AGONIST-INDUCED DESENSITIZATION OF β-ADRENERGIC RECEPTORS IN RAT MYOMETRIUM

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Received 8 October 1981

1. Introduction

Treatment of various cells with hormones leads to a subsequent loss of responsiveness to the hormone, a phenomenon commonly denoted as 'desensitization'. Specific catecholamine-induced refractoriness, viz., 'homologous desensitization' has been amply investigated [1–3] and was found to be associated with a net reduction of catecholamine-stimulated adenylate cyclase activity and with a net reduction in the number of β-adrenergic receptors. The observations in [4] strongly suggest that the agonist-specific desensitization in cultured astrocytoma cells initially occurs as a result of a rapid uncoupling of the β-adrenergic receptor from the adenylate cyclase which is followed by the loss of the desensitized receptors. The contribution of the uncoupling reaction to catecholamine-induced desensitization has also been reported with erythrocytes [5,6] and C_6 glioma cells [7].

In vitro, exposure of the oestrogen-dominated rat myometrium to either a β-adrenergic agonist or PGE_2 which enhanced intracellular cAMP generation [8,9] resulted in a subsequent refractory state of the tissue cAMP response to both stimuli [10]. The data revealed the existence of at least 2 distinct processes: a rapid event (5–10 min exposure), that results in a specific refractoriness to the inducing agent, and a non-specific process leading to a cross desensitization between isoproterenol and PGE_2, and vice versa, that can be detected only after prolonged exposure to the inducing agent, and that may involve cAMP as a mediator.

Abbreviations: [^{3}H]DHA, (−)[^{3}H]dihydroalprenolol; Gpp(NH)p, guanyl-5′-yl imidophosphate; MIX, 3-isobutyl-1-methylxanthine

Here, the consequences of isoproterenol selective desensitization of the rat myometrium are analysed at the membrane β-adrenergic receptor level. We show that the attenuated cAMP response to isoproterenol, when elicited by the agonist itself but not by PGE_2, is associated with both a reduction in the number of β-adrenergic binding sites, i.e., 'down regulation of receptors' and an impaired ability of the residual receptors to form a high affinity complex with the agonist, i.e. 'uncoupling process'.

2. Experimental

2.1. *Chemicals and sources:*

(−)Alprenolol and (+)alprenolol tartrate (Hassle); (−)propranolol and (+)propranolol hydrochloride (Imperial Chemical Pharma); (−)isoproterenol and (+)isoproterenol bitartrate (Sigma); phentolamine mesylate (Ciba); (±)isoproterenol hydrochloride (Fluka AG); Gpp(NH)p (Boehringer); PGE_2 (gift from Dr J. E. Pike, Upjohn Co.); (−)-[^{3}H]dihydroalprenolol (42 Ci/mmol) (New England Nuclear).

2.2. *Animals and tissue processing*

Uteri were obtained from oestrogen-pretreated rats (Wistar, 4–5 weeks old) and myometrium was prepared free of endometrium [8,10]. Myometrial strips (~50 mg) were routinely allowed to equilibrate in 2.0 ml Krebs bicarbonate buffer, containing 10 mM glucose for 25 min at 37°C (gas phase, 95% O_2 plus 5% CO_2) under continuous agitation and subsequently used for membrane preparation or further incubated for the different desensitization treatments.

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

2.3. *Preparation of crude membrane fractions*

Myometrial strips were homogenized with an Ultra-Turrax homogenizer in 10 vol. cold buffer consisting of 0.25 M sucrose, 1 mM $MgCl_2$, 5 mM Tris–HCl (pH 7.4) and centrifuged at 700 × *g* for 5 min. The sediment was washed by resuspension in the buffer and centrifugation at 700 × *g*. The pooled 700 × *g* supernatant fractions were centrifuged at 100 000 × *g* for 1 h. The resulting pellet was suspended in cold 50 mM Tris–HCl buffer (pH 7.4), 10 mM $MgCl_2$ at 4 mg protein/ml final conc. [11].

