ABSTRACT

Bladder overactivity (OAB) is a chronic and debilitating lower urinary tract (LUT) disorder that affects millions of individuals worldwide. LUT symptoms associated with OAB, such as urgency and urinary incontinence, cause a hygienic and social concern to patients, but their current pharmacological treatment is largely inadequate due to the lack of uroselectivity. Although OAB etiology remains multifactorial and poorly understood, increasing evidence indicates that serotonin [5-hydroxytryptamine (5-HT)] is an endogenous substance involved in the control of micturition at central and peripheral sites. In this study, we demonstrated the presence of three distinct 5-HT receptors localized at parasympathetic nerve terminals of the human bladder by measuring electrically evoked tritiated acetylcholine release in isolated detrusor strips. These prejunctional receptors, involved in both positive and negative feedback mechanisms regulating cholinergic transmission, have been characterized by means of three highly selective 5-HT antagonists for 5-HT4, 5-HT7, and 5-HT1A receptors, namely GR113808A ([1-[2-[(methylsulphonyl) amino] ethyl][4-piperidinyl][methyl1-methyl-1H-indole-3-carboxylate succinate], SB269970 [(R)-3-((4-(4-(2-pyrrolidinyl-1-sulfonyl)phenol hydrochloride), and WAY100635 [N-(2-((2-methoxypyphenyl)-1-piperaziny1)ethyl]-N-(2-pyridyl)-cyclohexane-carboxamide trichloride]. Under these conditions, we confirmed the facilitatory role of 5-HT4 heteroreceptors on acetylcholine release and revealed for the first time the occurrence of 5-HT4 and 5-HT1A heteroreceptors with a facilitatory and an inhibitory action, respectively. Our findings strengthen the novel concept for the use of recently patented selective 5-HT agonists and antagonists for the control of OAB dysfunctions associated with inflammatory conditions, although their therapeutic efficacy needs to be explored in the clinical setting.
the human detrusor, 5-HT potentiates the neurogenic contractions by activating the 5-HT\textsubscript{T} \text{1} receptor type (Candura et al., 1996). Since this enhancing effect of 5-HT is antagonized by the muscarinic antagonist atropine and the nerve conduction blocker tetrodotoxin, it was suggested that precunutional 5-HT receptors located at parasympathetic postganglionic neurons are implicated into the control of acetylcholine (ACh) release (Corsi et al., 1991). Such an assumption, however, was based on postjunctional effects (contractions) only and not verified by assessment of changed neurotransmitter release. The measurement of the electrically evoked tritiated ACh (\textsuperscript{3}H\textsubscript{ACh}) release in human detrusor strips allowed us to provide direct evidence that 5-HT\textsubscript{1} heteroreceptors are implicated in this stimulation of ACh release and to ascertain the involvement of other subtypes of 5-HT receptors in the control of the cholinergic neurotransmission in the human bladder.

### Materials and Methods

**Drugs and Chemicals.** GR113808A and ondansetron were kindly supplied by GlaxoSmithKline (Verona, Italy). Methiothepin maleate was purchased from Torcis Cookson Inc. (Bristol, UK), and 5-carboxamidotryptamine maleate (5-CT) was obtained from RBI (Natick, MA). Hemicholinium-3,5-hydroxytryptamine creatinine sulfate, ketanserin tartrate salt, SB269970, and WAY100635 were purchased from Sigma-Aldrich (St. Louis, MO), and [methyl-\textsuperscript{3}H]choline was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

