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REVIEW ARTICLE

Agonist-induced desensitisation of β_3-adrenoceptors: Where, when, and how?

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β_3-Adrenoceptor agonists have proven useful in the treatment of overactive bladder syndrome, but it is not known whether their efficacy during chronic administration may be limited by receptor-induced desensitisation. Whereas the β_2-adrenoceptor has phosphorylation sites that are important for desensitisation, the β_3-adrenoceptor lacks these; therefore, it had been assumed that β_3-adrenoceptors are largely resistant to agonist-induced desensitisation. While all direct comparative studies demonstrate that β_3-adrenoceptors are less susceptible to desensitisation than β_2-adrenoceptors, desensitisation of β_3-adrenoceptors has been observed in many models and treatment settings. Chimeric β_2- and β_3-adrenoceptors have demonstrated that the C-terminal tail of the receptor plays an important role in the relative resistance to desensitisation but is not the only relevant factor. While the evidence from some models, such as transfected CHO cells, is inconsistent, it appears that desensitisation is observed more often after long-term (hours to days) than short-term (minutes to hours) agonist exposure. When it occurs, desensitisation of β_3-adrenoceptors can involve multiple levels including down-regulation of its mRNA and the receptor protein and alterations in post-receptor signalling events. The relative contributions of these mechanistic factors apparently depend on the cell type under investigation. Which if any of these factors is applicable to the human urinary bladder remains to be determined.

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1 | INTRODUCTION

In 1967, Lands, Arnold, McAuliff, Luduena, and Brown (1967) proposed a subdivision of **β-adrenoceptors** into **β_1** and **β_2**. Soon thereafter, it emerged that some β-adrenergic-like responses were not mediated by either of these two subtypes (Furchgott, 1972). This included lipolytic responses in rat white adipose tissue (Harms, Zaagsma, & van der Wal, 1974) and smooth muscle relaxation responses in rat colon (Bianchetti & Manara, 1990) and human urinary bladder (Nergardh, Boreus, & Naglo, 1977), which looked to be mediated by β-adrenoceptors based on the order of potency of catecholamines. However, the existence of a third subtype, the **β_3-adrenoceptor**, only became fully accepted after it was cloned in 1989 (Emorine et al., 1989). β_3-adrenoceptors have a more restricted expression pattern in humans than β_1- or β_2-adrenoceptors (Michel & Gravas, 2016) and have a unique and species-dependent ligand recognition profile, including a low affinity for **propranolol** and several other classic antagonists previously considered to block all β-adrenoceptor subtypes (Cernecka,

Sand, & Michel, 2014). Some agonists including **mirabegron** and **solabegron** have higher affinity for the human than the rodent β_3-adrenoceptor, whereas others including BRL 27,344 and ritobegron have higher affinity for the rodent receptor. Similarly, some agonists including **CL 316,243** have greater efficacy at the rodent than the human receptor (Baker, 2005). While β_3-adrenoceptors play a key role in lipolysis in rodents, particularly in brown adipose tissue, brown adipose tissue is largely lacking in adult humans, and lipolysis in white adipose tissue involves β_3-adrenoceptor only to a minor extent, if at all, in humans (Lönnqvist et al., 1993). Finally, the mouse β_3-adrenoceptor gene has splice variants, whereas the human orthologue does not (Evans, Papaioannou, Hamilton, & Summers, 1999).

Recently, β_3-adrenoceptor agonists have been introduced clinically for the treatment of the overactive bladder syndrome. While they effectively improve bladder symptoms and are well tolerated (Chapple, Cardozo, Nitti, Siddiqui, & Michel, 2014; Ohlstein, von Keitz, & Michel, 2012; Yoshida, Takeda, Gotoh, Nagai, & Kurose, 2018), they are not curative, implying a need for long-term treatment. Other β-adrenoceptor subtypes exhibit desensitisation upon extended exposure to an agonist, for instance, in the use of β_2-adrenoceptor agonists for treatment of preterm labour (Engelhardt et al., 1997; Frambach et al., 2005; Michel, Pingsmann, Nohlen, Siekmann, & Brodde, 1989). This can limit the effectiveness of this treatment for women with preterm labour (The Canadian Preterm Labor Investigators Group, 1992) and animal models thereof (Caritis, Chiao, & Kridgen, 1991; Lye, Dayes, Freitag, Brooks, & Casper, 1992). β_2-adrenoceptors also exhibit desensitisation upon chronic use in patients with asthma (Salpeter, Ormiston, & Salpeter, 2004), although it is debatable whether this is a treatment-limiting effect. Therefore, the question arises as to whether β_3-adrenoceptors similarly undergo agonist-induced desensitisation and how this affects their therapeutic efficacy in long-term use.

The β_2-adrenoceptor is the prototype for studying agonist-induced regulation of GPCRs (Lefkowitz, 1998). Agonist-induced β_2-adrenoceptor desensitisation can occur within seconds to minutes, which is often studied by determining the proxy parameter, **cAMP** production or receptor internalisation (January et al., 1997). It can also occur after more prolonged agonist exposure (hours to days), which often involves down-regulation of the receptor at the protein level as a result of internalisation followed by lysosomal degradation and/or reduced mRNA abundance, which in turn can be due to decreased transcription and/or mRNA stability (Bouvier et al., 1989). Moreover, it can involve regulation of G protein expression (Adie & Milligan, 1994) and the function of **ACs** (Feldman, 1989; Medina-Martinez & Garcia-Sainz, 1993) and **PDEs** (Ortiz, Dasi, Cortijo, & Morcillo, 2000). The desensitising stimulus may be endogenous catecholamines, which exhibit elevated levels in disease states such as congestive heart failure (Maisel et al., 1989; Thomas & Marks, 1978), but may also be exogenously administered catecholamines and xenobiotics, for example, for the treatment of preterm labour (Engelhardt et al., 1997; Frambach et al., 2005; Michel et al., 1989) or congestive heart failure (Mauro & Mauro, 1986). While the mechanisms found to underlie agonist-induced β_2-adrenoceptor desensitisation have been qualitatively confirmed for many other GPCRs, the relative roles of the contributing mechanisms as well as the time courses of desensitisation differ among GPCRs. Even closely related receptors such as β_2- and β_1-adrenoceptors may exhibit differential regulation (Marullo, Nantel, Strosberg, & Bouvier, 1995; Michel, Feth, Sundermann, Rascher, & Brodde, 1993).

Based on the relative lack of phosphorylation sites in its C-terminus (Emorine et al., 1989; Lelias et al., 1993; Muzzin et al., 1991; Nahmias et al., 1991), it was originally assumed that β_3-adrenoceptors are resistant to agonist-induced desensitisation. While this assumption was supported by some early studies (Granneman, 1992; Nantel et al., 1993), later work shows that desensitisation can occur in at least some settings (Nantel, Bouvier, Strosberg, & Marullo, 1995). Therefore, this review will concentrate on tissues and cell types in which β_3-adrenoceptor desensitisation occurs upon prolonged agonist exposure, and what possibly differentiates these from those where desensitization does not occur. We will discuss desensitisation of tissue and cell responses, including those of signal transduction, and the underlying molecular and cellular mechanisms. We will also discuss the implications of β_3-adrenoceptor desensitisation for chronic treatment with corresponding agonists. Of note, some studies reporting on desensitisation of responses apparently occurring via β_3-adrenoceptors have not unequivocally established that this receptor is really mediating the response under investigation, for example, in neonatal rat cardiomyocytes. For ease of reading, descriptions of β_3-adrenoceptor ligands and cell lines mentioned in the manuscript are shown in Table 1.

2 | DESENSITISATION AFFECTING CELL/TISSUE FUNCTION

Desensitisation of cell and tissue function mediated by β_3-adrenoceptors has been explored in a small number of studies based on pretreatment of animals in vivo or of isolated tissue ex vitro (Table 2). In an early study, hamsters were treated for 6 days with osmotic minipumps delivering **noradrenaline** or with daily injections of noradrenaline or **adrenaline**, followed by assessment of lipolytic responses to **isoprenaline**, noradrenaline, adrenaline, the $\beta_{2/3}$-adrenoceptor agonist BRL 37,344, or the $\beta_{1/2}$-antagonist and partial β_3-adrenoceptor agonist **CGP 12,177** in isolated adipocytes (Carpene et al., 1993). While responses to these agonists were unchanged upon treatment, those to the β_1-adrenoceptor agonist **dobutamine** and the β_2-adrenoceptor agonist **procaterol** were reduced, indicating that in contrast to β_1- and β_2-adrenoceptors, the β_3-adrenoceptor was refractory to desensitisation. Similarly, oxygen consumption in response to the β_3-adrenoceptor agonist FR 149,175 remained stable after 2 weeks of treatment with the same compound in Zucker fatty rats (Hatakeyama et al., 2004).