2.4. *Binding assay of [^{3}H]DHA*

Membrane proteins (~0.4 mg) were incubated with the indicated concentrations of [^{3}H]DHA (1–15 nM) for 15 min at 30°C in 50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, in 400 μl final vol. When present, Gpp(NH)p was at 100 μM. At the end of the incubation, triplicate 100 μl aliquots were diluted with 4 ml ice-cold buffer and immediately filtered through Whatman GF/C glass fiber filters under reduced pressure. Filters were washed 3 times with 5 ml cold buffer, dried and the bound radioactivity determined by scintillation counting. Specific binding was defined as the difference between the amount of [^{3}H]DHA bound in the absence (total binding) and the presence (non-specific binding) of 10 μM unlabeled propranolol. In all results, bound [^{3}H]DHA refers only to specific binding which approximated 80% and 90% of the total binding, for 15 nM and 3 nM of tracer, respectively.

2.5. *Desensitization treatment*

Following the 25 min equilibration period, incubation of myometrium strips in Krebs buffer at 37°C was further continued with or without the addition of the indicated agonist for various times depending on the experiment. The tissue strips were subsequently washed 3 times with 10 ml hormone-free buffer, transferred to 2.0 ml fresh buffer, and allowed to equilibrate 5 min at 37°C before the preparation of particulate fractions for [^{3}H]DHA binding assay.

2.6. *Assay of cAMP levels*

In some experiments, the above-treated tissues were rechallenged with 0.3 μM isoproterenol in the presence of 275 μM MIX for estimation of cAMP accumulation essentially as in [10].

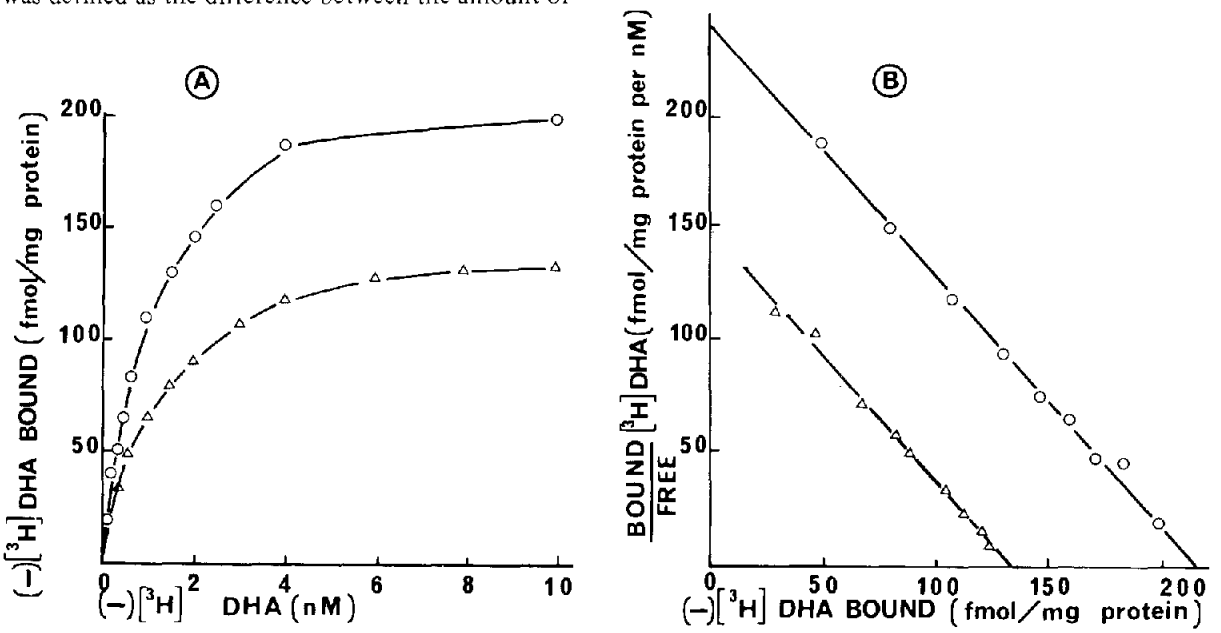

Fig.1. Specific binding of [^{3}H]DHA to myometrial membranes as a function of [^{3}H]DHA concentration. (A) Membranes (1 mg/ml) prepared from control (○) and from myometrial strips pre-treated for 1 h with 10 μM isoproterenol (△) were incubated with the indicated concentrations of [^{3}H]DHA and specific binding was determined as in section 2. Each value is the mean of triplicate determinations. (B) Scatchard plot of [^{3}H]DHA binding to membranes from control (○) and pre-treated myometrium (^). The intercept on the abscissa is equal to the maximal binding capacity (B_{max}) and the slope is equal to $-1/K_d$.

