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SHORT COMMUNICATION

Suitability of muscarinic acetylcholine receptor antibodies for immunohistochemistry evaluated on tissue sections of receptor gene-deficient mice

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Abstract Acetylcholine (ACh) is a major regulator of visceral function exerting pharmacologically relevant effects upon smooth muscle tone and epithelial function via five types of muscarinic receptors (M1R-M5R). In this paper, we assessed the specificity of muscarinic receptor (MR) antibodies in immunohistochemical labelling on tissue sections by analysing specimens from wild-type and respective gene-deficient mice. Of 24 antibodies evaluated in this study, 16 were tested at 18 different conditions each, and eight of them in 21 different protocols, resulting in a total number of 456 antibody/protocol combinations. Each of them was tested at four antibody dilutions at minimum, so that finally, at least 1,824 conditions were evaluated. For each of them, dorsal root ganglia, urinary bladder and cross-sections through all thoracic viscera were investigated. In all cases where the antigen was available, at least one incubation condition was identified in which only select cell types were immunolabelled in the positive control but remained unlabelled in the pre-absorption control. With two exceptions (M2R antibodies), however, all antibodies produced identical immunohistochemical labelling patterns in tissues taken from corresponding gene-deficient mice even when the pre-absorption control in wild-type mice suggested specificity. Hence, the present data demonstrate the unpleasant fact that reliable immunohistochemical localisation of MR subtypes with antibodies is the exception rather than the rule. Immunohistochemical detection of MR subtype localisation in tissue sections of peripheral organs is limited to the M2R subtype utilising the most commonly used methodological approaches.

Keywords Acetylcholine · Muscarinic receptor · Immunohistochemistry · Specificity · Receptor knockout strains · Mouse

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Introduction

Acetylcholine (ACh) is a major regulator of visceral function. It exerts its action via two basically different types of receptors, i.e. ionotropic nicotinic ACh receptors and metabotropic muscarinic ACh receptors (MR). The latter are of particular pharmacological relevance since they predominantly confer cholinergic control of smooth muscle tone and epithelial function in many viscera, including airways, gastrointestinal and urinary tract. Five MR subtypes have been cloned, termed M1R-M5R in the following, each of them displaying a distinct pharmacological profile. Activation of subtypes M1R, M3R and M5R is linked to the IP_3/Ca^{2+} pathway, and M2R/M4R stimulation inhibits adenylyl cyclase, although MR subtypes may couple to more than one signal transduction pathway. Organ- and tissue-specific distribution and effects of MR subtypes make them a widely used pharmacological target

for treatment of common diseases such as chronic obstructive pulmonary disease (Gosens et al. 2006; Gross and Skorodin 1984) and overactive bladder (Mansfield et al. 2005; Michel and Barendrecht 2008, Tong et al. 2006; Tyagi et al. 2006), and M2R dysfunction is associated with bronchial asthma (Bowerfind et al. 2002; Costello et al. 1998). On this background, detailed knowledge on the cellular distribution of MR subtypes in viscera does not only promote our basic knowledge on organ function but is also valuable to design new and understand current anticholinergic therapeutic strategies. It is widely accepted that immunohistochemistry of tissue sections offers the most powerful and convenient tool to address this aspect close to the in situ situation. In the course of our investigations of cholinergic innervation and non-neuronal visceral cholinergic systems, we are addressing this issue for more than 10 years now. Having the opportunity to validate the specificity of immunohistochemical labelling on tissue sections taken from respective gene-deficient mice, however, we noted an unexpectedly high degree of non-specific immunohistochemical labelling produced by MR antibodies despite the classical specificity control, i.e. pre-absorption of the antibody with corresponding antigen, suggested specificity. Although being primarily negative in nature, we here report these data since they may be helpful to others not having access to the knockout mouse strains or working with other species than mice where gene-deficient samples are not available.