In mouse ileum, a 4- or 24-hr treatment with 1 $mg \cdot kg^{-1}$ of the β_3-adrenoceptor-selective agonist CL 316,243 reduced maximum relaxation in response to freshly added CL 316,243 by 52% and 56%, respectively, whereas the potency of the agonist was not affected;

TABLE 1 Characterisation of β_3-adrenoceptor (AR) ligands and cell lines mentioned in this article

β_3-Adrenoceptor ligands	
Ligand	**Property**
BRL 26,830	β_3-AR-selective agonist
BRL 37,344	β-AR with poor selectivity for β_3-AR
CGP 12,177	β_1/β_2-adrenoceptor antagonist; partial β_3-AR agonist; β_1/β_2-AR radioligand, lower affinity for β_3-AR
CL 316,243	β_3-AR-selective agonist
D7114	β_3-AR-selective agonist
Fenoterol	β_2-AR-selective agonist
FR 149,175	β_3-AR-selective agonist
Iodocyanopindolol	β_1/β_2-AR radioligand, lower affinity for β_3-AR
Isoprenaline	β-AR antagonist, all subtypes
L 748,337	β_3-AR-selective antagonist/biased agonist; also radioligand
Mirabegron	β_3-AR-selective agonist
Ritobegron	β_3-AR-selective agonist
Ro 16-8714	β_3-AR-selective agonist
Salbutamol	β_2-AR-selective agonist
SB 206,606	β_3-AR radioligand
Solabegron	β_3-AR-selective agonist
SR 59,119	β_3-AR-selective agonist
SR 59,230	β-AR antagonist, all subtypes
Cell lines	
Cell line	**Origin**
3T3-F442	Mouse adipocyte
CHO	Hamster ovary
CHW	Hamster fibroblast
HEK 293	HEK
L	Mouse sarcoma
Ltk^-	Mouse fibroblast
M1	Mouse kidney cortical duct
SK-N-MC	Human neuroblastoma

Note: For references on ligands, see Baker (2005, 2010) and Hoffmann, Leitz, Oberdorf-Maass, Lohse, and Klotz (2004).

a 1 hr pretreatment did not affect the subsequent relaxation response (Hutchinson et al., 2000). These data indicate the occurrence of desensitisation upon long-term stimulation but not shorter time stimulation. In vivo treatment with the antagonist **SR 59,230** (1 $mg \cdot kg^{-1}$) for 4 hr increased maximum relaxation to freshly added CL 316,243 by 15% with a minor increase in agonist potency (pEC_{50} from 7.27 to 7.49). In contrast, a 4-hr in vivo treatment with 3 $mg \cdot kg^{-1}$ forskolin did not affect relaxation responses to CL 316,243. Of note, the changes that occurred following treatment with CL 316,243 occurred without changes in receptor binding or mRNA, whereas the changes with SR 59,230A were associated with an increase in β_3-adrenoceptor mRNA. These data were interpreted as showing agonist-induced desensitisation of β_3-adrenoceptor tissue function and sensitisation by an antagonist. The lack of effect of forskolin may indicate that the agonist-induced desensitisation may not be cAMP-mediated.

Relaxation of rat isolated pulmonary artery was assessed after pretreatment with vehicle or 0.3-$mg \cdot kg^{-1} \cdot day^{-1}$ isoprenaline for 7 days (Davel et al., 2015). As an internal control of the action of isoprenaline, the treatment induced right and left ventricular hypertrophy. The authors used phenylephrine-contracted artery rings to test responses to various vasodilators including the β-adrenoceptor agonists, isoprenaline, metaproterenol, and the β_3-adrenoceptor-selective agonist mirabegron, the endothelium-dependent **ACh**, and the receptor-independent **NO** donor **nitroprusside**. Whether this represents a β_3-adrenoceptor-mediated response has not been fully established. Pretreatment with isoprenaline did not alter relaxation responses to any of the three β-adrenoceptor agonists or to nitroprusside but caused some degree of sensitisation of the ACh response (E_{max} from 78.4% to 92.7%, pEC_{50} 7.03 to 7.39). These findings were interpreted to represent a lack of β-adrenoceptor desensitisation, including that of β_3-adrenoceptors based on the finding that the response to mirabegron was unaffected, but enhanced NO modulation of vascular tone after isoprenaline treatment. In line with this interpretation, NO levels and cGMP content as well as immunoreactivity for **endothelial NOS** in the pulmonary artery were increased after isoprenaline treatment. Thus, in vivo studies in three different tissues of two species did not yield a consistent pattern with desensitisation being observed in one but not two other studies; moreover, duration of agonist exposure appears to be a critical factor.

Desensitisation of β_3-adrenoceptor-mediated smooth muscle relaxation following ex vivo pretreatment of organ strips with agonists has been explored in two studies. Pretreatment of human near-term myometrial strips for 5 or 15 hr with β_2-adrenoceptor agonist salbutamol right-shifted the concentration–response curve to freshly added salbutamol, indicating desensitisation of the β_2 component of the response, but did not affect relaxation by the β_3-adrenoceptor agonist SR 59,119; pretreatment with SR 59,119 for the same times did not affect either response, indicating resistance to desensitisation of the β_3-adrenoceptor component (Rouget et al., 2004). Pretreatment of rat urinary bladder strips with isoprenaline or the β_2-adrenoceptor agonist **fenoterol** for 6 hr led to a desensitisation of the β_2-adrenoceptor component of the relaxation (response to fenoterol), whereas pretreatments with isoprenaline, fenoterol, CL 316,243, or mirabegron caused less if any desensitisation of the β_3-adrenoceptor component of relaxation (response to CL 316,243 or to mirabegron; Michel, 2014).

Thus, most studies on tissue and cell responses other than signal transduction failed to detect desensitisation of β_3-adrenoceptor responses. However, the total number of studies on cell and tissue responses other than signal transduction is too limited to draw generalisable conclusions in the face of heterogeneity of species, tissues, and treatment regimens that were investigated.

TABLE 2 In vivo and in vitro studies on desensitisation of β_3-adrenoceptor function

Tissue	Species	Response	Intervention	Outcome	Reference
In vivo treatments					
White adipocytes	Hamster	Lipolysis	Noradrenaline 5 $\mu g \cdot min^{-1} \cdot kg^{-1}$ infusion, 6 days	No change	Carpene et al., 1993
White adipocytes	Rat	Oxygen consumption	FR 149,175, 0.1–3.2 $mg \cdot kg^{-1}$ twice daily orally, 2 weeks	No change	Hatakeyama, Sakata, Takakura, Manda, & Mutoh, 2004
Ileum	Mouse	Relaxation	CL 316,243, 1 $mg \cdot kg^{-1}$ injection, 1 hr	No change	Hutchinson, Evans, & Summers, 2000
			CL 316,243, 1 $mg \cdot kg^{-1}$ injection, 4 or 24 hr	Reduced	
Pulmonary artery	Rat	Relaxation	Isoprenaline 0.3 $mg \cdot kg^{-1} \cdot day^{-1}$ injection, 7 days	No change	Davel, Victorio, Delbin, Fukuda, & Rossoni, 2015
In vitro treatments					
Myometrium	Human	Relaxation	SR 59,119, 10 μM, 5 or 15 hr	No change	Rouget et al., 2004
Urinary bladder	Rat	Relaxation	Isoprenaline, CL 316,243, mirabegron 10 μM each, 6 hr	No change	Michel, 2014

Note: For details, see Section 2.

3 | β_3-ADRENOCEPTOR-INDUCED DESENSITISATION OF cAMP SIGNALLING

Stimulation of AC leading to the formation of cAMP is the canonical signalling pathway of β-adrenoceptors, including the β_3-adrenoceptor subtype (Bylund et al., 1994). While stimulation of AC can be measured directly in studies with cell membrane preparations, for convenience, most investigators have explored desensitisation of β_3-adrenoceptor-mediated cAMP formation as cAMP accumulation in intact cells. However, use of cAMP accumulation assays has limitations that may affect the interpretation of the resulting data. Most importantly, cAMP is readily degraded by PDEs. Most investigators try to limit the impact by including PDE inhibitors such as **IBMX** in their assays. However, how well such inhibitors prevent cAMP degradation and whether they may have other effects, for example, by acting on adenosine receptors, is not well understood for most model systems (Michel & Seifert, 2015). Moreover, accumulating cAMP can leave cells, making intracellular concentrations lower than they should be and concomitantly act on adenosine receptors to inhibit cAMP formation (Pacini, Sanders-Silveira, & Godinho, 2018). Due to a combination of these two factors, cellular cAMP accumulation is not necessarily linear over time. An alternative only rarely used in desensitisation studies are FRET- or BRET-based cAMP probes (Barak et al., 2008), but this has been used in only one study in the field (Milano et al., 2018). Another complication of AC and cAMP accumulation assays is that β_3-adrenoceptors not only couple to G_s proteins to stimulate AC but can also couple to G_i proteins to inhibit it (Germack & Dickenson, 2006; Hadi et al., 2013; Sato et al., 2012; Soeder et al., 1999). Thus, unless G_i proteins are inhibited, for instance, by pretreatment with pertussis toxin, AC and cAMP accumulation assays reflect a net effect of stimulation via G_s and inhibition via G_i; in some models, this net effect may be an inhibition rather than a stimulation of cAMP accumulation (Germack & Dickenson, 2006). Of note, changes in cAMP formation may at least partly result from changes in the expression or function of G proteins, AC, or PDEs (see Section 1), but only a few studies have explored this. These factors must be taken into account in the interpretation of desensitisation data at the AC/cAMP level.

