraffin sections were rehydrated and endogenous peroxidase activity was quenched. Sections were then immunostained using the PAP (peroxidase anti-peroxidase) technique (Sternberger, 1975). Sections were incubated overnight at 4°C with a rabbit anti-TSPO polyclonal antibody (1:100), raised against amino acids 9–27 of TSPO which are conserved across species (Li et al., 2001), as described previously (Ostuni et al., 2004; 2007). Peroxidase-conjugated secondary antibody was purchased from Dakopatts (Trappes, France). Sections were counterstained with Mayer's haematoxylin (EMS, Hatfield, PA, U.S.A.). The specificity of the immunohistochemical signals was confirmed by performing staining in the absence of the primary antibody and with immuno-absorbed primary antibody (8 μg of recombinant

18 kDa TSPO proteins/ml of diluted antibody). DBI immunolocalization was investigated using an anti-(bovine DBI) antibody, as described previously (Ostuni et al., 2004).

Paraffin-embedded sections for confocal microscopy were rehydrated and subjected to dual immunofluorescent staining for TSPO and VDAC (voltage-dependent anion channel). Briefly, sections were incubated for 20 min with goat serum and incubated overnight at 4°C with rabbit anti-TSPO antibody (1:120). Sections were then incubated with Alexa Fluor® 488-coupled anti-rabbit antibody (Molecular Probes, Eugene, OR, U.S.A.) for 60 min (1:200) and, after washing, incubated with mouse anti-VDAC antibody (1:500; Molecular Probes). Following a 60 min incubation in Alexa Fluor® 546-coupled anti-mouse antibody (1:150; Molecular Probes), sections were washed and mounted in a hydrophilic anti-fade medium (Fluoromount G; EMS). Control staining was carried out by omitting the primary antibody. Staining was visualized with a Zeiss LSM 510 confocal microscope using a ×63 oil immersion lens. The fluorescence emitted was measured at 514 nm for Alexa Fluor® 488 and 622 nm for Alexa Fluor® 546. Images were deconvolved using a step-size of 0.4 μm and a pinhole value fixed at 1 μm. For image analysis, LSM 510 image software (Zeiss) was used to select specific fields (with identical intensity profiles for each fluorochrome) and to collect the results (histograms, fluorograms and dye co-localization).

Ultrastructure analysis

Submandibular glands pieces were fixed in 2.5 % glutaraldehyde for 60 min at room temperature and post-fixed in 1 % osmium tetroxide for 60 min. Tissue was then dehydrated and embedded in LX-112 resin. Sections were contrasted with 2 % uranyl acetate and observed with a JEOL 1200 EX electron microscope, operated at 80 kV accelerated voltage.

Post-embedding immunocytochemistry (gold labelling)

Pieces of submandibular glands were fixed in a mixture of 4 % paraformaldehyde and 0.2 % glutaraldehyde for 2 h at room temperature. After tissue dehydratation, it was embedded in LX-112 resin and cut with an ultramicrotome (Ultracut, Reichert-Jung). Sections were placed on to nickel grids and processed according to the manufacturer's instructions (Aurion, Wageningen, The Netherlands) for gold labelling. The dilution of the primary antibody was 1:160 and incubation was performed at 4°C overnight. The dilution of gold conjugate reagent was 1:30 and incubation was performed at room temperature for 2 h. Observations were made in the same conditions as described above.

Radioligand binding

Mitochondria-enriched fractions were obtained by differential centrifugation. Briefly, homogenization of submandibular glands give the whole-homogenate preparation, then the nuclear fraction was collected in the first pellet (600 *g* for 10 min) and the mitochondria-enriched fraction in the second pellet (10 000 *g* for 20 min), whereas the post-mitochondrial fraction was in the supernatant. ^{3}H-labelled PK 11195 binding to cellular membranes was carried out as described previously (Ostuni et al., 2004, 2007). The K_d and B_{max} values for PK 11195 were determined by analysis of the saturation isotherms using the following equation, where Y is the bound ligand:

$$Y = B_{max} \times [PK11195]^{b} / (K_d^{h} \times [PK11195]^{h})$$

A b (Hill coefficient) value of 1 ± 0.1 was obtained. Drug-displacement experiments were performed with membranes incubated in the presence of a constant radioligand concentration (3 nM ^{3}H-labelled PK 11195) and various concentrations of unlabelled ligand (0–30 μM). IC_{50} values for the various ligands were determined by curve fitting using the following equation, where S is the unlabelled concentration:

$$Y = 100 \times (IC_{50})^{nH} / (IC_{50}^{nH} \times S^{nH})$$

A b value of 1 ± 0.1 was obtained in all fittings. Protein concentrations were quantified using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) with BSA as a standard.