Materials and methods

The following MR gene-deficient ($^{-/-}$) mice and respective corresponding wild-type strains were used: M1R$^{-/-}$, M2R$^{-/-}$, M3R$^{-/-}$, M4R$^{-/-}$, M5R$^{-/-}$ and M2/3R$^{-/-}$. Their generation and characterisation have been described in detail earlier (Gomeza et al. 1999a, b; Fisahn et al. 2002; Yamada et al. 2001a, b). Animals were killed by isoflurane inhalation and then either subjected to perfusion fixation with (a) 4% phosphate-buffered formaldehyde (PFA; formaldehyde solution prepared by freshly dissolving paraformaldehyde), (b) Zamboni's fixative (15% saturated picric acid, 2% paraformaldehyde in 0.1 M phosphate buffer), (c) periodate–lysine–paraformaldehyde (PLP) solution (2% paraformaldehyde, 10 mM $INaO_4$, 75 mM L-lysine), or organs were freshly dissected and shock-frozen in isopentane cooled with liquid nitrogen. All steps and variations of the tissue processing and immunohistochemical steps are depicted as flow diagram in Fig. 1. Blocking solutions used to saturate non-specific protein binding sites were (1) 10% horse serum, 0.5% Tween 20, 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS; 0.005 M phosphate buffer with 4.48 g/l NaCl), (2) 5% BSA, 5% normal goat serum in PBS and (3) 50% horse serum in PBS with doubled NaCl concentration. Secondary antibodies used in this study were Cy3-conjugated donkey anti-rabbit Ig (Chemicon, Temecula, CA, USA), Cy3-conjugated donkey anti-goat Ig (Chemicon), Cy3-conjugated donkey anti-guinea pig Ig (Dianova, Hamburg, Germany), Cy3-conjugated donkey anti-rat Ig (Dianova), fluorescein isothiocyanate (FITC)-conjugated donkey anti-guinea pig Ig (Dianova), FITC-conjugated mouse anti-goat Ig (Dianova) and FITC-conjugated goat anti-rabbit Ig (Cappel, Karlsdorf, Germany).

Two different protocols for pre-absorption of the primary antibody with corresponding antigen were applied: (1) overnight incubation at 4°C with 20 μg peptide/ml antibody at working concentration and (2) incubation at room temperature for 4 h with 200 μg peptide/ml antibody at working concentration. Antibodies for which antigen was available and antibody working concentrations are listed in Table 1. Antigens were from the same source as the respective antibody.

All sections were coverslipped in 1:1 carbonate-buffered glycerol at pH 8.4 to reduce fading. Section were evaluated with epifluoresence microscopes (Zeiss Axioplan 2 imaging; Zeiss, Jena, Gemany; BX50, Olympus, Hamburg, Germany) equipped with appropriate filter sets.

Results

Of 24 antibodies evaluated in this study, 16 were tested at 18 different conditions each and eight of them in 21 different protocols (Table 1, Fig. 1), resulting in a total number of 456 antibody/protocol combinations. Each of them was tested at four antibody dilutions at minimum, so that finally, at least 1,824 conditions were evaluated. For each of them, dorsal root ganglia, urinary bladder and cross-sections through all thoracic viscera including oesophagus, heart, large blood vessels (aorta, caval veins), thymus, brown fat tissue, peripheral nerves (vagus nerve, phrenic nerve), extrapulmonary airways and lungs were investigated. For select antibodies, other segments of the gastrointestinal tract and the oviduct were additionally studied.

The incubation protocol had significant impact in that none of the antibodies produced identical immunohistochemical labelling pattern throughout all protocols. For each of those where the corresponding peptide was available for pre-absorption, however, at least one incubation condition was identified in which only select cell types were immunolabelled in the positive control but remained unlabelled in the pre-absorption control (Fig. 2). In the majority of cases, such results were obtained with cryosections taken from shock-frozen tissue and dipped in acetone or isopropanol before immunolabelling. Aldehyde fixation, either before sectioning or applied on cryosections,