3. Results and discussion

3.1. *Characteristics of (−)[^{3}H]DHA binding in particulate fractions of rat myometrium*

The presence of β-adrenergic receptors on oestrogen-pretreated rat myometrial membranes had been demonstrated by the ability of catecholamines to induce relaxation and intracellular cAMP accumulation [8]. Here, β-adrenergic receptors were directly monitored in the particulate fractions by binding of the radiolabeled antagonist [^{3}H]DHA. As shown in fig.1, the specific binding of [^{3}H]DHA was a saturable process. The equilibrium dissociation constant (K_d) determined by Scatchard analysis was found equal to 0.9 ± 0.1 nM. One class of binding sites was characterized with a maximal number of 200 ± 12 fmol [^{3}H]DHA bound/mg of membrane protein. Specific binding of [^{3}H]DHA to the particulate fraction reached equilibrium within 5 min at 37°C and was rapidly reversible: 80% of bound [^{3}H]DHA was dissociated within 5 min after addition of unlabeled alprenolol. Dissociation experiments (not shown) revealed no discrepancy between isotopic dilution and isotopic + chemical dilutions which agrees with the absence of cooperativity between binding sites. In the presence of 3 nM [^{3}H]DHA, specific binding was linear with membrane protein at 0.3–2 mg/ml.

The identity of [^{3}H]DHA binding sites with β-adrenergic receptors was confirmed by competition binding experiments (fig.2). The β-adrenergic agonist, (−)-isoproterenol and antagonists (−)-propranolol and (−)-alprenolol caused a complete and dose-dependent decrease of [^{3}H]DHA binding with a respective apparent dissociation constant 'K_d' of 0.11 μM, 0.0017 μM and 0.0008 μM (calculations were made according to [12] from the concentrations causing 50% decrease of [^{3}H]DHA binding). The α-adrenergic antagonist phentolamine, was ineffective in competing for the binding sites.

A fundamental property of the β-adrenergic agonists, not shared by antagonists, is the ability to allow formation of a high affinity state of β-adrenergic receptor which is shifted to a lower affinity state in the presence of guanyl nucleotides. In contrast, antagonist binding to the receptor is characterized by a single affinity form insensitive to guanyl nucleotides. The 'shift' from the high to the low affinity form of the agonist–receptor complex is normally associated with an increase in the agonist induced adenylate activity and thus reflects a 'coupled state' whereas in

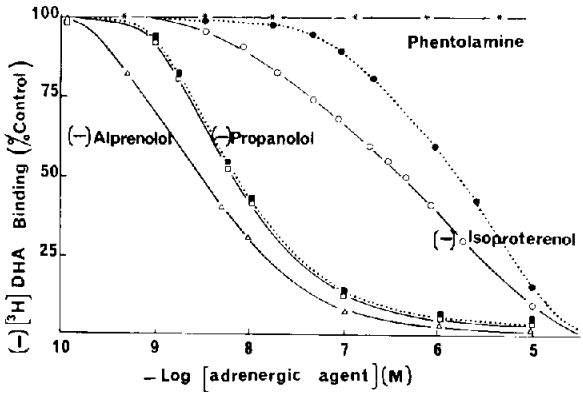

Fig.2. Displacement of bound [^{3}H]DHA by adrenergic agents in myometrial membranes: effect of Gpp(NH)p. Membrane preparations were incubated with 3 nM [^{3}H]DHA and various concentrations of (−)isoproterenol, (−)alprenolol (−)propranolol and ( )phentolamine, in the absence (----) or presence (. . .) of 100 μM Gpp(NH)p. Results are expressed as the percentage of the specific [^{3}H]DHA binding in the absence of any competing agent (170 fmol/mg protein). Experimental points are the mean of 2–6 different determinations.