Desensitisation studies at the level of AC/cAMP accumulation have been reported in four models representing endogenously expressed β_3-adrenoceptors, rat adipose tissue, neonatal rat cardiomyocytes, human myometrium, and human SK-N-MC neuroblastoma cells (Table 3). In rat white adipose tissue, stimulation for 1 hr with isoprenaline or BRL 37,344 did not desensitise the β_3-adrenoceptor component of AC stimulation but did markedly desensitise the β_1-adrenoceptor component of the response (Granneman, 1992). In a follow-up study, pretreatment with isoprenaline or the β_3-adrenoceptor agonist BRL 26,830 in vivo, reduced BRL 37,344-induced AC stimulation ex vivo (Granneman & Lahners, 1992). However, AC responses evoked by the G protein stimulator NaF were not altered, suggesting that desensitisation may have occurred at the receptor level. Cold exposure reduced AC responses to BRL 37,344 in brown adipose tissue of young and senescent rats, although to a smaller extent in the latter (Scarpace et al., 1999), indicating that the ability to undergo desensitisation within a given system may be modulated by additional factors such as ageing. In that model, desensitisation also extended to AC stimulation induced by GTP but not to that mediated by forskolin, suggesting that it may at least partly have occurred at the G protein level. Thus, data obtained in rat adipose tissues are not consistent.

In neonatal rat cardiomyocytes, treatment with noradrenaline markedly reduced cAMP accumulation in response to noradrenaline, isoprenaline, dobutamine, or procaterol; responses deemed to occur via β_1- and/or β_2-adrenoceptors (Germack & Dickenson,

TABLE 3 Studies on desensitisation of β_3-adrenoceptor-mediated cAMP formation.

Intervention	Outcome	Reference
In vivo treatments of animals		
Rat white adipose tissue		
Isoprenaline 2 × 200 μg or BRL 26,830, 2-mg injection, 8 hr	Decrease	Granneman & Lahners, 1992
Rat brown adipose tissue		
Cold exposure, 48 hr	Decrease	Scarpace, Matheny, & Thümer, 1999
In vitro treatments of tissues and cells natively expressing β_3-adrenoceptor		
Rat brown adipocytes		
Isoprenaline 100 μM, 1 hr	No change	Granneman, 1992
Neonatal rat cardiomyocytes		
Noradrenaline 100 μM, 24 hr	No change	Germack & Dickenson, 2006
Human myometrium		
SR 59,119, 10 μM, 5 or 15 hr	No change	Rouget et al., 2004
SK-N-MC human neuroblastoma cells		
Isoprenaline 100 μM, 1 hr	Decrease	Chaudhry & Granneman, 1994
Isoprenaline 10 μM, 30 min	No change	Curran & Fishman, 1996
In vitro treatments of cells transfected with β_3-adrenoceptors		
Ltk^- cells		
Isoprenaline 10 μM, 30 min	No change	Nantel et al., 1993
Isoprenaline 10 μM, 0–24 hr	No change	Nantel et al., 1995
CHW cells		
Isoprenaline 100 μM, 30 min	No change	Liggett, Freedman, Schwinn, & Lefkowitz, 1993
Isoprenaline 10 μM, 30 min	No change	Nantel et al., 1993
Isoprenaline 10 μM, 0–24 hr	No change	Nantel et al., 1995
CHO cells		
Isoprenaline 100 μM, 24 hr	Decrease	Chambers et al., 1994
Isoprenaline 100 μM, 1 hr	No change	Chaudhry & Granneman, 1994
Isoprenaline 10 μM, 15 min to 6 hr	Decrease	Candelore et al., 1996
Isoprenaline 10 μM, 24 hr	No change	Michel-Reher & Michel, 2013
Isoprenaline 10 μM, 24 hr	Decrease	Okeke, Michel-Reher, & Michel, 2018
HEK 293 cells		
Isoprenaline 100 μM, 1 hr	Decrease	Chaudhry & Granneman, 1994
Isoprenaline 10 μM, 24 hr	Decrease	Vrydag, Alewijnse, & Michel, 2009
Isoprenaline 10 nM to 10 μM, 2–24 hr	Decrease	Michel-Reher & Michel, 2013
Isoprenaline, L 755,507, CL 316,243, solabegron 10 μM, 24 hr	Decrease	Okeke, Michel-Reher, & Michel, 2017
Mouse renal epithelial cells		
Mirabegron 10 nM, 1–12 hr	No change	Milano et al., 2018
Mirabegron 10 nM, 24 hr	Decrease	

Note: cAMP accumulation has been measured as proxy for cAMP formation in some studies. Studies with transfected cells are based on human receptor except for Chaudhry and Granneman (1994), who used both rat and human receptors with similar results. For details, see Section 3.

2006). When β_1- and β_2-adrenoceptors had been blocked during pretreatment, stimulation by isoprenaline turned into inhibition, apparently a β_3-adrenoceptor-mediated and pertussis toxin-sensitive response. This inhibitory β_3-adrenoceptor response did not exhibit desensitisation. In isolated human myometrial strips, a 15 hr pretreatment with 10 μM **salbutamol** reduced cAMP accumulation in response to freshly added salbutamol, reflecting desensitisation of the β_2-adrenoceptor in this tissue; however, pretreatment with either salbutamol or SR 59,119 did not affect cAMP responses to freshly added SR 59,119, suggesting that the β_3-adrenoceptor in this

tissue did not undergo desensitisation (Rouget et al., 2004). In SK-N-MC cells, treatment with 100 μM isoprenaline for 1 hr was reported to desensitise the β_3 component of AC stimulation (Chaudhry & Granneman, 1994), whereas treatment with 10 μM isoprenaline for 30 min did not (Curran & Fishman, 1996). However, interpretation of data from SK-N-MC cells is complicated by their co-expression of β_1-adrenoceptors, which also undergo agonist-induced desensitisation (Michel et al., 1993). Thus, desensitisation and a lack thereof were both reported in tissues and cells with native expression of β_3-adrenoceptors However, the number of models being studied is too small to allow robust conclusions, particularly since each model has been studied only once.

Given the heterogeneous results in models endogenously expressing β_3-adrenoceptors, several investigators have turned to transfected cells as simpler models, potentially allowing more detailed dissection of underlying molecular pathways (Table 3). Upon expression in mouse fibroblast Ltk$^-$ cells, two studies from the same group of investigators reported a lack of change in maximum AC stimulation upon pretreatment with isoprenaline for 30 min to 24 hr, but under at least some conditions, agonist potency decreased in pretreated cells (Nantel et al., 1993, 1995). In contrast, a marked reduction of maximum responses was observed in Ltk$^-$ cells transfected with β_2-adrenoceptors. A chimeric receptor consisting of a β_3-adrenoceptor with substitution of its third cytoplasmic loop and carboxyl-terminal tail for the corresponding region of the β_2-adrenoceptors resulted in an intermediate phenotype with a minor reduction in maximum AC stimulation along with a major reduction in agonist potency. Thus, these two regions of the receptor may play a role in desensitisation but apparently do not entirely explain the difference between the two subtypes. Upon expression of β_3-adrenoceptors in Chinese hamster fibroblast CHW cells, a largely similar observation was reported by the same investigators (Nantel et al., 1993, 1995); no change in maximum AC responses upon pretreatment with isoprenaline for 30 min to 24 hr, whereas these responses were markedly reduced in cells transfected with β_2-adrenoceptors. However, an additional experiment provided an interesting insight: if cAMP accumulation in intact cells was studied, a time-dependent desensitisation of the β_3-adrenoceptor response became detectable; while this was smaller than that of the β_2 response, it pointed to the possibility that desensitisation in intact cells may involve changes in cAMP breakdown or extrusion. Overall, these results indicated that the β_3-adrenoceptor was less prone to desensitisation than the β_2-adrenoceptor for stimulation times varying between 30 min and 24 hr and that, in addition to a relative lack of phosphorylation sites present in the third intracellular loop and the carboxyl tail of the β_2-adrenoceptor, other determinants contribute to the relative resistance of the β_3-adrenoceptor to desensitisation. Little agonist-induced desensitisation of cAMP formation has also been observed in murine renal cortical collecting duct cells transfected with the human β_3-adrenoceptor (Milano et al., 2018).

Data obtained from transfected CHO cells yielded more variable data. Desensitisation of cAMP accumulation was observed in some studies with short-term (Candelore et al., 1996) and long-term

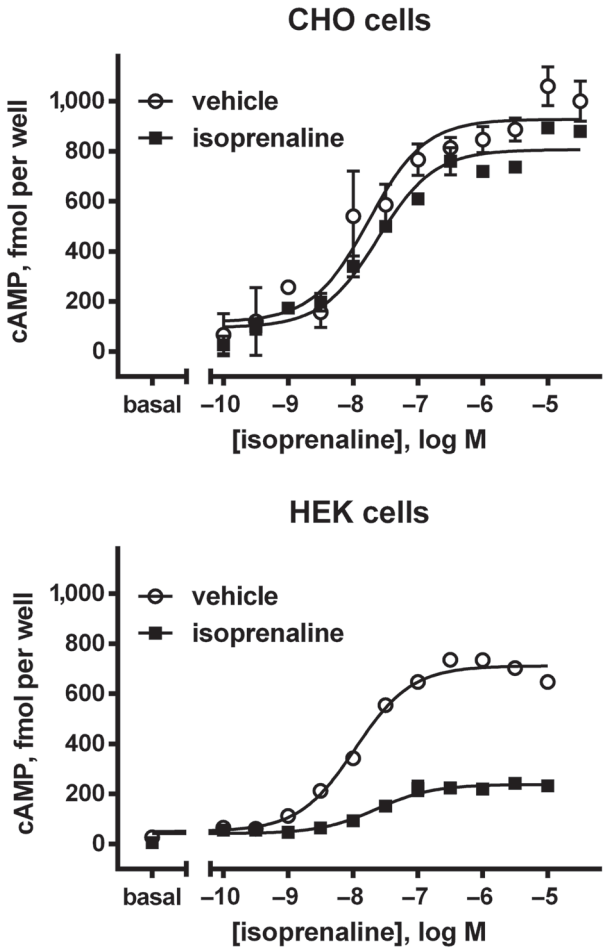

FIGURE 1 Desensitisation of cAMP accumulation mediated by human β_3-adrenoceptors transfected into CHO and HEK 293 cells. Cells were treated for 24 hr with 10 μM isoprenaline or vehicle, followed by washout and exposure to fresh isoprenaline. Desensitisation was observed in HEK 293 but not in CHO cells. Reproduced with permission from Michel-Reher and Michel (2013)