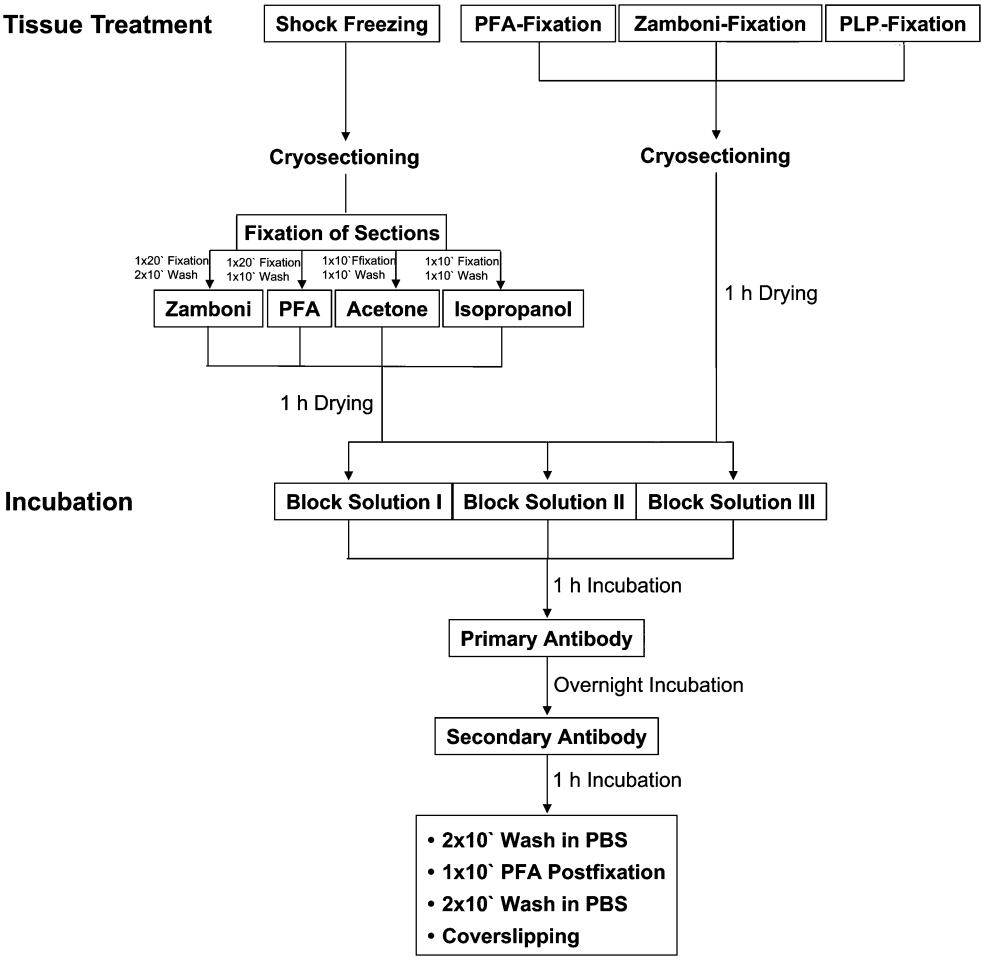

Fig. 1 Flow diagram of variations of the immunohistochemical procedure that have been applied to all antibodies tested in this study (Table 1)

often resulted in weaker and more general immunohistochemical labelling that persisted after pre-absorption.

With two exceptions, all MR antibodies tested in this study produced identical immunohistochemical labelling patterns in tissues taken from mice with gene deficiency for the MR subtype under study and the corresponding wild-type strain. This also applied to antibody/protocol combinations in which the pre-absorption control suggested specificity (Fig. 2). In view of the sequence homologies between MR subtypes, we tried to explore the possibility that immunohistochemical labelling in the respective knockout strain may be caused by antibody binding to another MR subtype expressed by the same cell type. As a model, we chose the airway smooth muscle which is known from binding and pharmacological studies to express both M2R and M3R but not other MR subtypes to a significant amount (Fayon et al. 2005; Gosens et al. 2006; Roffel et al. 1988). Hence, even in case of missing subtype selectivity of MR antibodies, there shall be no airway smooth muscle labelling in sections taken from M2R/M3R double knockout mice. This, however, did not match the actual observation (Fig. 2).

In contrast, the rat monoclonal M2R antibody 367 (Levey et al. 1995) labelled airway smooth muscle and the cell membrane of a subpopulation of dorsal root ganglion neurons and atrial and pulmonary vein cardiomyocytes in wild-type but not in M2R knockout mice (Fig. 2). This labelling was particularly bright using the protocol of tissue freezing, cryosectioning, on-slide fixation with acetone and pre-incubation with blocking solution I (10% horse serum, 0.5% Tween 20, 0.1 BSA in PBS). Use of blocking solutions II and III only slightly reduced labelling intensity, and replacement of acetone by isopropanol for on-slide fixation still resulted in useful labelling but at further reduced intensity. Much weaker labelling, but in the same distribution pattern, was observed after aldehyde fixation. In addition to that seen in the lung, specific staining, as judged from its absence in tissues taken from M2R-deficient mice, was observed in all parts of the gastrointestinal tract. In the ampulla of the oviduct, however, this antibody intensely labelled the apical part of ciliated cells even in M2R/M3R double knockout mice to the same extent as in wild-type mice (Fig. 2). Occasionally, such labelling of ciliated cells in