different 'uncoupled systems', the receptor displays a uniform low affinity for the agonist which is not affected by guanylnucleotides [13–17]. Data of fig.2 illustrate that the β-adrenergic receptors of the rat myometrium exhibit binding characteristics of 'coupled receptors'. The displacement curve of isoproterenol for [^{3}H]DHA binding is indeed shifted to the right when binding was assayed in the presence of 100 μM GppNHp with a 5-fold reduction in the agonist affinity (K_d for isoproterenol 0.11 ± 0.01 and 0.5 ± 0.07 μM, $p < 0.05$, in the absence and presence of guanylnucleotide, respectively). Under similar conditions, the displacement curve for propranolol was not altered by Gpp(NH)p. Similar binding properties have been described for the β-adrenergic receptors in the adult rat uterus at different stages of the oestrus cycle [18].

3.2. *Modulation in the number and properties of β-adrenergic receptors in desensitized myometrium*

Exposure of myometrial strips to isoproterenol leads to a subsequent decrease in the β-adrenergic response in terms of cAMP [10]. Data of fig.1 illustrate that this phenomenon is also accompanied by a reduction in the number of β-adrenergic receptors in the membranes. The total number of [^{3}H]DHA

binding sites in the membranes prepared from tissue exposed to isoproterenol, 10 μM, 1 h at 37°C, represent only 70% of the initial number of binding sites in control membranes. Scatchard analysis of the data indicated that the K_d of [^{3}H]DHA for binding to control (0.9 ± 0.1 nM) and desensitized (1.1 ± 0.15 nM) membranes are virtually identical. The time courses of β-adrenergic cAMP response and of β-adrenergic receptor losses appear to be similar (fig.3). Both phenomena were already detectable within 10 min exposure to the agonist and reached a plateau value after 30–45 min pretreatment. Total [^{3}H]DHA binding sites in desensitized membranes averaged 140 ± 10 *vs* 200 ± 12 fmol/mg protein for control ($p < 0.05$). Of importance was the observation that the extent of β-adrenergic receptor losses was consistently lower than the decline in the isoproterenol response in terms of cAMP. No further decrease in the number of β-adrenergic receptors could be obtained by prolongation of pretreatment of the myometrium with isoproterenol.

Performing binding assays in the presence of 100 μM Gpp(NH)p had no effect on the apparent number of [^{3}H]DHA binding sites in membranes of desensitized myometrium. In contrast to the substantial restoration of [^{3}H]DHA binding sites in the presence of guanyl nucleotides in membranes from isoproterenol desensitized myometrium [19], the degree of [^{3}H]-DHA binding loss in the present study was identical under all assay conditions. Our results clearly indicate that residually bound agonist cannot account for the reduction in the binding capacity caused by incubation of the intact myometrium with isoproterenol. Indeed, Gpp(NH)p which lowers agonist affinity (fig.2) should have led to rapid dissociation of the agonist from the receptors [1]. The phenomenon (fig.3) is rather consistent with a down regulation of β-adrenergic receptors in the desensitized myometrium. As also shown in fig.3, prior treatment of the myometrium with PGE_2, under conditions where the subsequent responsiveness to isoproterenol was markedly attenuated, resulted in no change in the density of β-adrenergic receptors ($p < 0.4$).

Fig.4 illustrates the agonist binding properties of β-adrenergic receptors for control and post-desensitized membrane fractions. Following tissue incubation with isoproterenol (10 μM, 1 h), concomitant to the loss of [^{3}H]DHA binding sites (30%), the ability of the residual membrane receptors to form a high affinity state seems to be impaired (fig.4A). The latter observation is consistent with the notion of uncoupling between the receptor and the nucleotide regulatory component [4,5,15]. That is, in the absence of Gpp(NH)p, the K_d for isoproterenol determined in competition binding studies with membranes from the desensitized tissue (0.3 ± 0.03 μM), is shifted to values 3–4-fold greater than that observed in control membranes ($p < 0.05$). The apparent affinity for iso-