In vivo experiments

Rats were anaesthetized with urethane (1.2 g/kg of body mass; intraperitoneally) after an overnight fast for 12 h. The ducts of both submandibular glands were exposed and cannulated with fine glass cannulae (Ohlin and Perec, 1965). Secretion was induced by injection of methacholine (1, 3 or 10 μg/kg of body mass) or noradrenaline (3, 10, 30 or 60 μg/kg of body mass) through the femoral vein. Before each injection (15 min), the other femoral vein was infused with PK 11195 (25 nM) or vehicle (0.9 % NaCl and 0.1 % ethanol) at the same infusion rate. The saliva secreted was collected for 3 min and weighed. Samples were then stored at −18°C until quantification of total protein was carried out using the phenol reagent method (Lowry et al., 1951) or K^{+}, Na^{+} and Cl^{-} concentrations were determined using an electrolyte analyser (AVL910 electrolyte analyzer, AVL Scientific, Roswell, GA, U.S.A.).

Statistics

Results are expressed as the means ± S.E.M. Group means were compared using a one-way ANOVA or a Student's t test. $P < 0.05$ was considered significant.

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References

Alho, H., Varga, V. and Krueger, K.E. (1994) Expression of mitochondrial benzodiazepine receptor and its putative endogenous ligand diazepam binding inhibitor in cultured primary astrocytes and C-6 cells: relation to cell growth. Cell Growth Differ. **5**, 1005–1014

Anholt, R.R., De Souza, E.B., Oster-Granite, L. and Snyder, S.H. (1985) Peripheral-type benzodiazepine receptors: autoradiographic localization in whole-body sections of neonatal rats. J. Pharmacol. Exp. Ther. **233**, 517–526

Basile, A.S., Lueddens, H.W. and Skolnick, P. (1988) Regulation of renal peripheral benzodiazepine receptors by anion transport inhibitors. Life Sci. **42**, 715–726

Baum, B.J. (1987) Neurotransmitter control of secretion. J. Dent. Res. **66**, 628–632

Beinlich, A., Strohmeier, R., Kaufmann, M. and Kuhl, H. (2000) Relation of cell proliferation to expression of peripheral benzodiazepine receptors in human breast cancer cell lines. Biochem. Pharmacol. **60**, 397–402

Beurdeley-Thomas, A., Miccoli, L., Oudard, S., Dutrillaux, B. and Poupon, M.F. (2000) The peripheral benzodiazepine receptors: a review. J. Neurooncol. **46**, 45–56

Braestrup, C. and Squires, R.F. (1977) Specific benzodiazepine receptors in rat brain characterized by high-affinity (^{3}H)diazepam binding. Proc. Natl. Acad. Sci. U.S.A. **74**, 3805–3809

Casellas, P., Galiegue, S. and Basile, A. S. (2002) Peripheral benzodiazepine receptors and mitochondrial function. Neurochem. Int. **40**, 475–486

Castle, D. and Castle, A. (1998) Intracellular transport and secretion of salivary proteins. Crit. Rev. Oral Biol. Med. **9**, 4–22

Costa, B., Salvetti, A., Rossi, L., Spinetti, F., Lena, A., Chelli, B., Rechichi, M., Da Pozzo, E., Gremigni, V. and Martini, C. (2006) Peripheral benzodiazepine receptor: characterization in human T-lymphoma Jurkat cells. Mol. Pharmacol. **69**, 37–44

Culty, M., Li, H., Boujrad, N., Amri, H., Vidic, B., Bernassau, J.M., Reversat, J.L. and Papadopoulos, V. (1999) *In vitro* studies on the role of the peripheral-type benzodiazepine receptor in steroidogenesis. J. Steroid Biochem. Mol. Biol. **69**, 123–130