Table 1 Characteristics of primary antibodies

Antigen	Host species, code	Source
M1-receptor aa 451–460	Rabbit AS-3701S	Biotrend, Cologne, D
M1-receptor[1] N-terminal peptide, human	Goat SC-7471	Santa Cruz, California, USA
M1-receptor aa 227–353, human	Rabbit #AMR-001	Alomone labs, Jerusalem, Israel
M1-receptor[a] aa 227–353	Rabbit #AB5164	Chemicon, Temecula, CA, USA
M1-receptor aa 435–448, human/rat/mouse	Rabbit 001	own
M1-receptor aa 435–448, human/rat/mouse	Rabbit 002	own
M1-receptor[a] aa 330–343	Guinea pig GP20a	H. Kurzen, Mannheim, D
M2-receptor aa 225–356, human	Rabbit #AMR-002	Alomone labs, Jerusalem, Israel
M2-receptor aa 457–466	Rabbit AS3721S	Biotrend, Cologne, D
M2-receptor aa 225–359 fused to GST (i3 loop)	Rat, monoclonal IgG2a, mAB367	Chemicon, Temecula, CA, USA
M2-receptor aa 225–356	Rabbit AB5166-50ULa	Chemicon, Temecula, CA, USA
M3-receptor[a] aa 580–589	Rabbit AS-3741S	Biotrend, Cologne, D (R&D)
M3-receptor[a] aa 461–479, rat	Rabbit #AMR-006	Alomone labs, Jerusalem, Israel
M3-receptor[a] aa 572–589	Rabbit R66136	J. Wess, Bethesda, USA
M3-receptor aa 572–589	Rabbit R66431	J. Wess, Bethesda, USA
M3-receptor second cytoplasmatic domain, human	Rabbit #AB9453	Chemicon, Temecula, CA, USA
M3-receptor[a] aa 271–480	Rabbit H-210 (sc-9108)	Santa Cruz, California, USA
M3-receptor aa 347–362, human/rat/mouse	Rabbit 001	own
M3-receptor aa 347–362, human/rat/mouse	Rabbit 002	own
M3-receptor[a] aa 290–304	Guinea pig GP19b	H. Kurzen, Mannheim, D
M4-receptor aa 220–394, recombinant	Rabbit H-175 (sc-9109)	Santa Cruz, California, USA
M4-receptor i3 loop (human), fused to GST	Mouse, monoclonal MAB1576	Chemicon, Temecula, CA, USA
M4-receptor aa 469–478	Rabbit AS-3761S	Biotrend, Cologne, D (R&D)
M5-receptor aa 519–531	Rabbit AS-3781S	Biotrend, Cologne, D (R&D)

[a] Antibodies are tested additionally with PLP fixation

wild-type and knockout mice was also visible in the airways, but not to that extent as seen in the oviduct.

Knockout-proof specific labelling was also obtained with some batches of the commercially available rabbit polyclonal M2R antiserum AB5166-50ULa (Table 1), whereas other batches from the same supplier resulted in identical labelling patterns in wild-type and knockout mice.

Discussion

The present data demonstrate the unpleasant fact that reliable immunohistochemical localisation of MR subtypes with antibodies is the exception rather than the rule, based upon the persistence of MR immunolabelling in tissue sections taken from organs of gene-deficient mice. It is important to note in this context that the strategies to generate the knockout strains M1R, M3R and M5R here included deletion of the start codon, and no residual ligand binding or function have been detected in these strains (Fisahn et al. 2002; Yamada et al. 2001a, b). The M2R- and M4R-deficient strains were generated by replacing the region between the third transmembrane domain and C terminus of the third intracellular loop and between the middle of the second transmembrane domain and the N terminus of the third intracellular loop, respectively, by the PGK-neomycin resistance gene (Gomeza et al. 1999a, b). Again, no residual ligand binding or function have been detected in these strains. It has to be noted, however, that the rabbit polyclonal M2R antibody AS3721S and M4R antibodies were raised against peptide sequences located N-terminally to the replaced sequences.