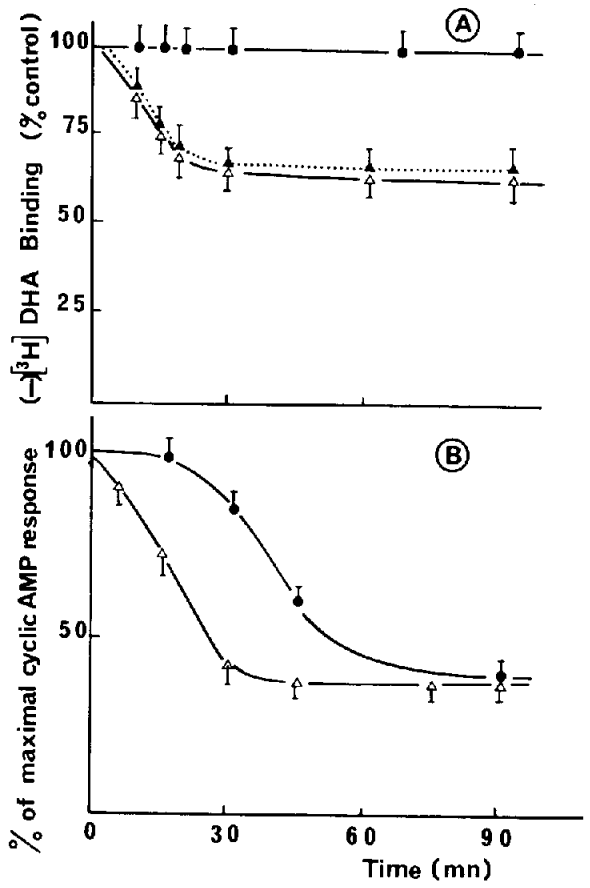

Fig.3. Time courses of decrease in β-adrenergic receptor density and isoproterenol-stimulated cAMP accumulation during incubation of rat myometrium with isoproterenol or PGE_2. Tissues were incubated with 10 μM isoproterenol (△,▲) or 30 μM PGE_2 (●). At the indicated times, tissues were washed free of hormone and used (A) for membrane preparation and binding assay with 15 nM [^{3}H]DHA in the absence (△) or presence (▲) of 100 μM Gpp(NH)p or (B) for rechallenge incubations with 0.3 μM isoproterenol before cAMP determinations as in section 2. For control tissue specific [^{3}H]DHA binding was 198 ± 15 fmol/mg protein and cAMP response to isoproterenol was 102 ± 10 pmol cAMP/mg protein. Results are expressed as the percentage of control binding (A) and control cAMP response (B). Values represent the mean ± SE of 4–6 expt.

