EDITORIAL FOCUS

Do β₃-adrenoceptor agonists cause urinary bladder smooth muscle relaxation by inhibiting acetylcholine release?

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β₃-ADRENOCEPTOR (β₃-AR) agonists such as mirabegron are a novel drug class to treat overactive bladder syndrome (OAB) (4). Classic concepts have assumed that β₁-AR agonists work by directly acting on a β₁-AR located in the plasma membrane of smooth muscle cells in the detrusor of the urinary bladder (15). Recently, this concept has been critiqued increasingly (7) because mirabegron, the only β₃-AR agonist currently in clinical use, has an EC₅₀ for relaxation of isolated detrusor strips of 588–776 nM in humans and 288–5,113 nM in rats, with comparable values in mice and monkeys (12), but maximum plasma levels upon therapeutic dosing reach only 83–167 nM (9). On the other hand, several cell types potentially involved in the control of detrusor smooth muscle tone have been reported to express β₃-AR and/or be responsive to β₃-AR agonists. These include the urothelium, afferent nerves, interstitial cells of Cajal, blood vessels supplying the urinary bladder, and the major pelvic ganglion (7, 12, 14). However, none of these has it been shown that mirabegron works at concentrations of ~100 nM. Therefore, uncertainty remains as to which cell type or combination of cell types mediates detrusor smooth muscle relaxation in response to systemic administration of β₃-AR agonists.

A related receptor, the β₂-AR facilitates release of noradrenaline from sympathetic nerve endings, but its effects on acetylcholine release from parasympathetic nerve endings have remained controversial, as both enhancing and inhibiting effects have been reported. Recently, it has been shown that β₂-AR agonists can inhibit not only electrical field stimulation-induced contraction of isolated human detrusor strips but also acetylcholine release in this preparation (6). Sources of acetylcholine in the urinary bladder include not only parasympathetic nerves but also nonneuronal release from the urothelium (16). In the preparation studied by D’Agostino et al. (6), acetylcholine release occurred primarily from nerves, as it was concentration-dependently inhibited by tetrodotoxin. Importantly, mirabegron had high potency for inhibition of contraction (EC₅₀ 123 nM) and of acetylcholine release (EC₅₀ 129 nM) in that study. Although the high potency of mirabegron for relaxation may refute the non-smooth muscle hypothesis (7), the observation that inhibition of contraction and acetylcholine release exhibited similar potency raised the possibility that the latter may be the cause of former.

A recent article in the journal by Silva et al. (13) confirms that β₂-AR agonists can substantially inhibit electrical field stimulation-induced acetylcholine release from an isolated human detrusor preparation and that such inhibition is prevented by a selective β₂-AR antagonist. Notably, mirabegron was effective in this model already at a concentration of 100 nM. Because β₂-AR agonists, similar to other β-AR subtypes, couple to stimulation of cAMP formation and cAMP is degraded to adenosine, Silva et al. (13) tested whether adenosine may mediate inhibition of acetylcholine release observed in the presence of β₂-AR agonists. Indeed, isoprenaline and mirabegron increased extracellular adenosine concentrations in the detrusor strips. Antagonism of A₁ adenosine receptors by 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) or blockade of the equilibrative nucleoside transporters with dipyridamole or S-(4-nitrobenzyl)-6-thioinosine (NBTI) prevented inhibition of acetylcholine release, suggesting that it did not necessarily occur via a β₂-AR located in the nerve ending but rather indirectly by intermediate formation of adenosine subsequent activation of A₁ inhibitory receptors. The physiological basis of this observation was demonstrated by in vivo experiments in anesthetized rats, in which DPCPX, dipyridamole, and NBTI reversed the decrease in voiding frequency caused by isoprenaline. To further substantiate the mechanistic basis of an indirect inhibition of acetylcholine release, these authors (13) also performed immunohistochemical staining of β₂-AR and vesicular acetylcholine transporter, a marker of cholinergic neurons. Given the limited target selectivity of many β₃-AR antibodies, they used a previously proposed approach (2) to concomitantly stain by multiple antibodies targeted against different epitopes of the β₂-AR. However, little colocalization of the β₂-AR and acetylcholine transporter was observed, suggesting that the receptor is expressed primarily by cells other than parasympathetic nerve endings.