It is worth noting that for most of the antibodies, the false-positive labelling would not have become evident if tissue from gene-deficient mice were unavailable because it could be successfully prevented by liquid-phase pre-absorption of the antibody with the antigen used for immunisation. In the absence of paired samples of which one is missing the protein of interest, i.e. in most cases wild-type and gene-deficient mice, this pre-absorption control is the most powerful control for specificity in immunohistochemistry. Still, it cannot provide final proof of the chemical identity of the antigen labelled in the tissue section (Forssmann et al. 1981) as confirmed also by the present set of data. The successful pre-absorption control documents that the labelling of structures in the tissue section occurred by antibody binding to the tissue section via the variable region of its F_{ab} fragment. This may be caused by stretches of amino acid sequences shared between two proteins, as it often occurs among members of a protein family or receptors isoforms. However, we do not have evidence that this is the underlying cause of the

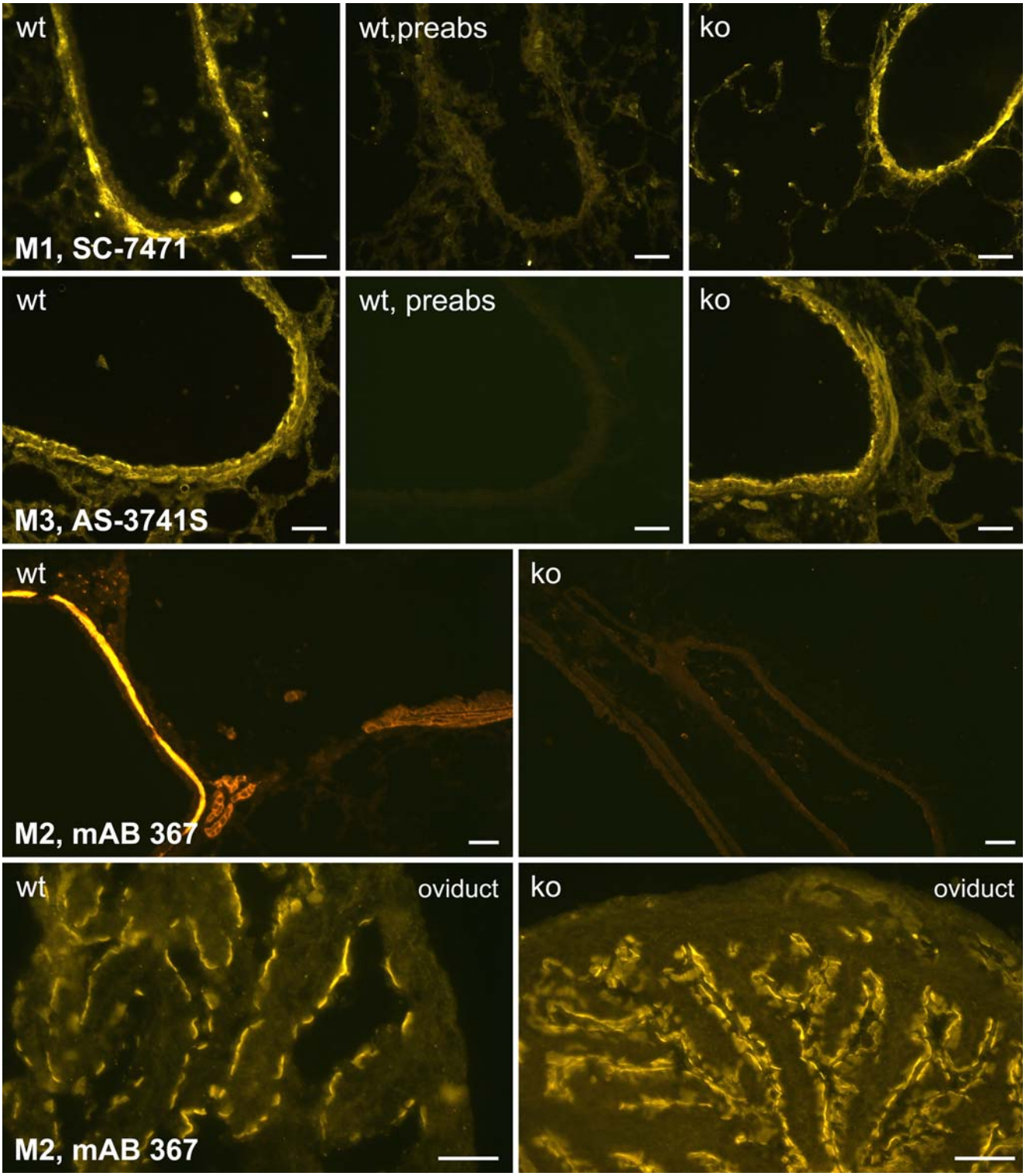

Fig. 2 Immunohistochemical labelling obtained with MR antibodies on mouse lung sections and oviduct, Cy3-conjugated secondary antisera. Both the M1R antibody SC-7471 and the M3R antibody AS-37415 label bronchial smooth muscle in wild-type mice (*wt*). This staining can be prevented by liquid-phase pre-absorption (*preabs*) with the respective corresponding antigen but still persists in sections taken from M1R deficient (SC-7471) and M2/3R double-knockout (AS-3741S) animals (*ko*). Immunohistochemical labelling obtained with rat monoclonal M2R antibody 367 of bronchial smooth muscle and pulmonary vein cardiomyocytes in wild-type animals (*wt*) is absent in corresponding knockouts (*ko*), demonstrating specificity. The same antibody, however, exhibits non-M2R-specific labelling of ciliated epithelial cells of the oviduct

false-positive reactions observed in the present study. In cases where the antibodies were raised against short peptides of known sequence, database search did not reveal sequence identities to other known proteins. With respect to possible cross-reactivity between MR subtypes, we were able to directly address this issue on airway smooth muscle and still observed immunohistochemical MR labelling in sections taken from mice lacking both receptors subtypes expressed by airway smooth muscle, i.e. M2R and M3R. Hence, the endogenous antigen bound by the antibody is not an MR isoform. There are well-documented examples, however, that false-positive immunohistochemical labelling that passes the pre-absorption control can result from ionic interactions even in the absence of sequence homologies at the amino acid level (Grube 1980), which may have occurred under the present conditions as well.