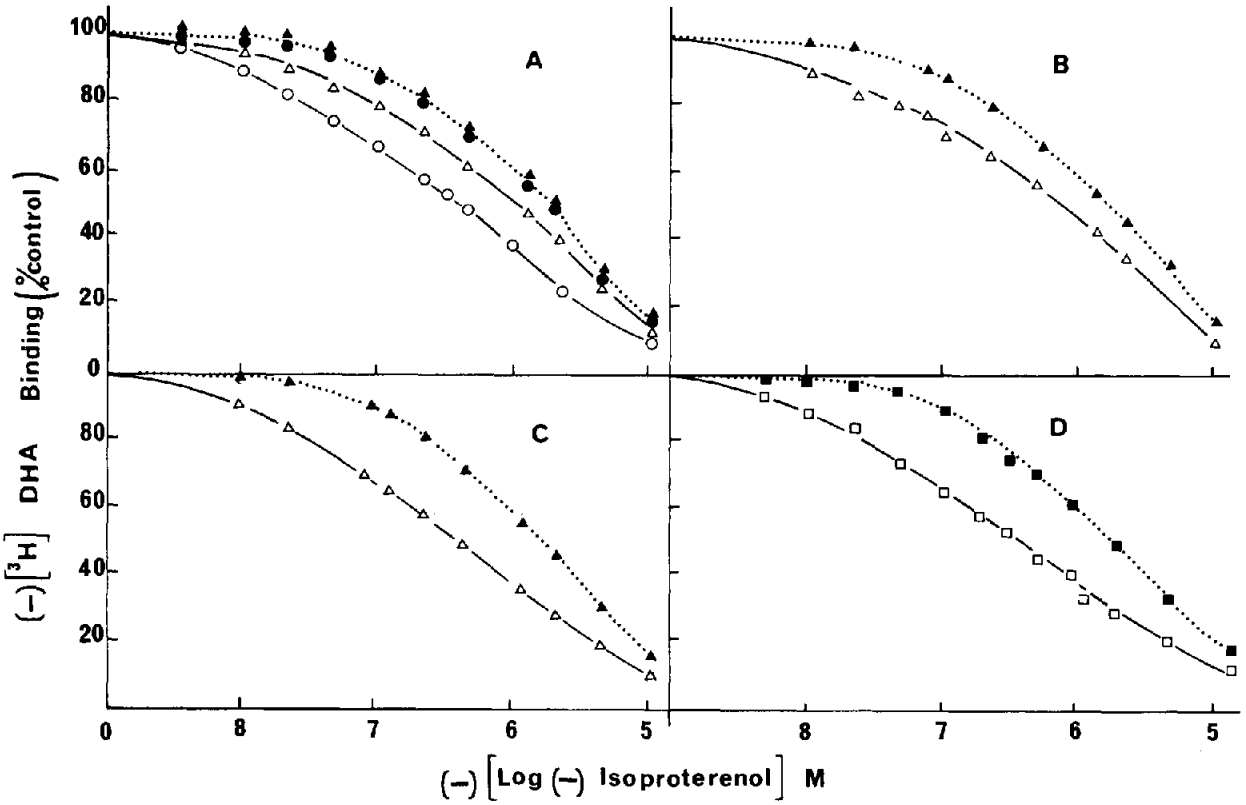

Fig.4. Concentration effect curves for inhibition of [^{3}H]DHA binding by (−)isoproterenol in membrane preparations from control, desensitized and resensitized tissues. Myometrial strips were incubated in the absence (○,●) or presence (△,▲) of 10 μM isoproterenol for 1 h (A), 5 min (B) or in the presence (□,■) of 30 μM PGE_2 (D). Tissues were then washed free of hormone and membrane fractions prepared as in section 2. Binding assays were carried out with 3 nM [^{3}H]DHA in the absence (open symbols) and presence (closed symbols) of 100 μM Gpp(NH)p. For (C) tissues were incubated 1 h with 10 μM isoproterenol washed as above and subsequently incubated in 5 ml hormone-free buffer for 90 min prior to preparation of membranes, and assay of [^{3}H]DHA binding in the absence (△) and presence (▲) of 100 μM Gpp(NH)p. Data are expressed as percent of maximal specific binding (which averaged: (A) control 165 ± 15, desensitized 110 ± 10; (B) 118 ± 12; (C) 112 ± 12; (D) 160 ± 10 fmol [^{3}H]DHA bound/mg membrane protein). Values represent the pooled means from 3–6 similar expt.

proterenol in the presence of Gpp(NH)p is similar for control and desensitized membranes. As a result, Gpp(NH)p effects a smaller reduction (1.6-fold) in the apparent affinity for isoproterenol than that observed for receptors in membranes of control cells (4–5-fold) (fig.3,4). Fig.4B shows that the impairment in the ability of the receptor to form a high affinity state is already detectable following 5 min incubation with isoproterenol but the effect on the degree of uncoupling was less pronounced. In the latter case the K_d for isoproterenol was shifted to a value of 0.22 ± 0.04 μM (significantly different from control, $p < 0.01$), with a 2.2-fold reduction in the K_d in the presence of Gpp(NH)p. In contrast, the agonist binding properties to the β-adrenergic receptor in membranes derived from myometrium pretreated with PGE_2 (10 μM, 1 h) were undistinguishable from those of control tissue (fig.4D).

Fig.5 and 4C demonstrate the ability of the myometrium to recover following isoproterenol induced desensitization. On washing myometrial preparations pre-treated with 10 μM isoproterenol for 1 h and further incubated in a hormone-free medium, a time-lag preceded the onset of recovery of isoproterenol responsiveness in terms of cAMP accumulation, in agreement with [10]. Nevertheless, loss of membrane receptors was still not recovered after 2 h incubation of intact tissue in the absence of the agonist, under

