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ORIGINAL ARTICLE

Rat β_3-adrenoceptor protein expression: antibody validation and distribution in rat gastrointestinal and urogenital tissues

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Abstract β_3-Adrenoceptors play important roles in the regulation of urogenital and probably gastrointestinal function. However, despite recent progress, their detection at the protein level has remained difficult due to a lack of sufficiently validated selective antibodies. Therefore, we have explored the selectivity of two antibodies for the detection of rodent β_3-adrenoceptors in immunoblots and immunohistochemistry. Of two reportedly promising candidates, antibody AB15688 did not exhibit subtype selectivity in immunoblots. In contrast, the antibody Sc1473 exhibited at least some selectivity in immunoblots and more promising results in immunocytochemical and immunohistochemical stains in cells transfected with cloned β-adrenoceptor subtypes and in rat and mouse tissues. In a systematic screening of rat gastrointestinal and urogenital tissues, Sc1473 produced selective staining in the epithelial cell lining of the stomach and the urothelium of ureter and bladder. We conclude that the two tested antibodies are inappropriate or at least insufficient for immunoblotting applications, but Sc1473 appears to be useful for immunohistochemical detection of β_3-adrenoceptor protein in rodent tissues. The β_3-adrenoceptor protein exhibits a distinct expression pattern in the rat gastrointestinal and urogenital tract, which is at least partly in line with previously reported functional data.

Keywords β_3-Adrenoceptors · Antibody AB15688 · Antibody Sc1473 · Bladder · Gut · Ureter

Introduction

β_3-Adrenoceptor agonists have recently been shown to be effective in the treatment of patients with overactive bladder syndrome (Ohlstein et al. 2012; Chapple et al. 2014). For a better understanding of their potential desirable and adverse effects, a comprehensive evaluation of the β_3-adrenoceptor expression pattern at the protein level is desirable but until now remains ill-defined (Michel et al. 2010). The only human tissue in which a large body of data exists on β_3-adrenoceptors is the target tissue for the clinically used compounds, i.e. the urinary bladder, and even in the bladder, most of the expression information is inferred from functional studies (Michel and Vrydag 2006; Igawa et al. 2012).

Progress in the identification of β_3-adrenoceptor protein expression is hampered by lack of suitable tools such as radioligands or antibodies (Vrydag and Michel 2007). Specifically, it has been shown that many antibodies against β_3-adrenoceptors (Pradidarcheep et al. 2009; Cernecka et al. 2012), other β-adrenoceptor subtypes (Hamdani and van der Velden 2009) or other G-protein-coupled receptors in general (Pradidarcheep et al. 2008; Michel et al. 2009; Adams et al. 2012; Beermann et al. 2012; Neumann et al. 2012; Yu and Hill 2013; Cecyre et al. 2014; Talmont and Mouledous 2014;

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Böhmer et al. 2014), in many, if not most cases, lack selectivity for their cognate receptors when tested based on stringent criteria. A well-validated monoclonal antibody against β_3-adrenoceptors (Chamberlain et al. 1999) unfortunately never became available commercially and no longer is available from the original investigators.

For human β_3-adrenoceptors, recent efforts have identified two antibodies which, at least to some degree, appear suitable for selective labelling (Limberg et al. 2010; Cernecka et al. 2012). For rodent β_3-adrenoceptors, the antibody AB15688 was proposed to be selective based on immunocytochemical experiments in transiently transfected Chinese hamster ovary (CHO) cells (Kullmann et al. 2009). Moreover, the antibody Sc1473 has been reported to yield a specific band in mouse heart, which was absent in β_3-adrenoceptor knockout mice (Bundgaard et al. 2010). As the latter antibody had been found to be invalid for use with human β_3-adrenoceptors (Pradidarcheep et al. 2009), we hypothesized that β_3-adrenoceptor selectivity may be species-dependent, similar to that of some small molecule ligands at this receptor (Nahmias et al. 1991; Liggett 1992; van Wieringen et al. 2013). Therefore, the present study was designed to further evaluate the target selectivity of AB15688 and Sc1473 for the rodent β_3-adrenoceptor. As Sc1473 proved more promising in these experiments, we have used it to systematically explore the tissue expression pattern of β_3-adrenoceptors in the gastrointestinal and urogenital tract of the rat.

Material and methods

Cell lines and tissue sources

Chinese hamster ovary (CHO) cells stably transfected with rat β_1-, β_2- or β_3-adrenoceptors at presumed physiological levels (150, 630 and 200 fmol/mg protein, respectively) (Hatanaka et al. 2013) were kindly provided by Dr. Masahi Ukai (Astellas, Tsukuba, Japan) and cultured in Ham's F-12 medium containing 10 % (*v*/*v*) foetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin and 0.2 mg/ml geneticin. In some cases, CHO cells stably transfected with the human β-adrenoceptor subtypes were used, which had been obtained from Dr. Carsten Hoffman (University of Würzburg, Germany) and cultured similarly (Niclauß et al. 2006).

Confluent 15-cm dishes of CHO cells expressing β-adrenoceptors were washed with phosphate-buffered saline (PBS) and harvested by scraping. Cells were collected by centrifugation and homogenized with an Ultra-Turrax T8 (IKA Labortechnik, Staufen, Germany). Cell membranes were collected by a further centrifugation at 50,000*g* for 20 min at 4 °C and resuspended by homogenization in 10 mM Tris-HCl, 1.5 mM EDTA and 0.5 mM dithiothreitol (TED) buffer (Sigma-Aldrich, Zwijndrecht, The Netherlands), pH 8.0).