A crucial finding of the present study is cell-type-specific distribution of an antigen causing false-positive labelling, i.e. false-positive labelling may occur in one organ (in our case, oviduct) even when an antibody (here, rat monoclonal anti-M2R 367) used at the very same conditions at tissue sections of other organs (respiratory and gastrointestinal organs, heart, dorsal root ganglia) taken from the same individual gene-deficient animal specifically recognised only the respective receptor. As a consequence, specificity of an antibody cannot be generally claimed from a model system but needs to be evaluated for each application. Vice versa, however, it cannot be deduced from false-positive labelling observed in one particular organ (or species) that the antibody will be unsuitable for investigation of all other organs as well, as the same example demonstrates.

The importance of not only the antibody but all steps of tissue and section processing for specificity of immunolabelling is often underestimated, and many published reports lack sufficient methodological information of how tissue was processed for analysis (True 2008). However, all steps critically influence the outcome (Deutsch et al. 2008; Lorincz and Nusser 2008), as is also fully supported by our present data. Despite having tested 456 antibody/protocol combinations, we cannot claim that all possible methodological variations have been evaluated. Pepsin treatment of aldehyde-fixed tissue, for example, has been shown to be necessary to abolish immunohistochemical NMDA receptor 2A-subunit labelling of membranes in the hippocampus in the respective knockout mouse, whereas identical cytoplasmic labelling was obtained in wild-type and knockout mice without pepsin treatment (Watanabe et al. 1998). Hence, we are currently exploring the effects of enzymatic digestion and other antigen retrieval methods on MR immunohistochemistry, but a beneficial effect has not become evident so far. In a previous study, we identified a methodological variation utilising a pre-absorption step on tissue sections of knockout mice that resulted in specific immunohistochemical MR labelling of mouse bladder urothelium (Zarghooni et al. 2007), but this method was not generally applicable to all conditions and even failed to localise MR subtypes in the underlying urinary bladder smooth muscle. Still, we do not exclude the possibility that one of the antibodies used here and resulting in false-positive labelling in all conditions tested so far may prove to be useful under conditions that have not been evaluated yet.

In conclusion, the present data demonstrate that immunohistochemical detection of MR subtype localisation in tissue sections of peripheral organs is limited to the M2R subtype utilising the most commonly used methodological approaches. Hence, previous immunohistochemical reports on cellular localisation of MR subtypes have to be critically reevaluated under this aspect, including own studies (Haberberger et al. 1999, 2000) conducted before respective knockout strains were available.

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