pretreatment (Chambers et al., 1994; Okeke et al., 2018), whereas no such desensitisation was observed in other studies with short-term (Chaudhry & Granneman, 1994) or long-term pretreatment (Michel-Reher & Michel, 2013; Figure 1). In HEK 293 cells, five studies consistently demonstrated agonist-induced desensitisation of AC activity and cAMP accumulation with rat (Chaudhry & Granneman, 1994) and human receptors (Chaudhry & Granneman, 1994; Michel-Reher & Michel, 2013; Okeke et al., 2017; Vrydag et al., 2009; Figure 1). Desensitisation was also observed with a truncated version of the human receptor that lacked the six C-terminal amino acids encoded by the second exon, which include two serines potentially serving as acceptor sites for phosphorylation. Thus, these and studies with chimeric receptors expressed in Ltk$^-$ cells question the role of the C-terminus of the β_3-adrenoceptor in desensitisation responses.

In conclusion, agonist-induced desensitisation of β_3-adrenoceptor-stimulated cAMP formation is consistently absent in some models, for example, Ltk$^-$ cells, and consistently present in others, for example, HEK 293 cells, whereas inconsistent data have been reported in several other models including CHO cells. However, in studies where it was

compared with the β_2-adrenoceptor, the β_3-adrenoceptor was generally found to be less prone to desensitisation than its closely related subtype. These findings, as well as limited findings on cell/tissue function other than signal transduction (see Section 2), highlight the probability that the presence of β_3-adrenoceptor desensitisation is largely determined by the cell type in which it is expressed.

3.1 | Changes in G proteins, AC, and PDEs

The formation of cAMP upon β-adrenoceptor stimulation is modulated by the abundance and activity of α-subunits of G_s and G_i and of AC isoforms, whereas the fate of the cAMP formed is modulated by the abundance and activity of PDEs. Studies with β_1- and β_2-adrenoceptors have shown that prolonged agonist exposure may cause a down-regulation of the α-subunit of G_s and/or an up-regulation of the α-subunit of G_i proteins (Adie & Milligan, 1994; Eschenhagen et al., 1992; Kimura, Miyamoto, & Oshika, 1993; Marzo et al., 1991). Therefore, several groups have explored whether a similar regulation also occurs upon β_3-adrenoceptor stimulation. To test this involvement functionally, one group treated rats with isoprenaline or BRL 26,830 and found that AC responses to freshly added BRL 37,344 were reduced in adipose tissue; whereas, responses to the direct G protein activator NaF were not altered, suggesting that the attenuated response to the agonist did not occur at the G protein or AC level (Granneman & Lahners, 1992). Others exposed young rats to cold temperature and found that responses to BRL 37,344 and the direct G protein stimulator GTP were similarly desensitised in white adipose tissue, whereas those to forskolin were not, suggesting that desensitisation may have largely occurred at the G protein level (Scarpace et al., 1999). Similar changes did not occur in senescent rats.

More direct assessments have been based on immunoblotting of G protein α-subunits. In CHO cells transfected with human β_2- or β_3-adrenoceptors, a 7-hr treatment with isoprenaline concentration-dependently reduced immunoreactivity of G_s by approximately 50% (Chambers et al., 1994). The time course of the down-regulation of G_s was comparable with both receptor subtypes, but the concentration–response curve exhibited a lower potency for down-regulation via the β_3-adrenoceptor. This could explain the observed desensitisation of isoprenaline-stimulated AC activity, as no changes in β_3-adrenoceptor protein expression were observed. A study in HEK 293 cells transfected with human β_3-adrenoceptors reported a reduction of G_s immunoreactivity upon a 24-hr treatment with isoprenaline, but the extent of this down-regulation was only about 20% (Michel-Reher & Michel, 2013). Within the same study, isoprenaline treatment reduced immunoreactivity of G_{i1} by about 50% but did not change that of G_{i2}, G_{i3}, or $G_{q/11}$. As cAMP formation induced by forskolin desensitised to a similar extent as that by isoprenaline upon pretreatment with isoprenaline, the down-regulation of $G_{s\alpha}$ was unlikely to be a major contributor to functional desensitisation of the receptor. Of note, the authors reported immunoreactivity of G protein α-subunits based on loaded protein content, that is, without correcting for a reference protein. In a follow-up communication on re-analysis of the same data (Michel-Reher & Michel, 2015), isoprenaline treatment in these cells was found to reduce GAPDH immunoreactivity by approximately 30%. Had they normalised to GAPDH expression, as many authors do, the down-regulation of G_s and G_{i1} would have been masked, and the lack of significant regulation of G_{i3} would have turned into an apparent up-regulation. A study with in vivo pretreatment of rats with isoprenaline for 7 days reported a lack of change of expression of $G_{i1/2}$ and G_{i3} protein in pulmonary artery, when normalised for α-actin expression (Davel et al., 2015). Taken together, two studies in cell lines consistently reported a down-regulation of G_s upon treatment with isoprenaline, but it is unclear to what extent this may have contributed to the observed desensitisation of cAMP formation. However, in vitro and in vivo studies did not find an up-regulation of G_i, suggesting that this did not contribute to desensitisation.

In one of the above studies, performed in HEK 293 cells, pretreatment with isoprenaline reduced cAMP responses to forskolin to a similar extent as those to freshly added isoprenaline, suggesting that desensitisation of the cAMP formation could fully be explained by an alteration in the level of AC (Michel-Reher & Michel, 2013). Reduced AC activity was confirmed to have a major role in desensitisation in a follow-up study from these investigators (Okeke et al., 2017). However, the same investigators observed no change in forskolin-stimulated relaxation in rat urinary bladder after 6 hr of agonist treatment (Michel, 2014); similarly, rat ileum relaxation induced by forskolin was also found to be unaltered after 4 hr of agonist treatment (Hutchinson et al., 2000). A study in pulmonary artery demonstrated that a 7 day pretreatment of rats with isoprenaline reduced **PDE5** expression (Davel et al., 2015). While this may be relevant to enhanced endothelial NOS activity in the same study, it is unlikely to have contributed to β-adrenoceptor function because PDE5 is selective for cGMP, not cAMP; accordingly, relaxation responses to agonists including the β_3-adrenoceptor selective mirabegron were unchanged. An in vitro study with human myometrium did not detect changes in PDE4 upon pretreatment with β_2-selective salbutamol or β_3-adrenoceptor selective SR 59,119 (Rouget et al., 2004). Taken together, these studies suggest that prolonged stimulation of β_3-adrenoceptors can change the activity and/or expression of AC or PDEs, but the number of reports available is insufficient for a robust conclusion in the face of the divergent outcomes across models.

The local intracellular concentration of cAMP resulting from the net effect of the above factors leads to activation of more distal signalling responses such as activation of PKA or **Epac** or modulation of expression of various transcription factors. However, these have been investigated only rarely. One study used CHW and mouse sarcoma L cells, each transfected with either β_2- or β_3-adrenoceptors and treated for 10 min or 24 hr with isoprenaline (Nantel et al., 1995). In CHW cells, both short and extended treatment increased PKA activity, irrespective of which subtype had been stimulated. However, in L cells, both subtypes increased PKA activity only after short but not after prolonged stimulation, again similarly with both subtypes.

In conclusion, some studies have presented evidence that components of the signal transduction chain downstream of the β_3-adrenoceptor may exhibit desensitisation upon prolonged agonist exposure. However, such changes do not appear consistent across model systems and have been studied in too few instances to allow robust conclusions.

β_3-adrenoceptors couple not only to stimulation of cAMP formation but also to that of other signalling pathways, including phosphorylation of the MAPKs **ERK** and **p38**, and to extracellular acidification rates (Gerhardt, Gros, Strosberg, & Issad, 1999; Hadi et al., 2013; Robay, Toumaniantz, Leblais, & Gauthier, 2005; Sato, Horinouchi, Hutchinson, Evans, & Summers, 2007, 2008; Soeder et al., 1999; Steinle, Booz, Meininger, Day, & Granger, 2003). Interestingly, only one report published in abstract form has explored desensitisation of signalling pathways other than cAMP formation (Okeke et al., 2018). While the authors detected desensitisation of ERK phosphorylation in transfected CHO cells, this exhibited a different pattern from that of cAMP accumulation within the same series of experiments. These data suggest that different signalling responses to β_3-adrenoceptor stimulation may undergo differential regulation upon agonist exposure.