Heart, gall bladder and brown adipose tissue from FVB and β_3-adrenoceptor knockout mice (six males, three females each, 6–7 weeks old) as originally generated by Susulic et al. (1995) were provided by Dr. Roger Summers (Monash University, Melbourne, Australia). Other tissue for the validation studies was from male C57/BL/6C mice (body weight approximately 28 g) and from male Wistar rats (strain HsdCpb:WU, body weight approximately 250 g), both obtained from Harlan (Boxmeer, The Netherlands). Tissues for the mapping in gastrointestinal and urogenital tract were obtained from Wistar rats (six males, four females, 4–5 weeks old) obtained from the animal facility at the Academic Medical Center) and fed ad libitum. Animals were killed by instant decapitation under an O_2/CO_2 daze.

Immunoblotting

Frozen mouse and rat tissues were homogenized in buffer containing 150 mM NaCl, 0.1 % (*v*/*v*) Triton X-100, 50 mM Tris-HCl (pH 8.0) and protease inhibitor cocktail kit 100× (#78410; Thermo Scientific, Delft, The Netherlands). The homogenate was centrifuged at 8,800*g* for 10 min and the supernatant taken. The total protein concentration was determined according to Bradford (Bradford 1976). Membrane fractions from transfected CHO cells were prepared as reported previously (Cernecka et al. 2012).

Each sample containing 20, 30, 50 or 100 μg of total protein was dissolved in 5× Laemmli buffer (Laemmli 1970), boiled for 5 min at 95 °C, separated by 4–12 % (*w*/*v*) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blotting, we controlled protein transfer using Ponceau red staining of the membrane. Immunoblots were blocked with 5 % (*w*/*v*) non-fat dry milk in Tris-buffered saline (TBS) containing 0.1 % (*v*/*v*) Tween 20 for 2 h at room temperature. Then, the immunoblots were incubated overnight at 4 °C with TTBS with 5 % (*w*/*v*) milk containing the primary antibody Sc1473 (batch #B2411, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:100 or AB15688 (batch PSO 1443466, Chemicon International, Temecula, CA, USA) at a dilution 1:2000. Proteins were visualized after 1 h of incubation with horseradish peroxidase-conjugated rabbit antigoat (1:5000; Sigma-Aldrich) or rabbit antichicken secondary antibody (1:5000; Sigma-Aldrich) in Tween-containing TBS with 5 % (*w*/*v*) milk, respectively, followed by chemiluminescent imaging (Lumi-Light^{PLUS} Western Blotting Substrate, #12015196001, Roche Applied Science, Almere, The Netherlands).

Immunocytochemistry

CHO cells were placed in 16- or 96-well plates coated with collagen IV (Sigma-Aldrich). Cells were fixed with 3.6 % (*v*/*v*) formaldehyde in PBS at room temperature for 5 min and then permeabilized with 0.1 % (*v*/*v*) Triton X-100 (Sigma-Aldrich) in PBS for 10 min; dehydrated through 30, 50 and 70 % (*v*/*v*) ethanol; and rinsed with 3 % (*v*/*v*) H_2O_2 in PBS, respectively. Subsequently, the cells were blocked with 1 % (*w*/*v*) bovine serum albumin (BSA) in PBS for 30 min and incubated overnight at 4 °C with primary antibody Sc1473 (1:200) in PBS containing 0.1 % (*w*/*v*) BSA. Next, after being washed with PBS, the cells were incubated with alkaline phosphatase-conjugated rabbit antigoat secondary antibody (1:50; Sigma-Aldrich) for 1 h at room temperature. Staining of alkaline phosphatase-conjugated secondary antibody was developed with NBT/BCIP Stock Solution (Roche Applied Science, Mannheim, Germany) in 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl and 0.05-M $MgCl_2$ solution, which produced a dark-blue staining. Images were taken at ×10 magnification using a Nikon Eclipse TE2000-U fluorescence microscope (Plan Fluor ELWD ×20 objective, Nikon DXM1200F digital camera) with NIS Elements AR 2.30 software.

Immunohistochemistry

For the validation experiments, frozen mouse and rat tissues were sectioned at 7 μm. Tissue slides were fixed with 3.6 % (*v*/*v*) formaldehyde. After 5 min of fixation, the sections were permeabilized with 0.1 % (*v*/*v*) Triton X-100 in TBS for 10 min. Subsequently, endogenous peroxidases were blocked by incubating the sections in 1 % (*v*/*v*) H_2O_2 in TBS for 20 min. Next, the slides were blocked with 10 % (*v*/*v*) normal donkey serum in TBS for 30 min and incubated overnight at 4 °C with primary antibody Sc1473 in TBS containing 1 % (*w*/*v*) BSA. Different dilutions for each tissue were tested. After being washed with TBS, the tissue slides were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Staining of horseradish peroxidase-conjugated secondary antibody (antigoat, 1:100, Jackson ImmunoResearch, Newmarket, Suffolk, UK) was developed with 3,3′-diaminobenzidine (DAB) solution (0.25 g Tris, 0.06 g Na_2HPO_4, 0.0125 g NaCl, pH 7.8, 30 mg DAB in 30 ml Tris buffer), which produced a dark-brown staining. Afterwards, counterstaining with haematoxylin was performed. Images were taken at ×10 magnification. Staining with haematoxylin/eosin of all tissues was also performed.