**Tissue Preparation.** Specimens from the anterior part of the urinary bladder dome were obtained from 62 male patients (average age, 69) undergoing total cystectomy due to bladder base malignancy. The study was approved by the Ethics Committee of General Hospital of Voghera (Pavia, Italy). Specimens were transported to the laboratory in oxygenated Krebs’ solution (120 mM NaCl, 4.7 mM KCl, 0.6 mM MgSO\textsubscript{4}, 25 mM NaHCO\textsubscript{3}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 2.0 mM CaCl\textsubscript{2}, and 10 mM glucose) maintained at 5 to 6°C. Muscular strips (20 mm long, 4 mm wide), free of serosal and mucosal layers and with a good alignment of the muscle bundles, were prepared and stored overnight at 4°C. Strips were then mounted isometrically in 2-ml organ baths superfused with Krebs’ solution at 37°C bubbled with 95% O\textsubscript{2} and 5% CO\textsubscript{2}. The tissues were allowed to equilibrate for 45 min apart. Aliquots (1 ml) of the superfusate were added to 3 ml of Ultima Gold (PerkinElmer Life and Analytical Sciences). Quench correction curves were established, and external standardization was used for counting efficiency. Both resting and stimulation-evoked outflow of radioactivity was expressed in disintegrations per second (bequerels) per gram of tissue. The increase of the release caused by stimulation was obtained from the difference between the total tritium outflow during 3-min stimulation plus the following 12 min (stimulation outflow period) and the calculated spontaneous outflow. The decline for the spontaneous outflow was calculated by fitting a linear regression line to the values (expressed in bequerels per gram) of 3-min samples before and after the stimulation outflow period. The drugs were added 10 (5-HT agonists) or 30 (5-HT antagonists) min before the onset of S\textsubscript{1}. The prejunctional effect of 5-HT was expressed as S\textsubscript{2}/S\textsubscript{1} ratio in comparison with the equivalent ratio obtained in the absence of the drug (control experiments).

The EFS effectiveness in producing neuronal cholinergic smooth muscle contractions (Tonini et al., 1994) was recorded with a force displacement transducer and displayed on a polygraph (Battaglia Rangoni, Bologna, Italy). This allowed us to record simultaneously in each experiment both pre- and postjunctional effects. Postjunctional effects evoked by EFS were assessed as the mean of six peak contractions evoked by S\textsubscript{1} and S\textsubscript{2}.

**Data Analysis.** Values from individual experiments were averaged, and the S.E.M. values were calculated. Concentration-response curves for prejunctional effects of 5-HT and 5-CT were constructed in the absence and in the presence of 5-HT antagonists. Drug potency estimates were evaluated as −log EC\textsubscript{50} (negative log of the molar concentration-producing half-maximal effect) by nonlinear curve fitting (GraphPad Prism, version 3.02; GraphPad Software Inc., San Diego, CA). Affinity (pA\textsubscript{4}) and apparent affinity (pK\textsubscript{D}) estimates were calculated according Schild plot analysis and the method of Furchgott (1972), respectively. The differences between groups of data were evaluated by Student’s unpaired t test. A p value less than 0.05 was considered statistically significant.

### Results

In human isolated detrusor strips, EFS produced a contractile response (7.65 ± 1.57 g, n = 27) and a marked \textsuperscript{3}H outflow (S\textsubscript{1}, 8354 ± 1243 Bq/g dry tissue) (Fig. 1, A and C). The contractile effect was cholinergic and neuronal in origin (Tonini et al., 1994), and the \textsuperscript{3}H outflow reflected the release of \textsuperscript{[3]H}ACh from neural stores as previously demonstrated (D’Agostino et al., 2000). The S\textsubscript{2}/S\textsubscript{1} ratio, 0.805 ± 0.04 in control experiments, was not affected significantly following exposure to any of the 5-HT antagonists. Conversely, 5-HT (1 nM–10 \mu M) evoked a concentration-related decrease of \textsuperscript{[3]H}ACh release by 7.92 ± 4.7% at 1 nM; pA\textsubscript{4} = 7.27 (D’Agostino et al., 2000). The facilitatory [\textsuperscript{3}H]ACh release curve of 5-HT was shifted to the right in the presence of 10 to 60 nM GR113808A, a potent and selective 5-HT\textsubscript{4} antagonist, with a progressive depression of the maximal response (approximately 50% at 60 nM; n = 4, Fig. 2), indicating a noncompetitive (func-
tional) antagonism. An apparent affinity value ($pK_b$) of 9.50 ± 0.05 was estimated.