4 | CHANGES IN β_3-ADRENOCEPTOR PROTEIN EXPRESSION, PHOSPHORYLATION, AND LOCALISATION

Two main tools have been used for the detection of receptor expression at the protein level, radioligand binding, and immunodetection; while the former is easier to quantify, the latter often allows better spatial resolution in histological or cytological studies. However, both approaches can be problematic when applied to β_3-adrenoceptors. Standard β-adrenoceptor radioligands such as **[^{3}H]-CGP 12,177** or **[^{125}I]-iodocyanopindolol** have much lower affinity for β_3-adrenoceptors as compared to β_1- and β_2-adrenoceptors (Niclauß, Michel-Reher, Alewijnse, & Michel, 2006). The high concentrations required to use them as labels for β_3-adrenoceptors imply saturation of the other two subtypes, high non-specific binding and binding to non-β-adrenoceptor sites including **5-HT receptors** (Hoyer, Engel, & Kalkman, 1985a,b) and a protein later identified as a member of the TM9SF multi-spanning membrane protein family (Sugasawa et al., 2001, 1997). Thus, only one group has extensively used [^{3}H]-CGP 12,177 in intact transfected cells (Baker, 2005, 2010). In transfected cells, not expressing any of the other subtypes, [^{125}I]-iodocyanopindolol appears more suitable and is routinely used (Emorine et al., 1989; Hoffmann et al., 2004; Niclauß et al., 2006; Vrydag et al., 2009). It has also been used in some tissues with native expression of the receptor, but the considerable non-specific binding has limited interpretation of the data (Hutchinson, Chernogubova, Sato, Summers, & Bengtsson, 2006; Rathi et al., 2003; Roberts, Molenaar, & Summers, 1993; Rouget et al., 2005; Schneider & Michel, 2010). [^{3}H]-SB 206,606, a tritiated form of the active RR-enantiomer of the agonist BRL 37,344, has been introduced as a β_3-adrenoceptor-selective radioligand (Muzzin et al., 1994) but the reported K_d values of 38–93 nM limit its use. Accordingly, [^{3}H]-SB 206,606 has only rarely been used (Deng et al., 1996) but has been employed in desensitisation studies (Klaus et al., 1995). More recently, a tritiated version of the antagonist **L 748,337** has been validated as a β_3-adrenoceptor-selective radioligand (van Wieringen, Michel-Reher, Hatanaka, Ueshima, & Michel, 2013). Its affinity of 2–3 nM makes [^{3}H]-L 748,337 the radioligand of choice for the labelling of human β_3-adrenoceptors but due to a lower affinity in rodents, may be less useful in mice and rats, species that are often used to study β_3-adrenoceptors. [^{3}H]-L 748,337 has also been used in desensitisation studies of the human β_3-adrenoceptor (Michel-Reher & Michel, 2013).

While many β_3-adrenoceptor antibodies lack useful target specificity, some have recently been validated based on stringent criteria (Cernecka, Ochodnicky, Lamers, & Michel, 2012; Cernecka, Pradidarcheep, Lamers, Schmidt, & Michel, 2014; Kullmann et al., 2009; Limberg et al., 2010); however, even the usefulness of these antibodies is limited to some species and applications. A reported monoclonal antibody with good validation data (Chamberlain et al., 1999) unfortunately has never become widely distributed and is no longer available even from the original authors. Therefore, antibodies have only played a minor if any role in desensitisation studies. Use of transfected, tagged receptors may be an interesting alternative as they can be detected immunologically in a more robust way (Milano et al., 2018).

GPCRs can be present in a high- and low-affinity state for agonists (van Wieringen, Booij, Shalgunov, Elsinga, & Michel, 2013). The high-affinity state reflects the receptor-G protein coupled form and the low affinity, the uncoupled form of the receptor. Accordingly, desensitisation can also be reflected by a shift between these two states. Studies with transfected L cells expressing little if any of other subtypes did not find a shift in high- versus low-affinity state of the β_3-adrenoceptor upon treatment with 10 μM isoprenaline for 30 min, despite the presence of agonist-induced down-regulation of β_3-adrenoceptors (Nantel et al., 1993). A lack of agonist-induced shift of agonist affinity state was also observed with a chimeric receptor consisting of a β_3-adrenoceptor in which the third cytoplasmic loop and the C-terminus had been replaced with corresponding stretches of the β_2-adrenoceptor. In contrast, β_2-adrenoceptors exhibited a reduction in agonist high-affinity states upon agonist exposure.

Chronic exposure to agonists can lead to a reduction in total cellular receptor number, which has been widely observed with β_1- and β_2-adrenoceptors (Engelhardt et al., 1997; Matthews, Falckh, Molenaar, & Summers, 1996). Only few studies have explored this for the β_3-adrenoceptor, mostly using [^{125}I]-iodocyanopindolol as the radioligand (Table 4). Treatment with the β_3-adrenoceptor agonist Ro 16-8714 for 24–48 hr decreased receptor protein expression in brown adipose tissue in lean and obese Zucker rats (Revelli et al., 1992). In contrast, treatment with CL 316,243 for up to 24 hr did not reduce β_3-adrenoceptor protein expression in mouse ileum, despite causing desensitisation of relaxation responses (Hutchinson et al., 2000). Several studies in rats (Sun et al., 2011), dogs (Cheng et al., 2001), and patients with congestive heart failure (Moniotte et al., 2001; Napp et al., 2009) reported an increased abundance of β_3-adrenoceptor

TABLE 4 Studies on regulation of β_3-adrenoceptor protein and mRNA by agonist exposure

Intervention	Protein	mRNA	Reference
In vivo treatments of animals			
Rat brown adipose tissue			
Ro 16-8741, 0.87 mg·kg^{-1} orally, every 24 hr	Decrease	Decrease	Revelli, Muzzin, & Giacobino, 1992
Noradrenaline 2 × 250 μg injection, 8 hr	n.r.	Decrease	Granneman & Lahners, 1992
Isoprenaline 2 × 200 μg injection, 8 hr	n.r.	Decrease	
BRL 26,830, 2 × 2 mg injection, 8 hr	n.r.	Decrease	
Mouse brown adipose tissue			
CL 316,243, 1 mg·kg^{-1} injection, 1–24 hr	n.r.	Decrease[a]	Hutchinson et al., 2000
Rat white adipose tissue			
Noradrenaline 2 × 250 μg injection, 8 hr	n.r.	Decrease	Granneman & Lahners, 1992
Isoprenaline 2 × 200 μg injection, 8 hr	n.r.	Decrease	
BRL 26,830, 2 × 2 mg injection, 8 hr	n.r.	Decrease	
BRL 26,830, 10 mg·kg^{-1} injection, 4 hr	n.r.	Decrease	Granneman & Lahners, 1995
FR 149,175, 0.1–3.2 mg·kg^{-1} twice daily p.o., 2 weeks	n.r.	No change	Hatakeyama et al., 2004
Mouse white adipose tissue			
CL 316,243, 1 mg·kg^{-1} injection, 1–24 hr	n.r.	Decrease	Hutchinson et al., 2000
Mouse ileum			
CL 316,243, 1 mg·kg^{-1} injection, 1–24 hr	No change (63–77)	No change	Hutchinson et al., 2000
In vitro treatments of tissues and cells natively expressing β_3-adrenoceptor			
Neonatal rat cardiomyocytes			
Noradrenaline 100 μM, 24 hr	Increase	Increase	Germack & Dickenson, 2006
Noradrenaline 100 mM, 72 hr	n.r.	Increase	Watts et al., 2013
Hamster brown adipocytes			
Isoprenaline 1 μM, 2–8 hr	n.r.	Decrease	Klaus et al., 1995
Noradrenaline 1 μM, 2–8 hr	n.r.	Decrease	
D7114, 1 μM, 2–8 hr	n.r.	Decrease	
Mouse brown adipocytes			
Noradrenaline 100 nM, 24 hr	n.r.	Decrease	Bengtsson, Redegren, Strosberg, Nedergaard, & Cannon, 1996
3T3-F442 mouse adipocytes			
Isoprenaline 100 μM, 4–30 hr	Increase (39)	Increase	Thomas, Holt, Schwinn, & Liggett, 1992
Isoprenaline 100 μM, 2–8 hr	n.r.	Decrease	Granneman & Lahners, 1994
Isoprenaline 100 μM, 4 hr	n.r.	Decrease	Granneman & Lahners, 1995
Human myometrial strips			
Salbutamol or SR 59,119, 10 μM, 15 hr	n.r.	No change	Rouget et al., 2004
SK-N-MC human neuroblastoma cells			
Isoprenaline 100 μM, 2–6 hr	n.r.	No change	Granneman & Lahners, 1994
Isoprenaline 1–100 nM, 1–24 hr	No change (189)	n.r.	Curran & Fishman, 1996
In vitro treatments of cells transfected with β_3-adrenoceptor			
CHW cells			
Isoprenaline 100 μM, 6–24 hr	No change (5,900)	n.r.	Liggett et al., 1993
Isoprenaline 10 μM, 1–24 hr	No change (281)	No change	Nantel, Marullo, Krief, Strosberg, & Bouvier, 1994

(Continues)

TABLE 4 (Continued)

Intervention	Protein	mRNA	Reference
Isoprenaline 10 μM, 2–24 hr	No change (200–350)	n.r.	Nantel et al., 1995
CHO cells			
Isoprenaline 100 μM, 0–24 hr	No change (3,000)	n.r.	Chambers et al., 1994
HEK 293 cells			
Isoprenaline 100 μM, 1 hr	No change (750)	n.r.	Chaudhry & Granneman, 1994
Isoprenaline 10 μM, 24 hr	No change (306)	n.r.	Michel-Reher & Michel, 2013
L cells			
Isoprenaline 10 μM, 1–24 hr	Decrease (122)	Decrease	Nantel et al., 1994
Isoprenaline 10 μM, 3–24 hr	Decrease (200–350)	n.r.	Nantel et al., 1995
M1 cells			
Mirabegron 10 nM, 6–24 hr	Decrease	n.r.	Milano et al., 2018

Note: Where available, baseline β_3-adrenoceptor density (fmol·mg^{-1}·protein) is shown in parentheses. Note that some of the mRNA expression changes were transient, that is, not found at all time points investigated. Studies in transfected cells refer to the human receptor. For details, see Sections 4 and 5. n.r.: not reported.