For the studies systematically assessing β_3-adrenoceptor expression in rat gastrointestinal and urogenital tissues, immunohistochemistry was performed as previously described (Pradidarcheep et al. 2008). The digestive and urinary tracts were removed en bloc to retain the topographical relations. The specimens were fixed overnight by immersion in freshly prepared 4 % (*v*/*v*) formaldehyde in PBS at room temperature, dehydrated in a graded series of ethanol and embedded in paraplast. Serial 7-μm-thick sections were mounted on poly-L-lysine-coated slides and stored at 4 °C. For immunohistochemical analysis, the sections were deparaffinized in xylene, rehydrated in graded ethanol and washed in PBS. The sections were then autoclaved in 10 mM sodium citrate, pH 6.0, for 10 min to retrieve antigens and to inactivate endogenous alkaline phosphatase; cooled down at room temperature for at least 15 min; washed in PBS; blocked in 10 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25 % (*w*/*v*) gelatin and 0.05 % (*v*/*v*) Tween 20, pH 8.0 (TENG-T), containing either 10 % (*v*/*v*) normal goat, foetal calf or rabbit serum, for 30 min in a moist incubation chamber; incubated overnight (without prior washing) at room temperature with the Sc1473 antiserum dissolved in the blocking solution; washed three times in a mixture of PBS and 0.5 M sodium acetate to reduce non-specific binding; and incubated for 2 h at room temperature with alkaline phosphatase-conjugated goat antirabbit IgG (Dako Inc., Glostrup, Denmark). After incubation, the sections were rewashed once more as described. To reveal antibody binding, the sections were incubated with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (toluidine salt; Dako Inc. Glostrup, Denmark) diluted in 100 mM Tris, pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$ at room temperature. Antibody concentrations and staining times were chosen to assure a linear relation between antibody binding and staining intensity, and care was taken in that the maximum absorbance of the stain did not exceed an optical density of 0.8. After the reaction was stopped in bidistilled water, the sections were quickly dehydrated through an ascending series of graded ethanol, cleared in xylene and mounted in Entellan (Merck).

Quantitative polymerase chain reaction

Total RNA was isolated from individual rat tissues (colon, gut, urinary bladder, brown adipose tissue, lung and heart) and wild-type and β_3-adrenoceptor knockout mouse tissues (brown adipose tissue, gut and heart) using TRIzol reagent. Reverse transcription to cDNA was performed using Reverse Transcription System (#A3500, Promega, Leiden, The Netherlands); 1 μg RNA was used per reaction. Real-time PCR was performed with a MyiQ™ Single-Color detection system (Bio-Rad Laboratories Inc. Life Science Group, Hercules, CA, USA). The sequences of the primers are listed in the Table 1. Ribosomal protein S18 (18S rRNA) was used as a reference gene. Real-time PCR data were analyzed using the comparative cycle threshold (C_t, amplification cycle number) method; relative differences in gene expression between tissues are reported as $\Delta\Delta C_t$ values.

Table 1 Primer sequences used in RT-PCR and genotyping experiments

Target name	Primer	Sequence (5′-3′)	Product size
Mouse β_3-adrenoceptor			
	Forward	CGCCTTCCGTCGTCTTCTGTGTAG	178
	Reverse	GCTTCCTTGCTGGATCTTCACGGC	
Rat ribosomal subunit 18S			
	Forward	TGGGCGGCGGAAAATAGCCTT	271
	Reverse	CAGGTCCTCACGCAGCTTGTTGT	
Rat β_3-adrenoceptor			
	Forward	TTCCCAGCTAGCCCTGTTGCG	223
	Reverse	GCGGTTCCTGGGAACTCACTCTGA	
Neomycin cassette			
	Forward	CTGTCCGGTGCCCTG AAT	450
	Reverse	GATATTCGGCAAGCAGGCAT	

Genotyping by polymerase chain reaction

Tissue samples from β_3-adrenoceptor knockout and wild-type mice (gut and brown adipose tissue) were dissolved overnight at 55 °C in 300 μl of lysis buffer containing 100 mM Tris-HCl, pH 8.5; 5 mM Na_2EDTA, pH 8.0; 0.2 % (*w*/*v*) SDS; 200 mM NaCl and 100 μg/ml proteinase K. After dissolving, we added 300 μl isopropanol and mixed until precipitation was completed. Afterwards, the whole solution was centrifuged at 8,800*g* for 15 min. After supernatant removal, pellet was washed with 70 % (*v*/*v*) ethanol and dissolved in TE buffer. Samples were analyzed by PCR amplifying a 450-bp fragment of neomycin cassette using primers described in Table 1.

Results

AB15688 antibody evaluation

In membranes from mouse heart, AB15688 labelled a major band with an apparent molecular weight of 44 kDa, which was also the most prominent band (Fig. 1). This band was also seen in membranes from CHO cells expressing rat β_3-adrenoceptors and, much weaker, in those from CHO cells containing rat β_1- or β_2-adrenoceptors despite a similar protein loading (Fig. 1); however, the 44-kDa band was only one of many bands in membranes from CHO cells expressing any of the three receptors and, actually, even in the β_3-adrenoceptor expressing cells, one of the weaker bands.

In membrane preparations from various rat tissues, AB15688 also detected a band at 44 kDa, but in contrast to the murine heart and similar to CHO cells, this was only one of many bands, many of which were more prominent than the 44-kDa band (Fig. 1). The intensity of the 44-kDa band in rat tissues exhibited an apparent rank order of gut > brown adipose tissue > colon > urinary bladder > heart. In contrast, AB15688 did not label any specific band in membranes prepared from CHO cells transfected with the human β_3-adrenoceptor as compared to human β_1- or β_2-adrenoceptors (data not shown), indicating selectivity of the 44-kDa band for the rodent over the human receptor.