De Souza, E.B., Anholt, R.R., Murphy, K.M., Snyder, S.H. and Kuhar, M.J. (1985) Peripheral-type benzodiazepine receptors in endocrine organs: autoradiographic localization in rat pituitary, adrenal, and testis. Endocrinology **116**, 567–573

Gandolfo, P., Louiset, E., Patte, C., Leprince, J., Masmoudi, O., Malagon, M., Gracia-Navarro, F., Vaudry, H. and Tonon, M.C. (2001) The triakontatetraneuropeptide TTN increases $[Ca^{2+}]_i$ in rat astrocytes through activation of peripheral-type benzodiazepine receptors. Glia **35**, 90–100

Gavish, M., Bachman, I., Shoukrun, R., Katz, Y., Veenman, L., Weisinger, G. and Weizman, A. (1999) Enigma of the peripheral benzodiazepine receptor. Pharmacol. Rev. **51**, 629–650

Hardwick, M., Fertikh, D., Culty, M., Li, H., Vidic, B. and Papadopoulos, V. (1999) Peripheral-type benzodiazepine receptor (PBR) in human breast cancer: correlation of breast cancer cell aggressive phenotype with PBR expression, nuclear localization, and PBR-mediated cell proliferation and nuclear transport of cholesterol. Cancer Res. **59**, 831–842

Hirsch, T., Decaudin, D., Susin, S.A., Marchetti, P., Larochette, N., Resche-Rigon, M. and Kroemer, G. (1998) PK11195, a ligand of the mitochondrial benzodiazepine receptor, facilitates the induction of apoptosis and reverses Bcl-2-mediated cytoprotection. Exp. Cell Res. **241**, 426–434

Itzhak, Y., Baker, L. and Norenberg, M.D. (1993) Characterization of the peripheral-type benzodiazepine receptors in cultured astrocytes: evidence for multiplicity. Glia **9**, 211–218

Kawaguchi, M. and Yamagishi, H. (1996) Coupling of benzodiazepines and $GABA_A$ receptors in the salivary glands is a factor of drug-induced xerostomia. Int. Acad. Biomed. Drug Res. **11**, 291–296

Kuhlmann, A.C. and Guilarte, T.R. (2000) Cellular and subcellular localization of peripheral benzodiazepine receptors after trimethyltin neurotoxicity. J. Neurochem. **74**, 1694–1704

Krueger, K.E. and Papadopoulos, V. (1990) Peripheral-type benzodiazepine receptors mediate translocation of cholesterol from outer to inner mitochondrial membranes in adrenocortical cells. J. Biol. Chem. **265**, 15015–15022

Kujirai, M., Sawaki, K. and Kawaguchi, M. (2002) Inhibitory effect of diazepam on muscarinic receptor-stimulated inositol 1,4,5-trisphosphate production in rat parotid acinar cells. Br. J. Pharmacol. **137**, 945–952

Lacapere, J.J. and Papadopoulos, V. (2003) Peripheral-type benzodiazepine receptor: structure and function of a cholesterol-binding protein in steroid and bile acid biosynthesis. Steroids **68**, 569–585

Li, H., Yao, Z., Degenhardt, B., Teper, G. and Papadopoulos, V. (2001) Cholesterol binding at the cholesterol recognition/interaction amino acid consensus (CRAC) of the peripheral-type benzodiazepine receptor and inhibition of steroidogenesis by an HIV TAT-CRAC peptide. Proc. Natl. Acad. Sci. U.S.A. **98**, 1267–1272

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275

Martinez, J.R. (1987) Ion transport and water movement. J. Dent. Res. **66**, 638–647

Nayyar, T., Mukherjee, S. and Das, S.K. (1996) Modulation of binding characteristics of peripheral benzodiazepine receptors in vitamin A-deficient guinea pig lung. Biochem. Pharmacol. **51**, 1203–1209

O'Beirne, G.B., Woods, M.J. and Williams, D.C. (1990) Two subcellular locations for peripheral-type benzodiazepine acceptors in rat liver. Eur. J. Biochem. **188**, 131–138