[a]Decrease observed for β_{3a}- but not β_{3b}-adrenoceptor.

protein, although only some of them were based on validated antibodies. Results from a study in a rat model of colitis (Vasina et al., 2008) are difficult to interpret for two reasons. First, it is based on immunohistochemistry; while using a validated antibody (Cernecka, Pradidarcheep, et al., 2014), the use of immunohistochemistry resulted in qualitative rather than quantitative conclusions on expression levels. Second, treatment with the β_3-adrenoceptor agonist SR 58,611 for 7 days improved the colitis, and the disease associated down-regulation of the receptor, making it difficult to determine whether the apparent increase in receptor protein expression was a direct consequence of agonist exposure or reflected improvement in the pathology that had caused the down-regulation.

Others have used tissue samples or cell lines endogenously expressing β_3-adrenoceptors with ex vivo or in vitro exposure to agonist (Table 4). Surprisingly, treatment of murine adipocyte 3T3-F442 cells with isoprenaline time-dependently increased [^{125}I]-iodocyanopindolol binding (Thomas et al., 1992). A similar increase was observed in neonatal rat cardiomyocytes (Germack & Dickenson, 2006; Watts et al., 2013). Such agonist-induced increases in β_3-adrenoceptor expression may relate to the cAMP response elements in the receptor gene (Liggett & Schwinn, 1991; Thomas et al., 1992). In contrast, studies in human myometrial strips (Rouget et al., 2004) or in SK-N-MC neuroblastoma cells did not show any alterations in β_3-adrenoceptor protein expression upon agonist treatment (Curran & Fishman, 1996). The negative studies showed a down-regulation of β_1- or β_2-adrenoceptors. Taken together, studies with endogenously expressed rat, mouse, or human β_3-adrenoceptors showed highly heterogeneous results with up-regulation, no change, and down-regulation; however, whenever investigated, findings of corresponding mRNA were concordant with those of protein expression (Hutchinson et al., 2000; Revelli et al., 1992; Thomas et al., 1992).

Apparently because of the better detectability of β_3-adrenoceptor protein (see above), most investigators have explored agonist-induced regulation at the protein level in transfected cells. In line with the findings in tissues and cells natively expressing β_3-adrenoceptors, the results appear to be cell type-dependent (Table 4). A lack of agonist-induced down-regulation has consistently been reported in transfected CHW cells (Liggett et al., 1993; Nantel et al., 1995, 1994). While most studies have transfected a β_2-adrenoceptor as a positive control for a receptor that undergoes agonist-promoted down-regulation, a chimeric receptor that comprised the β_3-adrenoceptor up to proline-365 of the cytoplasmic tail and the C-terminus of the β_2-adrenoceptor was used in one study (Liggett et al., 1993). While the β_3-adrenoceptor did not exhibit down-regulation and the β_2-adrenoceptor did undergo a marked down-regulation, the chimeric receptor had an intermediate phenotype being closer to that of the β_2-adrenoceptor, providing additional insight into the molecular basis of down-regulation. A lack of β_3-adrenoceptor protein down-regulation has also been reported in transfected CHO cells (Chambers et al., 1994) and HEK 293 cells (Chaudhry & Granneman, 1994; Michel-Reher & Michel, 2013). In contrast, agonist-induced down-regulation of β_3-adrenoceptor protein has consistently been observed upon transfection in L cells (Nantel et al., 1995, 1994). This agonist-promoted down-regulation of the β_3-adrenoceptor was found to result from a reduction in the mRNA encoding the receptor and not a change in the half-life of the protein, pointing to a change in RNA stability as the underlying mechanism (Nantel et al., 1994; see below). Using tagged β_3-adrenoceptors, their down-regulation was observed in transfected murine renal epithelial cells; in a time course experiment, this was absent after 1–3 hr, moderate after 6–12 hr, and marked with the 24-hr treatment (Milano et al., 2018).

Other than down-regulation, the number of agonist accessible receptors can also change through an altered subcellular localisation. Thus, receptors may migrate from the plasma membrane to an intracellular compartment where they are no longer accessible to

hydrophilic agonists such as catecholamines. This process is termed internalisation, sequestration, or endocytosis. It is believed to mainly play a role in short-term re-sensitisation and has extensively been explored for β_2-adrenoceptors (Lefkowitz, 1998). Studies in SK-N-MC neuroblastoma cells natively expressing β_3-adrenoceptors (Curran & Fishman, 1996) and in transfected CHW cells (Liggett et al., 1993; Nantel et al., 1993) did not detect agonist-induced internalisation of the β_3-adrenoceptor, with concomitantly expressed β_1-adrenoceptors (Curran & Fishman, 1996) or transfected β_2-adrenoceptors (Nantel et al., 1993) serving as an internal positive control. A lack of internalisation was also reported upon expression of human β_3-adrenoceptors in mouse renal epithelial cells (Milano et al., 2018). The observed lack of β_3-adrenoceptor sequestration is in-line with the absence of phosphorylation sites in the C-terminus of the receptor deemed relevant for desensitisation (Emorine et al., 1989; Lelias et al., 1993; Muzzin et al., 1991; Nahmias et al., 1991), believed to be a molecular prerequisite for internalisation of the other β-adrenoceptor subtypes (Lefkowitz, 1998).

Phosphorylation of C-terminal serine and threonine residues by GPCR kinases (GRKs), also known as **β-adrenergic receptor kinases**, is considered as an important molecular step in the agonist-induced desensitisation of β_2-adrenoceptors since removal of these sites by site-directed mutagenesis resulted in largely diminished desensitisation (Bouvier et al., 1988; Bouvier, Leeb-Lundberg, Benovic, Caron, & Lefkowitz, 1987). Also, adding extra phosphorylation sites increases receptor–arrestin interaction (Zindel et al., 2015), an interaction believed to initiate the process of desensitisation by sterically interfering with G protein engagement and promoting receptor endocytosis via the association of β-arrestin with components of the endocytic machineries (Goodman et al., 1996; Laporte, Oakley, Holt, Barak, & Caron, 2000). The human (Emorine et al., 1989; Lelias et al., 1993), rat (Muzzin et al., 1991) and murine β_3-adrenoceptor (Nahmias et al., 1991) contain only few C-terminal serine or threonine residues, and those few are located in positions considered unfavourable for phosphorylation, leading to the hypothesis that this paucity in phosphorylation sites may underlie the generally observed reduced propensity of the β_3-adrenoceptor to undergo desensitisation when compared with the β_2-adrenoceptor. To test the functional consequence of this lack of phosphorylation sites, CHW cells were transfected with human β_2-adrenoceptor, β_3-adrenoceptor, or chimera thereof (Liggett et al., 1993). In whole-cell phosphorylation studies with such cells, β_2-adrenoceptor exhibited isoprenaline-induced phosphorylation whereas β_3-adrenoceptors did not, even if a GRK was overexpressed. A chimeric receptor, consisting of the β_3-adrenoceptor with the cytoplasmic tail of the β_2-adrenoceptor, however, exhibited phosphorylation. To directly test whether the difference in phosphorylation may be responsible for the different desensitisation observed between the two receptor subtypes, a series of β_3/β_2-adrenoceptor chimeras were expressed in HEK 293 cells and tested for their ability to undergo internalisation and β-arrestin recruitment (Angers, 2003). For this study, β-arrestin recruitment was measured by monitoring agonist-promoted BRET between β-arrestin and the receptors (Angers et al., 2000). In contrast to the β_2-adrenoceptor that promoted a robust recruitment of β-arrestin and was internalised upon agonist stimulation, no such recruitment or internalisation was observed for the wild-type β_3-adrenoceptor (Figure 2). This is consistent with the observation that the β_3-adrenoceptor does not undergo agonist-promoted phosphorylation (Liggett et al., 1993), a post-translational modification needed to favour high-affinity interaction with β-arrestin. The introduction of the second intracellular loop and carboxyl tail of the β_2-adrenoceptor into the β_3-adrenoceptor (β_3-3232) restored agonist-promoted internalisation to some extent (Jockers, Da Silva, Strosberg, Bouvier, & Marullo, 1996; Figure 2) but was insufficient to restore any detectable β-arrestin recruitment suggesting that a β-arrestin-independent mechanism may be involved in the internalisation of the β_3-3232. A similar lack of β-arrestin recruitment was also observed for the other chimeras tested (β_3-3233, β_3-3323, β_3-3332, β_3-3322, and β_3-3222). These data also show that linear determinants from the β_2-adrenoceptor, including phosphorylation sites, are not sufficient to instill the high-affinity interaction with β-arrestin and suggest that a more complex conformational rearrangement must be required. Interestingly, a chimeric receptor in which the carboxyl tail of the β_3-adrenoceptor was inserted in the β_2-adrenoceptor (β2-2223) showed a reduced but not abolished ability to recruit β-arrestin. This confirmed a role for the C-tail phosphorylation sites in the recruitment of β-arrestin to the β_2-adrenoceptor. However, it also indicated that while being important to stabilise a high-affinity interaction, it may not be essential for the initial recruitment. These findings are consistent with a