Sc1473 antibody evaluation

Confirming a previous report (Bundgaard et al. 2010), Sc1473 labelled a major band with an apparent molecular weight of 44 kDa in membranes from mouse heart, and this was the most prominent band detected in such membranes (Fig. 1). Sc1473 also labelled a 44-kDa band in membranes from CHO cells expressing rat β_3-adrenoceptors, while this was neither the only nor the most prominent band observed in those blots, it was clearly discernable, and the number and relative intensity of other bands were less than observed with AB15688 (Fig. 1). The 44-kDa band was not observed in membranes from CHO cells expressing rat β_1- or β_2-adrenoceptors despite a similar protein loading, whereas most other bands had a similar intensity in membranes from CHO cells expressing all three β-adrenoceptor subtypes (Fig. 1). In contrast and in confirmation of our previous findings (Pradidarcheep et al. 2009), Sc1473 did not label a 44 kDa or any other specific band in membranes prepared from CHO cells transfected with the human β_3-adrenoceptor as compared to human β_1- or β_2-adrenoceptors (data not shown). In rat tissues, Sc1473 antibody also detected a 44-kDa band, while it was only weak in urinary bladder and brown adipose tissue (Fig. 1), the labelling pattern of Sc1473 in rat tissues appeared to be more specific than with AB15688. In immunocytochemical experiments with CHO cells expressing the rat β-adrenoceptor subtypes, Sc1473 showed a high level of staining in CHO cells expressing rat β_3-adrenoceptors, whereas only little staining was observed in CHO cells expressing β_1- or β_2-adrenoceptors (Fig. 2), despite the very intensely staining common bands in immunoblots of membranes of the same cells (Fig. 1).

In reverse transcription (RT)-PCR experiments with rat tissues, β_3-adrenoceptor messenger RNA (mRNA) was detected in brown adipose tissue (BAT), urinary bladder, colon, gut and heart (C_t values of 24.8, 26.5, 28.3, 28.6 and 29.5, respectively); in contrast, much less, if any, β_3-adrenoceptor mRNA was detected in rat lung (C_t value 35.4), i.e. only 1/1552 of values in BAT, in line with previous reports that rat lung lacks β_3-adrenoceptor mRNA (Muzzin et al. 1991) or protein expression (Schneider and Michel 2010). Accordingly, Sc1473 did not yield detectable staining in rat lung in

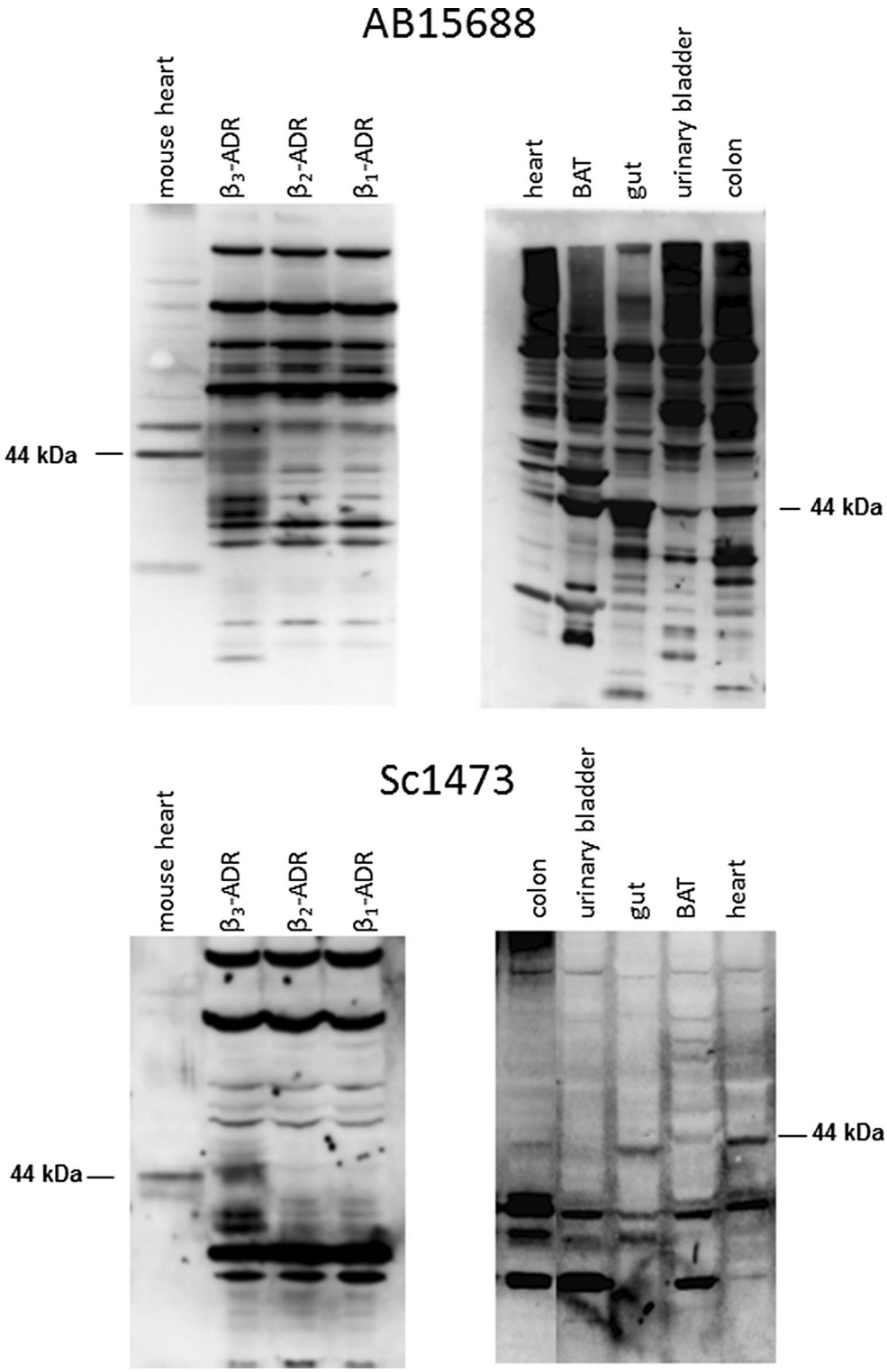

Fig. 1 Immunoblots with AB15688 (*upper panels*) or Sc1473 (*lower panels*) in membranes from CHO cells transfected with rat β_1-, β_2- or β_3-adrenoceptors and mouse heart (*left panels*) or in heart, brown adipose tissue (BAT), gut, urinary bladder and colon of the rat (*right panels*). Similar findings were obtained in at least one additional experiment