The findings of D’Agostino et al. (6) and of Silva et al. (13) in combination make a convincing point that β₃-AR agonists, including therapeutically achieved concentrations of mirabegron, can inhibit neuronal acetylcholine release in human detrusor. They support a hypothesis that (indirect) inhibition of acetylcholine release may be the mechanism for detrusor smooth muscle relaxation. However, it is not fully clear yet whether such inhibition of acetylcholine release indeed occurs exclusively indirectly via adenosine formation and A₁ adenosine receptor activation or whether it may also involve a neuronally expressed β₃-AR. Thus, another group of investigators has recently also reported immunohistochemical studies of β₃-AR and vesicular acetylcholine transporter in the human bladder (5). These authors have used the same approach of concomitant labeling with multiple antibodies targeting differ-
ent epitopes in the β3-AR, actually even the same antibodies. Despite using the same antibodies, Coelho et al. (5) reported very different findings. In their hands, β3-AR colocalized not only with the acetylcholine transporter but also with β3-tubulin, another marker of neurons; such colocalization would allow for a direct effect of β3-AR agonists on cholinergic neurons. The two studies also differ in other ways: Silva et al. (13) have detected β3-AR in human bladder in smooth muscle fibers and, to a lesser extent, in urothelium and suburothelium. In contrast, Coelho et al. (5) detected β3-AR primarily in nerve fibers in the mucosa and muscular layers of the bladder but not in urothelium or smooth muscle. The cholinergic fibers expressing β3-AR were found mostly in the suburothelium, where they mingled adrenergic fibers (staining positive for tyrosine hydroxylase) and peptidergic fibers (staining positive for calcitonin gene-related peptide). Earlier studies based on validated antibodies have reported β3-AR expression to a greater extent in urothelium than smooth muscle of the human bladder and also in sub-urothelial myofibroblast-like cells, intramural ganglia, Schwann cells, and intramural nerves (10). However, the latter study did not explore whether the nerves expressing β3-AR were sympathetic, parasympathetic, and/or peptidergic. Thus, various investigators using similar approaches and antibodies have obtained at least in part different results with regard to the localization of β3-AR in the human urinary bladder. The reasons for these divergent results are not fully clear. However, it should be noted that sensitivity and specificity of immunohistochemical staining depend not only on the antibody being used but also on other factors, including thickness of slices, fixation and denaturalization protocols (8), and type of microscopy. Therefore, it is possible that rather minor differences in experimental protocol may have led to major differences in staining pattern, making it difficult to determine in which cell types within the urinary bladder β3-AR are expressed at the protein level. As Coelho et al. (5) and Silva et al. (13) work within the same institution, a collaborative study between them appears to be an obvious approach to settle this issue.

The physiological question is whether and to which extent parasympathetic nerves contribute to OAB symptoms and are a target for its treatment. The original argument by Eastham et al. (7) is that mirabegron effects on smooth muscle occur at concentrations considerably exceeding those achieved in plasma of mirabegron-treated subjects (6, 13). On the other hand, D’Agostino et al. (6) also reported such low mirabegron concentrations to cause smooth muscle relaxation; why they found much higher potency of mirabegron in isolated human detrusor than several previous studies (12) remains to be determined. Of note is that the potency of β3-AR agonists to cause relaxation of detrusor smooth muscle can markedly differ depending on the stimulus used for inducing tone in the preparation (3).

The conundrum with the prejunctional parasympathetic nerve hypothesis is that parasympathetic nerves are physiologically active in the voiding phase of the micturition cycle, whereas OAB symptoms occur in the storage phase; acetylcholine released during the storage phase, e.g., by bladder distension, is believed to come largely from nonneuronal sources such as the urothelium (11). To settle such questions, additional studies are required that determine the potency of β3-AR agonists not only for relaxation of smooth muscle and inhibition of acetylcholine release but also for functional responses of other cell types implicated in the regulation of detrusor tone. It is not an improbable hypothesis that improvement of OAB symptoms after administration of β3-AR agonists is the net effect of concomitant action on multiple cell types, including urothelium, afferent nerves, and interstitial cells. Of note is that, as highlighted by the prejunctural findings of Silva et al. (13), release of ATP and/or adenosine could act as an intermediate player in all of these cell types; after all, the original concept of ATP as a cotransmitter was developed by Burnstock et al. (1) based largely on findings in the urinary bladder.

DISCLOSURES
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AUTHOR CONTRIBUTIONS
K.O. and M.C.M. conceived and designed research; K.O., S.G., and M.C.M. drafted manuscript; K.O., S.G., and M.C.M. edited and revised manuscript; K.O., S.G., and M.C.M. approved final version of manuscript.

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