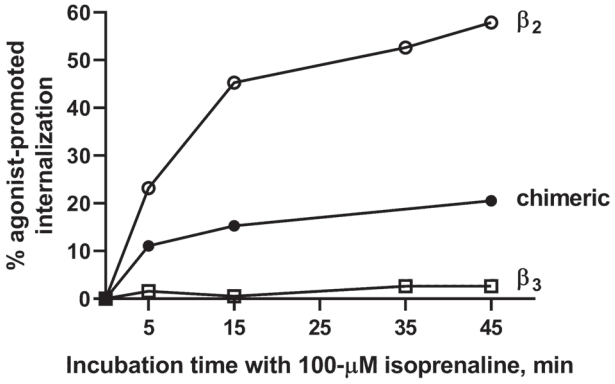

FIGURE 2 Time course of agonist-promoted internalisation of the β_3-, β_2-, and a β_3-3232 chimeric adrenoceptor. HEK 293 cells stably expressing the β_2-adrenoceptor, β_3-adrenoceptor, or the β_3-3232 (chimeric) were stimulated with 100 μM isoprenaline for the indicated times. Each of the constructs was tagged at their N-terminus with a Myc-tag allowing their immuno-detection using an anti-Myc antibody. Internalisation was assessed by flow cytometry and expressed as % loss of cell surface receptor immunoreactivity. Reproduced with permission from Angers (2003). The data show that in contrast to the β_2-adrenoceptor that undergoes a time-dependent internalisation upon isoprenaline stimulation, the β_3-adrenoceptor does not. However, introducing the second intracellular loop and the carboxyl-terminus of the β_2-adrenoceptor within the β_3-adrenoceptor backbone (β_3-3232) restored agonist-promoted internalisation to some extent

recent observation suggesting that β-arrestin may have a bitopic mode of interaction involving a distinct domain of the receptor (Cahill et al., 2017; Thomsen et al., 2016).

Taken together, these data demonstrate that the presence of agonist-induced down-regulation of β_3-adrenoceptor protein appears to be a cell type-dependent phenomenon, with a lack of down-regulation being the more frequent phenotype. In contrast to β_2-adrenoceptors, β_3-adrenoceptors apparently do not undergo agonist-induced internalisation. This may be related to their lack of C-terminal phosphorylation sites and their limited ability to bind β-arrestin.

5 | CHANGES IN β_3-ADENOCEPTOR mRNA EXPRESSION

Investigation of mRNA expression can serve to explore whether changes in protein expression can be explained by those of corresponding mRNA abundance. A small number of studies have applied them in this way in the β_3-adrenoceptor field (Germack & Dickenson, 2006; Revelli et al., 1992; Thomas et al., 1992). However, most investigators have explored alterations in β_3-adrenoceptor mRNA expression as a proxy for those of protein expression, presumably because mRNA levels are technically easier to quantify for β_3-adrenoceptors than those of functional protein (see Section 4). Four considerations apply when interpreting mRNA data with regard to functional receptor desensitisation. First, any reduction in mRNA abundance is only likely to translate into changes in receptor responsiveness after hours or days because the half-life of β_3-adrenoceptor protein has been estimated at 15–18 hr (Nantel et al., 1994) whereas that of β_3-adrenoceptor mRNA has been estimated at 30–90 min (Bengtsson et al., 1996; Granneman & Lahners, 1995; Klaus et al., 1995). Hence, changes in mRNA expression have mostly been studied in the context of extended (hours to days) exposure to agonists. Second, any change in mRNA abundance may result from those of transcription and/or mRNA stability. While altered mRNA expression upon agonist treatment mainly reflects mRNA stability for β_2-adrenoceptors (Danner, Frank, & Lohse, 1998; Hadcock, Wang, & Malbon, 1989), it appears to reflect transcription for β_3-adrenoceptors (Bengtsson et al., 1996; Granneman & Lahners, 1995). Of note, studies on transfected receptors provide limited insight into possible changes in receptor transcription as this parameter is typically not controlled by the native promoter of the receptor but rather by an artificial one such as that derived from cytomegalovirus. Hence, with few exceptions (Nantel et al., 1994), studies on agonist effects on β_3-adrenoceptor mRNA abundance have been reported in cells and tissues natively expressing the receptor. Third, the promotor region of the β_3-adrenoceptor contains four cAMP response elements (Liggett & Schwinn, 1991; Thomas et al., 1992) and an intron containing a putative enhancer element specific for adipose tissue (Granneman, Lahners, & Rao, 1992). This may at least partly explain tissue-selective regulation of mRNA expression. However, there are also less well-defined mechanisms leading to inhibition of transcription upon agonist exposure (Granneman & Lahners, 1995). The net effect of these mechanisms in each cell type is difficult to predict. Finally, the predictive value of mRNA levels for corresponding β_3-adrenoceptor protein expression has been questioned (Nantel et al., 1994).

Several investigators have treated lean and obese Zucker rats (Granneman & Lahners, 1992; Revelli et al., 1992), hamster (Klaus et al., 1995), or mice (Hutchinson et al., 2000) in vivo with agonists including isoprenaline, noradrenaline, BRL 26,830, CL 316,243, D7114, or Ro 16-8741 for 1–48 hr and mostly observed a down-regulation of β_3-adrenoceptor mRNA. In some cases, such down-regulation was transient and in mice only the β_{3a} was affected but not the β_{3b} splice variant of the receptor. Cold exposure, considered to stimulate a release of endogenous noradrenaline, also yielded a transient down-regulation (at 1–12 hr but not 24 hr) in mice (Bengtsson et al., 1996) and did not exhibit down-regulation at later time points (48–72 hr) in rats (Granneman & Lahners, 1992; Scarpace et al., 1999). Conversely, surgical denervation increased β_3-adrenoceptor mRNA expression in rats (Granneman & Lahners, 1992). Acute treatment with noradrenaline similarly reduced β_3-adrenoceptor mRNA expression in white and brown adipose tissues in rats (Granneman & Lahners, 1992). A study in mice confirmed a similar reduction in white and brown adipose tissues for the β_{3a}-adrenoceptor, but a reduction in β_{3b}-adrenoceptor mRNA was only observed in white adipose tissue (Hutchinson et al., 2000). A similar regulation of both murine splice variants has also been reported by others in vivo and in rat adipocyte 3T3-F442 cells (Granneman & Lahners, 1995). In contrast, treatment of Zucker fatty rats with the β_3-adrenoceptor agonist FR-149,175 did not change β_3-adrenoceptor mRNA expression (Hatakeyama et al., 2004).

Investigations with in vitro treatment of tissues and cells natively expressing β_3-adrenoceptors have yielded conflicting results. Increased mRNA expression was reported in 3T3-F442 mouse adipocytes following treatment with isoprenaline by some (Thomas et al., 1992), but a decreased expression was reported by others (Granneman & Lahners, 1995). The decrease was mimicked by treatment with 8-Br-cAMP and found to be due to reduced transcription and not to altered mRNA degradation. In neonatal rat cardiomyocytes, treatment with noradrenaline increased β_3-adrenoceptor mRNA expression (Watts et al., 2013). Similarly, in congestive heart failure, a condition associated with an increased sympathetic tone, an increased cardiac β_3-adrenoceptor mRNA expression is consistently exhibited in rats (Ozakca et al., 2013; Zhao et al., 2013), dogs (Cheng et al., 2001), and humans (Moniotte et al., 2001). In SK-N-MC human neuroblastoma cells, treatment with 8-Br-cAMP increased β_3-adrenoceptor mRNA expression, whereas treatment with isoprenaline had no effect (Granneman & Lahners, 1994). Similarly, dexamethasone and the PKC activator **phorbol myristate acetate** did not change β_3-adrenoceptor mRNA levels in SK-N-MC cells but reduced it in 3T3-F442 cells (Granneman & Lahners, 1995). This reduction by phorbol myristate acetate in 3T3-F442 cells was confirmed by other investigators (Feve, Pietri-Rouxel, El Hadri, Drumare, & Strosberg, 1995). Treatment of human myometrial strips with 10 μM salbutamol or SR 59,119 also failed to change β_3-adrenoceptor

mRNA expression (Rouget et al., 2004). Among the limited studies with transfected β_3-adrenoceptors, reduced mRNA expression was reported upon expression in L cells (Nantel et al., 1995) but no change with expression in CHW cells (Nantel et al., 1994).

In conclusion, data on agonist-induced regulation of β_3-adrenoceptor mRNA expression are inconclusive when interpreted across tissues (Table 4). However, they appear more consistent within a tissue, further supporting the view that regulation of β_3-adrenoceptor presence and function depends critically on cellular context, in the majority of cases yielding a down-regulation. Of note, agonist-induced up-regulation has also been reported in some cases. The molecular basis for this appears to involve cAMP-mediated inhibitory and stimulatory effects on β_3-adrenoceptor gene transcription, the balance of which may be cell type-specific.