immunohistochemical experiments but distinct staining in transitional epithelium and inner longitudinal muscularis of rat bladder (data not shown). Similarly, Sc1473 did not yield detectable signals in mouse lung, also known not to express β_3-adrenoceptor mRNA (Nahmias et al. 1991), but distinct staining in mouse heart located in the ventricular endocardium and in the close proximity of blood vessels (data not shown).

To extend the validation of Sc1473, we attempted to use β_3-adrenoceptor knockout mice (Susulic et al. 1995). The

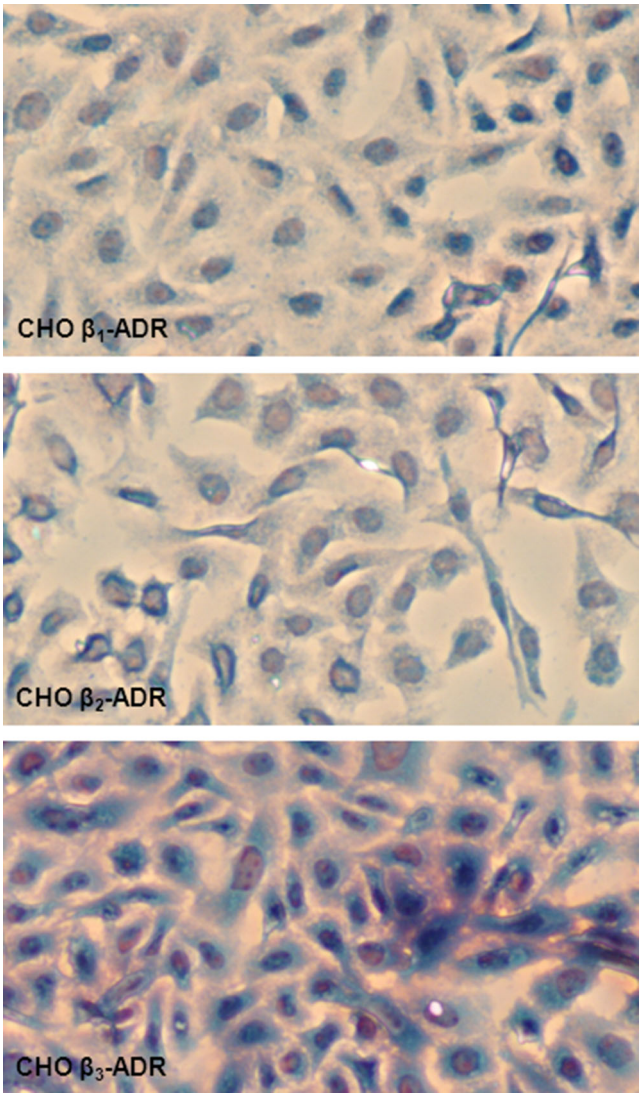

Fig. 2 Immunocytochemistry with antibody Sc1473 in CHO cells stably expressing rat β_1-, β_2- or β_3-adrenoceptors. Alkaline phosphatase-conjugated secondary antibodies were used yielding blue staining. Data are from a representative experiment performed at least twice with similar results

signals detected with Sc1473, raised against a C-terminal part of the receptor, were still present in tissues from these mice (suppl. Fig. 1). However, the location of the cassette leading to disruption of the mouse β_3-adrenoceptor gene in these mice may cause a functional knockout while still allowing for expression of the C-terminus of the receptor; i.e. the region against which Sc1473 had been raised. We confirmed the presence of the inserted cassette by genotyping of knockout in comparison to wild-type mice (suppl. Fig. 2). In RT-PCR experiments, mRNA corresponding to the C-terminus of the β_3-adrenoceptor was detected at comparable levels in BAT, gut and heart of knockout mice and wild-type mice (suppl. Fig. 3).

Expression of β_3-adrenoceptors in rat gastrointestinal and urogenital tissues

Staining patterns were reproducible within and between rats, and no sex differences were observed. The β_3-adrenoceptor was not expressed in the epithelium of the oesophagus, small intestine or colon but was found in the external mucous layer of the epithelium of the stomach (Fig. 3). The smooth muscle layer did not stain for the β_3-adrenoceptor. The urothelium of the ureters and bladder stained consistently positive for the β_3 receptor, whereas the urothelium of the urethra and the suburothelial glands, which are present in the urethral sphincter complex region, were negative (Fig. 4). The smooth muscle layer of the ureters and bladder was also negative.

Discussion

β_3-Adrenoceptors have recently been validated clinically as a target for the treatment of the overactive bladder syndrome (Ohlstein et al. 2012; Chapple et al. 2014). However, potential effects on other organ systems have been difficult to predict because little information is available about the expression of this receptor at the protein level throughout the body (Michel et al. 2010). This lack of data stems at least partly from a lack of suitable tools such as radioligands or antibodies (Vrydag and Michel 2007). Therefore, we have explored the selectivity of two commercially available antibodies directed at rodent β_3-adrenoceptors, AB15688 and Sc1473, in immunoblots and immunocytochemistry and histochemistry.