In the presence of 1 μM methiothepin, a putative 5-HT$_{1/2/3/7}$ receptor antagonist, the prejunctional inhibitory effect observed during the exposure of 1 nM 5-HT was abolished, whereas the facilitatory component of the 5-HT curve was not affected ($pEC_{50}$ value of 8.06 ± 0.04, $n = 5$). In such a condition of 5-HT$_{1/2/3/7}$ receptor blockade, GR113808A (3–30 nM) was able to antagonize in a competitive manner the facilitatory curve induced by 5-HT (Fig. 3). According to Schild analysis, a $pA_2$ value of 9.43 ± 0.27 with a slope of 1.06 ± 0.19 ($n = 20$) was calculated.

In the presence of 3 μM GR113808A, a concentration producing a nearly complete blockade of 5-HT$_4$ receptors, 5-HT (1 nM–1 μM) caused a concentration-dependent inhibitory effect on $[^3H]ACh$ release with a maximal decrease of 22.58% ± 6.37 ($n = 5$) and a calculated $pEC_{50}$ of 7.99 ± 0.69. In this condition, the inhibition of the $[^3H]ACh$ release was reversed yielding an enhancement (by about 20% at 10 μM concentration of 5-HT) (Fig. 4). The putative 5-HT$_{1/7}$ agonist 5-CT, in a 0.1 nM to 1 μM concentration range, caused dual effects on electrically evoked $[^3H]ACh$ release (Fig. 4), similar to 5-HT (maximal decrease of 23.05% ± 0.45, $n = 5$) but with an inhibitory potency ($pEC_{50}$ of 9.03 ± 0.05) 10-fold higher than that showed by 5-HT. Both the inhibitory and facilitatory component of the curve was not affected in the presence of 100 nM ketanserin, a putative 5-HT$_2$ antagonist, or 10 nM ondansetron, a selective 5-HT$_3$ antagonist, or 3 μM GR113808A (not shown). SB269970 (0.3–30 nM), a new potent and selective 5-HT$_7$ antagonist, counteracted in a concentration-dependent fashion only the facilitatory component produced by 5-CT (100 nM–1 μM) (Fig. 5) with a $pK_b$ value of 9.07 ± 0.05 ($n = 4$).

In condition of 5-HT$_7$ blockade by 30 nM SB269970, the inhibitory component of the curve produced by 0.1 to 10 nM 5-CT was antagonized in a competitive manner by the selective 5-HT$_{1A}$ antagonist WAY100635 (0.3–3 nM) (Fig. 6). The WAY100635 affinity was calculated by Schild plot analysis ($pA_2$ value, 9.81 ± 0.10; slope, 0.98 ± 0.01; $n = 4$).

**Discussion**

The modulatory role of 5-HT on parasympathetic function in the human detrusor has been investigated previously in functional experiments dealing with nerve-mediated contractile response to EFS. Under these conditions, a facilitatory role of 5-HT in enhancing cholinergic twitch response through 5-HT$_4$ receptor activation has been demonstrated (Tonini et al., 1994). This study allowed the demonstration by means of a direct $[^3H]ACh$ release measurement that the modulatory role of 5-HT on parasympathetic nerve terminals innervating the human detrusor is more complex than that previously thought. Indeed, we found that three different
5-HT receptor sites are operative prejunctionally; namely the 5-HT₄, 5-HT₇, and 5-HT₁A receptors. The first two receptor types, which are positively coupled to adenylyl cyclase, enhance [³H]ACh release, whereas the 5-HT₁A subtype, which is coupled to Gᵢ/o effector proteins, negatively controls transmitter release. The inhibitory control of [³H]ACh release prevails at subnanomolar concentrations of 5-HT, whereas the facilitatory effect is prominent at higher 5-HT concentrations.

Based on the evidence that the two opposite mechanisms are working simultaneously, the excitatory component was studied after blockade of the inhibitory component, and vice versa. In the presence of methiothepin, a 5-HT₁/₂/₃/₇ receptor antagonist, at 1 (●), 10 (○), and 60 nM (▲), 5-HT was able to reveal a concentration-dependent inhibitory effect (maximum effect, 23% versus control), which was followed by an excitatory effect at micromolar concentrations unrelated to 5-HT₁A receptor activation.