Ohlin, P. and Perec, C. (1965) Salivary secretion of the major sublingual gland of rats. Experientia **21**, 408–409

Oke, B.O. and Suarez-Quian, C.A. (1993) Localization of secretory, membrane-associated and cytoskeletal proteins in rat testis using an improved immunocytochemical protocol that employs polyester wax. Biol. Reprod. **48**, 621–631

Okubo, M. and Kawaguchi, M. (1998) Inhibitory regulation of amylase release in rat parotid acinar cells by benzodiazepine receptors. Eur. J. Pharmacol. **359**, 243–249

Ostuni, M.A., Ferrero, A.J., Bereciartu, A., Houssay, A.B. and Tumilasci, O.R. (2003) Modulation by somatostatin of rat submandibular salivary secretion. Arch. Oral Biol. **48**, 205–212

Ostuni, M.A., Marazova, K., Peranzi, G., Vidic, B., Papadopoulos, V., Ducroc, R. and Lacapere, J.J. (2004) Functional characterization and expression of PBR in rat gastric mucosa: stimulation of chloride secretion by PBR ligands. Am. J. Physiol. Gastrointest. Liver Physiol. **286**, G1069–G1080

Ostuni, M.A., Ducroc, R., Peranzi, G., Tonon, M.C., Papadopoulos, V. and Lacapere, J.J. (2007) Translocator protein (18 kDa) ligand PK 11195 induces transient mitochondrial Ca^{2+} release leading to transepithelial Cl^- secretion in HT-29 human colon cancer cells. Biol. Cell. **99**, 639–647

Papadopoulos, V. (1993) Peripheral-type benzodiazepine/diazepam binding inhibitor receptor: biological role in steroidogenic cell function. Endocr. Rev. **14**, 222–240

Papadopoulos, V., Baraldi, M., Guilarte, T.R., Knudsen, T.B., Lacapere, J.J., Lindemann, P., Norenberg, M.D., Nutt, D., Weizman, A., Zhang, M.R. and Gavish, M. (2006) Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol. Sci. **27**, 402–409

Perez, C., Cruciani, R., Rubio, M.C. and Stefano, F.J. (1985) Benzodiazepine binding sites in rat submaxillary gland: absence of markers of GABA system. Comp. Biochem. Physiol. C **82**, 451–456

Piludu, M., Rayment, S.A., Liu, B., Offner, G.D., Oppenheim, F.G., Troxler, R.F. and Hand, A.R. (2003) Electron microscopic immunogold localization of salivary mucins MG1 and MG2 in human submandibular and sublingual glands. J. Histochem. Cytochem. **51**, 69–79

Schneyer, L.H., Young, J.A. and Schneyer, C.A. (1972) Salivary secretion of electrolytes. Physiol. Rev. **52**, 720–777

Scully, C. (2003) Drug effects on salivary glands: dry mouth. Oral Dis. **9**, 165–176

Sternberger, L.A. (1975) Immunocytochemistry, Prentice Hall, New Jersey

Suzuki, S., Mifune, H., Kamimura, R., Yabuki, A., Obara, T., Matsumoto, M. and Tsuchiya, K. (2003) Fine structure of the parotid and mandibular glands of the cotton rat (*Sigmodon hispidus*). Exp. Anim. **52**, 441–444

Taketani, S., Kohno, H., Furukawa, T. and Tokunaga, R. (1995) Involvement of peripheral-type benzodiazepine receptors in the intracellular transport of heme and porphyrins. J. Biochem. (Tokyo) **117**, 875–880

Thaysen, J.H., Thorn, N.A. and Schwartz, I.L. (1954) Excretion of sodium, potassium, chloride and carbon dioxide in human parotid saliva. Am. J. Physiol **178**, 155–159

Yamagishi, H. and Kawaguchi, M. (1998) Characterization of central- and peripheral-type benzodiazepine receptors in rat salivary glands. Biochem. Pharmacol. **55**, 209–214

Yamagishi, H., Watanabe, M., Yazaki, K., Sawaki, K. and Kawaguchi, M. (2000) Pharmacological characterization of an 18-kDa protein associated with the peripheral-type benzodiazepine receptor in salivary glands. Jpn. J. Pharmacol. **82**, 110–115

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