According to the manufacturer's information, AB15688 has been raised in chicken against a synthetic peptide from the carboxyl terminus of the mouse β_3-adrenoceptor and is claimed to be suitable for immunoblotting studies with the rat and mouse receptor. In our immunoblotting experiments, AB15688 exhibited a clear band of 44 kDa in mouse heart membranes, the expected molecular size for the unglycosylated receptor. A 44-kDa band was also seen in immunoblots with membranes from CHO cells transfected with rat β_3-adrenoceptors but not in those transfected with rat β_1- or β_2-adrenoceptors. This band was also seen in five rat tissues with bona fide β_3-adrenoceptor expression. However, both in transfected CHO cells, largely independent of the transfected subtype, and in rat tissues, many additional bands were seen and several of them were more prominent than the 44-kDa band. Taken together, these data indicate that AB15688 exhibits some degree of selectivity for rodent β_3-adrenoceptors, more so in mice than in rats. However, in rat tissues and transfected hamster cells, it also labels many other targets. While AB15688 exhibited some specificity for β_3-adrenoceptors in immunocytochemistry experiments with CHO cells transfected with the rat β-adrenoceptor subtypes

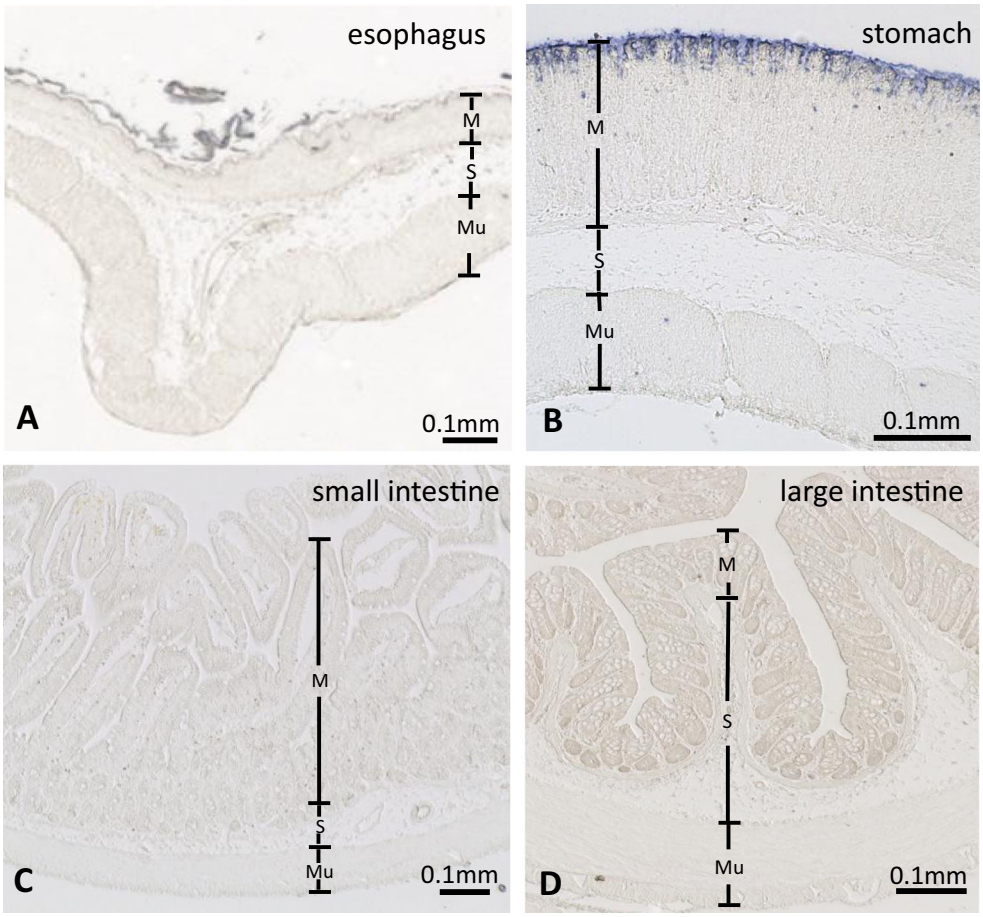

Fig. 3 β_3-Adrenoceptor staining pattern in the rat gastrointestinal tract (GIT). Alkaline phosphatase-conjugated secondary antibodies were used yielding blue staining; haematoxylin (red) was used for counterstaining. Note the presence of the receptor protein only in the lining epithelial cells (surface mucous cells) of the body part of stomach (**b**). All other GIT layers including mucosa (*M*), submucosa (*S*) and muscularis externa (*Mu*) in oesophagus (**a**), ileum (**c**) and rectum (**d**) did not show β_3-adrenoceptor staining. *Bar*=0.1 mm

(Kullmann et al. 2009), this application is not recommended by the manufacturer. For this reason, and also based on the many additional bands in tissue immunoblots, we have not used this antibody in our tissue mapping experiments in rats.

According to the manufacturer's information, Sc1473 has been raised in goats against the C-terminus of the murine β_3-adrenoceptor and is claimed to be reactive against mouse and rat receptors in immunoblotting, immunohistochemistry and ELISA applications. We have previously reported that this