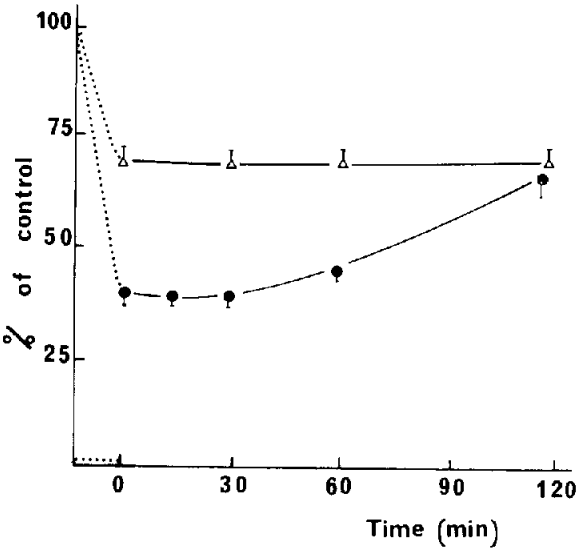

Fig.5. Time required for recovery of isoproterenol-induced refractoriness. Myometrial strips were initially exposed to 10 μM isoproterenol for 60 min as shown by the dotted line on the abscissa. The agonist was then removed by 3 successive washings. Tissues were subsequently incubated in hormone-free buffer and at the indicated times used for membrane preparation and binding assay with 15 nM [^{3}H]DHA (△) or for rechallenge incubations with 0.3 μM isoproterenol and cAMP determinations (•). The response of the treated tissue is expressed as the percent of the response of control tissue treated the same but without isoproterenol in the 60 min exposure period. Control values were 210 ± 15 fmol [^{3}H]DHA bound/mg membrane protein and 120 ± 12 pmol cAMP/mg protein in response to isoproterenol. Values represent the mean ± SE of 3 expt.

conditions where 40–50% of the ability of isoproterenol to stimulate cAMP accumulation was already restored (fig.5). Of interest was the observation that in this situation, the uncoupling phenomenon at the level of β-adrenergic receptors was substantially reduced (fig.4C). The membrane receptors restored their ability to form a high affinity state as well as their sensitivity to shift to a low affinity state in the presence of Gpp(NH)p. In the resensitized membrane K_d for isoproterenol in the absence and presence of the guanyl nucleotide (0.1 ± 0.01 and 0.45 ± 0.05 μM, respectively) were close to those of control tissues. Thus the recovery of normal agonist binding properties coincides with the progressive restoration of isoproterenol stimulated cAMP accumulation in the myometrium.

In conclusion, these findings imply that for the oestrogen-treated rat myometrium, the isoproterenol-induced self-refractoriness in terms of the cAMP response may readily be explained by both a reduction in the number of membrane receptors and the uncoupling process at the level of residual β-adrenergic receptors. Thus the percent loss in cAMP response to isoproterenol can reasonably be expected to exceed the percent loss of total receptor populations as measured by [^{3}H]DHA binding. It must be emphasized that the relation of the loss of β-adrenergic receptors to the uncoupling reaction is not entirely clear [4,5,7]. Nonetheless, if the time courses of development of refractoriness in the myometrium did not allow one to distinguish temporally, uncoupling from receptor losses, the kinetics of the reversibility process clearly demonstrate that the apparent conversion of 'uncoupled' to 'coupled' receptor is rapid compared to the recovery of lost receptors. Here, the restoration of normal receptor density following myometrium refractoriness has not been thoroughly investigated but appeared, as emphasized for other systems [3,4], a very slow process that may readily explain the incomplete recovery of cAMP response to isoproterenol even 4 h after removal of the agonist [10]. Finally, it is clear that both the uncoupling and the down regulation phenomena can solely and specifically be promoted by the agonist itself and do not seem apparently to be mediated by cAMP. Elevation of cAMP content of the myometrium by PGE_2 did not affect the level nor the agonist binding properties of the β-adrenergic receptors. These data support our interpretation [10] that a distinct mechanism is involved in the cross-desensitization caused by PGE_2 towards isoproterenol-cAMP response in the intact myometrium. The latter mechanism, as suggested by these observations, should be operating at a step distal to the membrane β-adrenergic receptors.

Acknowledgements

Supported in part by CNRS (L. A. 272, A. T. P. 4162), DGRST (79.9.0782) and Fondation pour la Recherche Médicale Française. Thanks are due to G. Thomas for technical assistance and to J. Jalicot and M. G. Guyon for preparing the typescript.

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