6 | EVIDENCE OF SYNTHESIS AND CONCLUSIONS

Based upon a lack of consensus phosphorylation sites in its C-terminal and third intracellular domain, it had been assumed that β_3-adrenoceptor-mediated functions are resistant to desensitisation. The above data demonstrate that resistance to desensitisation is present in many but not all tissues and cell types (Figure 3). When desensitisation is observed, it often is less pronounced and/or requires longer agonist exposure as compared to that of β_1- and β_2-adrenoceptors. Moreover, the presence of desensitisation and the molecular level at which it occurs depend strongly on the cell type in which the receptor is expressed.

6.1 | What are the molecular alterations underlying β_3-adrenoceptor desensitisation?

All available data suggest that β_3-adrenoceptors do not undergo internalisation upon agonist exposure (Curran & Fishman, 1996; Liggett et al., 1993; Milano et al., 2018; Nantel et al., 1993). Given that there is strong evidence that internalisation requires phosphorylation (Lefkowitz, 1998) and that such phosphorylation sites are missing in the β_3-adrenoceptor (Emorine et al., 1989), this is not too surprising.

Down-regulation of β_3-adrenoceptor protein upon agonist exposure has been reported only in one in vivo and several studies with transfection into Ltk^- cells, whereas most studies have found no change or even increases in receptor expression (Table 4). The technical difficulties of detecting β_3-adrenoceptors may explain this scarcity of data (see Section 4). Many more studies have demonstrated a down-regulation of β_3-adrenoceptor mRNA (Table 4), but in most cases, we do not know whether this translates into changes in expression of functional receptor protein. Apparently, reduced mRNA expression is caused by changes in transcription, not those of mRNA stability (Bengtsson et al., 1996).

Interestingly, desensitisation of β_3-adrenoceptor-mediated function can occur despite a documented lack of down-regulation of receptor protein (Chambers et al., 1994; Chaudhry & Granneman, 1994; Hutchinson et al., 2000; Michel-Reher & Michel, 2013), indicating that changes at the post-receptor level must contribute to desensitisation. Such changes at the level of G protein expression (Chambers et al., 1994; Michel-Reher & Michel, 2013), cAMP formation in the presence of direct G protein stimulators (Scarpace et al., 1999), and AC activity (Michel-Reher & Michel, 2013; Okeke et al., 2017) have been reported, but examples also exist where desensitisation was not accompanied by changes at these levels (Davel et al., 2015; Granneman & Lahners, 1992; Hutchinson et al., 2000; Scarpace et al., 1999).

β_3-adrenoceptor desensitisation can be induced in some models by agents not acting on the receptor, for example, forskolin (Michel-Reher & Michel, 2013) or agonists of other G_s-coupled GPCRs. This points to a role for cAMP in mediating desensitisation. However, the opposite can also occur; the β_3-adrenoceptor gene contains cAMP response elements (Liggett & Schwinn, 1991; Thomas et al., 1992), and an elevation of intracellular cAMP can increase receptor

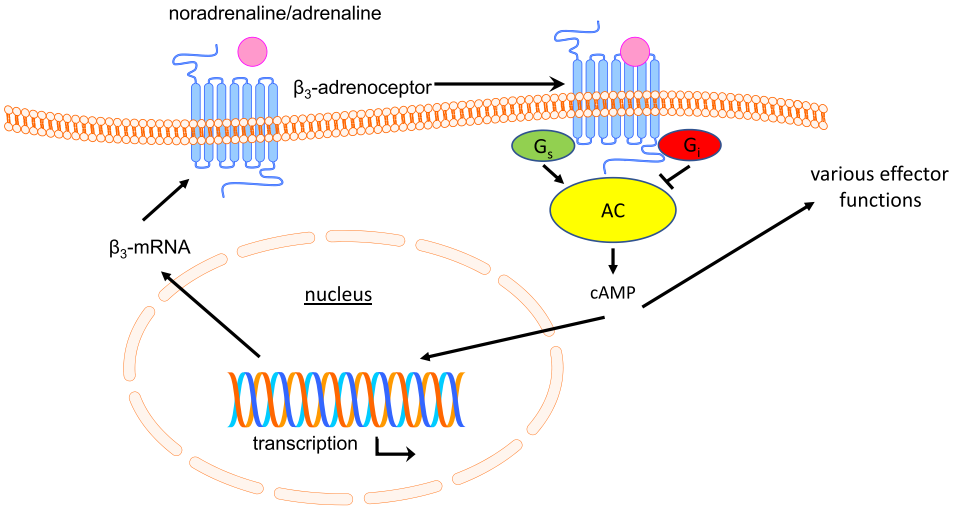

FIGURE 3 Schematic diagram of key aspects of β_3-adrenoceptor function and regulation. In many if not most tissues and cell types, these do not change upon prolonged agonist exposures, but in some, each of them may decrease (except for G_i, which may increase). For details, see main text

expression and/or function (Germack & Dickenson, 2006; Thomas et al., 1992). This raises the possibility that, at least in some cell types, the lack of desensitisation observed does not represent a true resistance but rather the net effect of ongoing sensitisation and desensitisation, but this has not been explored sufficiently for firm conclusions to be reached. Based on limited data, it appears that desensitisation may be independent of β_3-adrenoceptor gene polymorphisms (Vrydag et al., 2009). As β_3-adrenoceptors couple not only to cAMP formation but also to other signalling pathways such as ERK or p38 phosphorylation, it is surprising that with one exception (Okeke et al., 2018), desensitisation of these alternative signalling pathways has not been tested.

6.2 | Why is desensitisation tissue/cell type specific?

Both the presence of desensitisation and the level at which it occurs appear to be highly cell type-dependent. While dependency on cell type has been observed for desensitisation of several GPCR that bind arrestin (Tobin, Butcher, & Kong, 2008), it is not fully clear how this can be explained for β_3-adrenoceptors. The presence of an intron containing a putative enhancer element specific for adipose tissue (Granneman et al., 1992) may at least partly explain tissue-selective regulation of mRNA expression. Moreover, the presence of desensitisation is modulated within a tissue by additional factors such as age of the animal or innervation status. Understanding the molecular mechanisms underlying such differential desensitisation could help to devise strategies to exploit the presence or absence of desensitisation in a therapeutic manner.

6.3 | Desensitisation in disease states

Many GPCRs undergo changes in disease states (Insel, Tang, Hahntow, & Michel, 2007), a phenomenon that has perhaps best been studied for β_1-adrenoceptors, which are desensitised and down-regulated in patients with congestive heart failure and animal models thereof (Brodde, 1991; Hammond, 1993). However, the regulation of β_3-adrenoceptor expression in disease may differ as it appears to be up-regulated in heart failure at the mRNA and/or protein level (Cheng et al., 2001; Moniotte, Kobzik, et al., 2001; Napp et al., 2009; Ozakca et al., 2013; Sun et al., 2011; Zhang et al., 2005; Zhao et al., 2013). These data have led to the hypothesis that β_3-adrenoceptor agonists could be useful in patients with heart failure (Rasmussen, Figtree, Krum, & Bundgaard, 2009). An up-regulation of cardiac β_3-adrenoceptor expression at the mRNA level has also repeatedly (Amour et al., 2007, 2008; Dinçer et al., 2001; Kayki-Mutlu, Arioglu Inan, Ozakca, Ozcelikay, & Altan, 2014) but not unequivocally (Haley, Thackeray, Kolajova, Thorn, & DaSilva, 2015; Jiang et al., 2015) been observed in animal models of diabetes or of hypothyroidism (Arioglu, Guner, Ozakca, Altan, & Ozcelikay, 2010). Nevertheless, β_3-adrenoceptors were also reported to be down-regulated in an animal model of colitis, where treatment with an agonist improved symptoms and reversed the down-regulation (Vasina et al., 2008). However, our overall knowledge on changes in β_3-adrenoceptor expression and function in disease is too limited and needs expansion.

6.4 | Conclusions and clinical implications

Based on known β_3-adrenoceptor functions, it is anticipated that potential clinical uses of ligands for this subtype will be limited to agonists (Dehvari, da Silva Junior, Bengtsson, & Hutchinson, 2018; Michel, Ochodnicky, & Summers, 2010). For such uses, a relative resistance to desensitisation is likely to be a desirable property. For instance, desensitisation is a treatment-limiting factor in the use of β_2-adrenoceptor agonists in women with preterm labour (Engelhardt et al., 1997; Frambach et al., 2005; Michel et al., 1989). Tocolysis has also been proposed as an indication for β_3-adrenoceptor agonists (Bardou et al., 2007; Ursino, Vasina, Raschi, Crema, & De Ponti, 2009); however, whether tocolytic responses to β_3-adrenoceptor agonists are indeed resistant to desensitisation remains to be studied. The only validated clinical use of β_3-adrenoceptor agonists is the treatment of the overactive bladder syndrome (Chapple et al., 2014; Ohlstein et al., 2012). While the value of the reported lack of desensitisation in in vitro animal studies (Michel, 2014) remains to be determined, a 1-year study in patients presented no evidence of desensitisation for the same agonist (Chapple et al., 2013) but may not have included sufficiently early time points to assess desensitisation during the first 2 weeks of treatment. The key translational challenge of β_3-adrenoceptor desensitisation studies discussed here is the remarkable heterogeneity between species, tissues/cell types, and functional assessments; this makes it difficult to predict what will happen in the target species (humans) and tissue (e.g., urinary bladder or myometrium).

6.5 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Christopoulos et al., 2017; Alexander, Fabbro et al., 2017).

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CONFLICT OF INTEREST

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