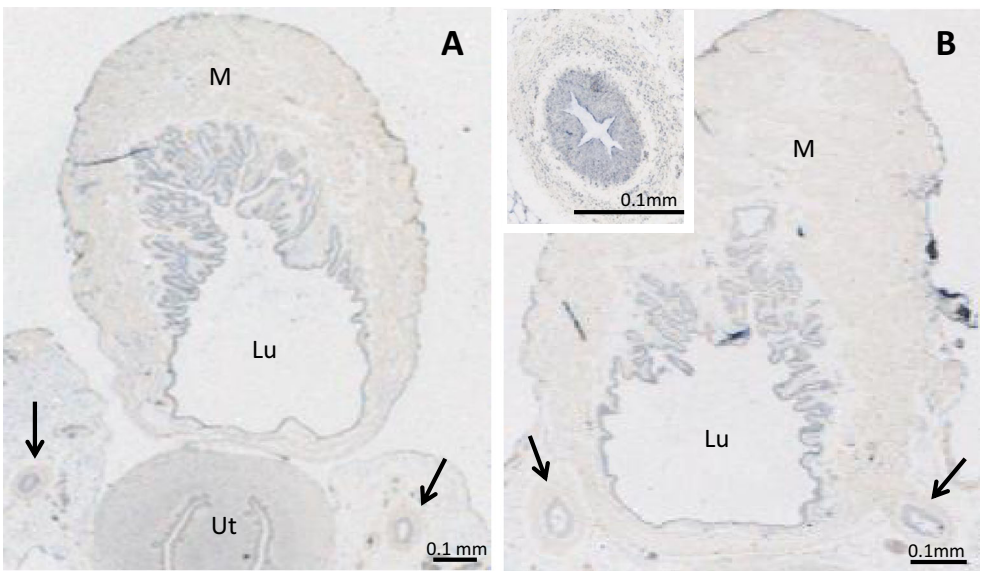

Fig. 4 β_3-Adrenoceptor staining pattern in the urinary bladder and ureter (*arrows*) of female (**a**) and male (**b**) rats. Alkaline phosphatase-conjugated secondary antibodies were used yielding blue staining; haematoxylin (red) was used for counterstaining. Note the presence of the receptor protein in the urothelium but not in the muscular wall (*M*) of the urinary bladder and ureter of both sexes. *Inset* in **b** shows the staining of β_3-adrenoceptor at the epithelial lining of the ureter at higher magnification. *Lu* bladder lumen, *Ut* uterus. *Bar*=0.1 mm

antibody does not detect any specific band in CHO cells transfected with human β_3-adrenoceptors (Pradidarcheep et al. 2009), as confirmed in the present study. On the other hand, it has been reported that Sc1473 labels a single band of approximately 44 kDa in mouse heart, which is absent in the hearts of β_3-adrenoceptor knockout mice (Bundgaard et al. 2010); in our initial validation experiments, we also observed this band in mouse heart. These data indicate that Sc1473 may have target selectivity in rodent species. Therefore, we have further explored the selectivity of this antibody using three types of negative controls, i.e. cells transfected with related receptor subtypes, native tissue lacking expression and tissues from knockout mice.

In our first approach, Sc1473 identified a 44-kDa band in CHO cells stably transfected with rat β_3-, but not β_1- or β_2-adrenoceptors, although several other bands were detected, which exhibited a similar density irrespective of the transfected receptor subtype. On the other hand, Sc1473 exhibited β_3-adrenoceptor selectivity in immunocytochemistry experiments using the same cell lines. This approach has also been used by others for validation of antibodies against rat (Kullmann et al. 2009) or human β_3-adrenoceptors (Limberg et al. 2010). While these data question the use of Sc1473 in immunoblot experiments, they were promising for use in immunohistochemical experiments in rodent tissues. Such differentiation can also be derived from the indirect comparison in our immunoblotting and the immunocytochemical experiments by (Kullmann et al. 2009) using AB15688. Apparently, more epitopes are exposed after extensive denaturation as done in immunoblots than in immunocytochemical or immunohistochemical studies, as previously also demonstrated for antibodies against creatine kinase (Lamers et al. 1989). Studies on glutamic acid decarboxylase also support the notion that linear structures expose different epitopes for antibody recognition than those found in complex three-dimensional conformations (Al-Bukhari et al. 2002). Thus, validation of a receptor antibody for one application does not necessarily provide validation for other uses; lack of selectivity in one application similarly does not exclude some degree of selectivity in another one, but that needs to be explicitly tested.

In our second approach, we have employed the rather restrictive expression of β_3-adrenoceptors in mammals (Michel et al. 2010). Thus, β_3-adrenoceptors have not been detected in rat (Muzzin et al. 1991) or mouse lung (Nahmias et al. 1991) at the mRNA level and in rats based on radioligand binding studies at the protein level (Schneider and Michel 2010); lack of β_3-adrenoceptor mRNA expression in rat lung was confirmed in the present study. Accordingly, Sc1473 stained neither rat nor murine lung but, in parallel experiments, exhibited distinct staining in mouse heart or rat urinary bladder, which are known to express corresponding mRNA. Thus, these two approaches provided evidence that Sc1473 exhibits target selectivity in rat and mouse tissues in immunohistochemistry.

In our third approach, we used β_3-adrenoceptor knockout mice originally developed by Susulic et al. (1995), and our data based on these mice are not easy to understand. Susulic et al. (1995) have replaced 306 bp of β_3-adrenoceptor coding sequence between *Nhe*I and *Xho*I corresponding to residue 120, in the middle of the third transmembrane domain, to residue 222, at the carboxy-terminal end of the fifth transmembrane domain with a PGK-neo-poly(A) vector. In homozygote knockout mice, β_3-adrenoceptor mRNA was not detected using a probe based on codon 120–222. In contrast, we did not detect a reduction in β_3-adrenoceptor mRNA in three tissues of knockout mice using a probe based on codon 621–680. This was a surprising finding, as it is unclear which promoter would drive expression of a C-terminal mRNA fragment. In line with comparable expression of β_3-adrenoceptor C-terminal mRNA in BAT, gut and heart of wild-type and knockout mice, the signal detected by Sc1473, directed against the C-terminus of the receptor, did not disappear in these mice. Experiments with β_2-adrenoceptors have demonstrated that truncated C-terminal fragments can express at the protein level and insert into the cell membrane (Kobilka et al. 1988). Hence, we consider these specific knockout mice unsuitable to explore target selectivity of antibodies directed against the C-terminus. However, we cannot explain why the 44-kDa band detected by Sc1473 in murine heart disappeared in knockout animals when studied by Bundgaard et al. (2010) but not by us. Of note, in line with our findings, it has recently been discussed whether the lack of disappearance of staining with antibodies directed against the C-terminus of the AT_{1a} angiotensin II receptor in corresponding knockout mice may represent false negative data because that disruption also did not eliminate the C-terminus (Premer et al. 2013). Thus, knockout approaches causing functional inactivation of a receptor but not eliminating expression of its C-terminus may be an inappropriate approach for the testing of antibodies raised against this part of the receptor. Whether such knockout animals represent inactivation of the receptor remains to be tested in functional experiments.