A dependable receptor analysis of both components of the curve was carried out by means of 5-CT, a 5-HT₁/₇ receptor-preferring agonist, with an affinity/potency higher than 5-HT (see Table 1). 5-CT caused a biphasic curve characterized by an inhibitory component followed by an excitatory component. The biphasic response caused by 5-CT was unaffected
by ketanserin, ondansetron, and GR113808A, thus excluding the involvement of 5-HT₂, 5-HT₃, and 5-HT₄ receptors (see Table 1 for antagonist receptor selectivity). Conversely, in the presence of SB269970, a potent and selective 5-HT₇ antagonist (Lovell et al., 2000; Thomas et al., 2000), the excitatory component of the curve was concentration-dependently inhibited up to the suppression at 30 nM. The calculated apparent affinity value (pKᵦ) of 9.07 indicates that the second excitatory component occurring at high 5-HT/5-CT concentrations is mediated by the 5-HT₇ receptor type. In the presence of 5-HT₇ receptor blockade, a pure 5-CT-mediated inhibitory curve was observed. This curve was concentration-dependently antagonized in a competitive manner by WAY100635, a selective 5-HT₁A receptor antagonist (Forster et al., 1995). The calculated affinity value (pA₂ of 9.81) characterized unambiguously the inhibitory 5-HT receptor as the 5-HT₁A subtype.

Our findings raise the intriguing possibility that 5-HT differentially regulates, via distinct 5-HT heteroreceptors, ACh release from parasympathetic nerve terminals, which, in turn, affects detrusor smooth muscle contraction. However, the importance of these 5-HT receptors in regulating cholinergic nerve activity and bladder tone in physiological conditions is presently unknown. In the bladder, the serotonergic innervation is scanty (de Groat and Booth, 1993; Hoyle and Burnstock, 1993); therefore, it is unlikely that neuronal 5-HT may substantially contribute to the regulation of parasympathetic function physiologically. This is in agreement with our results regarding the inefficacy of various 5-HT antagonists on ACh release. Conversely, the discovery of a double-positive serotonergic feedback mechanism together with a negative one, regulating the release of ACh, may have profound implications in the pathophysiological mechanisms underlying bladder disorders and therapy. In fact, since the 5-HT₄/5-HT₇-mediated potentiation of cholinergic transmission is experimentally prevalent when the levels of the agonist in the neural cleft are elevated, one can speculate that 5-HT might play a role in pathological conditions associated with enhanced turnover rates or increased levels from tissue sources. Although endogenous 5-HT is synthesized mostly by enterochromaffin cells in the gastrointestinal tract and, to a lesser extent, in the central and peripheral nervous system, inflammatory cells including mast cells are possible sources of 5-HT (Theoharides et al., 1982; Ford and Kava, 1997). It is noteworthy that mast cells are universally found in close proximity to nerves, where factors released from mast cells and neurotransmitters from nerves (i.e., ACh) (Spanos et al., 1996) are involved in a bidirectional communication, promoting also axon reflex via local ganglia to the spinal cord and then to the brain (Maurer et al., 2003). In this respect, a well documented mastocytosis within the muscle bundles and in close proximity to bladder nerve terminals has been detected under pathological conditions associated with interstitial cystitis, a sterile bladder inflammation of unknown etiology (Theoharides et al., 2001). Interstitial cystitis is character-
ized by a variety of OAB symptoms that include urinary frequency and urgency (Chancellor and Yoshimura, 2004). It is conceivable to assume that these symptoms, partly related to parasympathetic activation, are due to 5-HT released by mast cell degranulation since in the light of our results elevated levels of 5-HT cause a marked rise in the release of ACh, the most important excitatory transmitter in the human detrusor. Indeed, an altered control of peripheral cholinergic activity at both peripheral (this study) and supraspinal (D’Agostino et al., 2004) sites.


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