Previously, we have proposed that a receptor antibody must fulfil at least one of four criteria to be considered having acceptable target specificity (Michel et al. 2009). These included (a) disappearance of staining in knockout animals of the target receptor, (b) reduction of staining upon knock-down approaches such as siRNA treatment, (c) selectivity of staining in immunoblots or immunocytochemistry for the target receptor vs. related subtypes when expressed in the same cell line and/or (d) antibodies raised against multiple distinct epitopes of a receptor yielding very similar staining patterns. Our immunocytochemistry results with Sc1473 fulfil the third of these criteria. Moreover, in the absence of suitable knockout

animals, we have presented evidence that Sc1473 does not stain rodent tissues known to lack β_3-adrenoceptors, providing bona fide evidence that the second criterion is also met. Based on the above, we concluded that Sc1473 is the most promising among the currently tested and available antibodies against rodent β_3-adrenoceptors.

Therefore, we have used Sc1473 to explore expression of this receptor at the protein level in the gastrointestinal and urogenital tract of rats. Previous studies have only inconsistently detected β_3-adrenoceptor mRNA in rat ileum (Muzzin et al. 1991; Granneman and Lahners 1992; Evans et al. 1996; Roberts et al. 1999), whereas moderate to strong mRNA expression was reported in stomach (Granneman and Lahners 1994; Cohen et al. 1995; Evans et al. 1996) and colon (Evans et al. 1996; Roberts et al. 1999; Vasina et al. 2008). Functional data, although not fully conclusive, have indicated relaxation of rat stomach (Cohen et al. 1995), ileum (Roberts et al. 1999) and colon (Bianchetti and Manara 1990; Kaumann and Molenaar 1996) by β_3-adrenoceptor agonists. Against this background, it is noteworthy that Sc1473 did not stain smooth muscle in any of these gastrointestinal tissues. The only gastrointestinal tissue in which we observed staining was in the external mucous layer of the epithelium of the stomach.

Within the rat urogenital tract, urothelium of the ureter stained positive in our experiments. While we are not aware of β_3-adrenoceptor mRNA studies on rat or mouse ureter, its expression level in human ureter was reported to be low (Park et al. 2000; Matsumoto et al. 2013); moreover, in some species including humans, ureter relaxation by β_3-adrenoceptor agonists has been reported (Park et al. 2000; Matsumoto et al. 2013). Several studies have reported β_3-adrenoceptor mRNA in rat bladder, including specifically in its urothelium (Seguchi et al. 1998; Fujimura et al. 1999; Barendrecht et al. 2009; Kullmann et al. 2011). While relaxation of rat detrusor strips via β_3-adrenoceptors has consistently been reported in many studies (Michel and Vrydag 2006; Igawa et al. 2012; Michel 2014), we did not detect staining of rat bladder smooth muscle. Rather, we have β_3-adrenoceptor staining in rat bladder urothelium. Previous studies in rat (Kullmann et al. 2009) and human tissue (Limberg et al. 2010) detected some staining of bladder smooth muscle but much more prominent staining in the urothelium. While functional β-adrenoceptors have been demonstrated in the urothelium (Birder et al. 2002), the subtype mediating such effects has not been fully established. Nonetheless, it has been proposed that urothelium plays an important role in the clinical effects of β_3-adrenoceptor stimulation (Andersson et al. 2013). While the urothelial β_3-adrenoceptor may contribute to detrusor relaxation by β_3-adrenoceptor agonists in vivo, these data do not resolve the apparent contradiction of low to absent staining in smooth muscle in this study as well as in previous ones by other investigators in rat (Kullmann et al. 2011) and human bladder (Limberg et al. 2010) and the relaxation responses to β_3-adrenoceptor agonists in mucosa-denuded, isolated rat (Kullmann et al. 2011), porcine (Masunaga et al. 2010) or human bladder strips (Otsuka et al. 2008). Perhaps, a very small number of β_3-adrenoceptors are sufficient to elicit relaxation.

In conclusion, our data demonstrate that Sc1473 exhibits some degree of selectivity for rodent β_3-adrenoceptors in immunocytochemical and immunohistochemical studies but may be less suitable for use in immunoblotting; however, β_3-adrenoceptor antibodies with improved target selectivity remain desirable. Our data also indicate that knockout animals may yield false negative results in the specificity evaluation of antibodies. However, as the burden of proof lies on demonstrating specificity, this does not invalidate that lack of disappearance of staining in knockout animals is one of the strongest criteria to demonstrate target selectivity of an antibody. Staining patterns observed in rat gastrointestinal and urogenital tract are only partly in line with reported functional data. While we cannot exclude that this reflects issues with the Sc1473 antibody, it is also possible that small numbers of β_3-adrenoceptors in smooth muscle, too small to be reliably detected by immunohistochemistry, are sufficient to elicit some degree of smooth muscle relaxation.

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