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ENDOGENOUS NORADRENALINE MASKS BETA-ADRENERGIC RECEPTORS IN RAT HEART MEMBRANES VIA TIGHT AGONIST BINDING

VIVECA NERME,*† YVONNE SEVERNE,‡ TOMMY ABRAHAMSSON* and GEORGES VAUQUELIN‡

* Department of Pharmacology and Biochemistry, Hässle Cardiovascular Research Laboratories, S-431 83 Mölndal, Sweden; ‡ Institute of Molecular Biology, Free University Brussels (VUB), 65 Paardenstraat, B-1640 St Genesius Rode, Belgium

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Abstract—The tight binding of noradrenaline (NA) to β-adrenergic receptors was studied in membranes from the left ventricular myocardium of the rat. Addition of GTP (0.1 mM) to membrane preparations from control rats (NA concentration 8.5 ± 2.1 nM) caused a 4–35% (N = 8) increase ($P < 0.01$) in the number of specific binding sites of [^{125}I](−)pindolol. In contrast, addition of GTP did not cause any changes in the number of β-adrenergic receptors in heart membranes from reserpinized animals (NA concentration < 0.1 nM). In heart membranes from reserpinized animals, preincubation with NA (followed by washing) revealed a time- and concentration-dependent decrease with a maximum of 35–40% in the [^{125}I](−)pindolol-binding sites. This agonist-mediated decrease in the number of receptors was prevented if GTP was also present in the NA-preincubation medium. It is concluded that NA can undergo tight binding to β-adrenergic receptors in rat heart membranes. The heart-membrane preparations contain endogenous NA which, via tight agonist binding, is responsible for masking part of the β-adrenergic receptor population.

Catecholamine-mediated desensitization of β-adrenergic receptors quite often appears to be associated with a decrease in the number of receptors in the plasma membrane. This phenomenon of homologous receptor regulation occurs *in vivo* or in intact cell systems and has already been shown to be associated in several instances with internalization of the receptor [1, 2]. Several reports have, however, also described the ability of catecholamines to cause an apparent decrease in the number of receptors in erythrocyte-membrane preparations from the frog [3, 4]. The mechanism here is completely different from that in the *in vivo* situation [5]. The decrease in the number of receptors in isolated membranes appears to be related to the fact that functional coupling between the agonist-bound receptor and the adenylate cyclase-stimulating regulatory component (N_S)§ causes a conformational change in the receptors which is associated with tight binding of the agonist [6].

In erythrocyte membranes from the turkey, however, such tight agonist binding does not occur unless the agonist receptor–N_S complex is stabilized by the group-specific agent *N*-ethylmaleimide, probably by alkylation of the N_S [7]. This phenomenon can easily be demonstrated experimentally by the fact that a combination of agonist and *N*-ethylmaleimide causes a decrease in β-adrenergic receptor sites available to the radioligand binding, whereas neither compound has an effect *per se* [8]. This apparent decrease in the number of receptors in frog and turkey erythrocyte membranes has previously been interpreted as, in the former case, a model for densensitization [3] and, in the latter, the ability of *N*-ethylmaleimide to inactivate agonist-bound receptors [8]. However, it is quite possible that both these phenomena occur via the same mechanisms of tight agonist binding [6, 7]. The similarity in mechanisms also appears from the fact that in both cases the decrease in the number of receptors is prevented by the presence of GTP [9, 10], known to mediate dissociation of N_s from the agonist-bound receptors [2].

The presence of endogenous catecholamines in heart-membrane preparations has previously been indicated by the ability of β-adrenergic antagonists [11], and also reserpine [12, 13], to decrease the basal adenylate cyclase activity. Owing to endogenous catecholamines the phenomenon of tight agonist binding might also occur spontaneously in membrane preparations from the brain or sympathetically innervated tissues. In the present study we demonstrate that endogenous noradrenaline can undergo tight binding to heart-membrane β-receptors and thus mask part of the receptor population.

MATERIALS AND METHODS

Male Sprague Dawley rats (250–300 g) from Möllegaard, Denmark were used. To deplete catecholamines, animals were treated with reserpine 2.0 mg/kg i.p., 18 hr and 0.5 mg/kg i.p. 4 hr before the experiment. The animals were killed by a blow on the neck.

† To whom all correspondence should be addressed.

§ Abbreviations: NA, noradrenaline; N_S, adenylate cyclase-stimulating regulatory component; IPIN, [^{125}I](−)Pindolol; IPR, isoprenaline; k_d, equilibrium dissociation constant; n_H, Hill coefficient.

Membrane preparations

The free wall of the left ventricle was thoroughly rinsed in ice-cold buffer A (20 mM Tris–HCl in 154 mM NaCl, pH 7.5). The muscle was freed of valvular connective tissue, weighed and homogenized in 15 ml buffer A with a Brinkman Polytron (Setting 7 for 15 sec). The homogenates were filtered through double layers of nylon mesh and centrifuged 3 times at 30,000 *g* at 4°. The pellets from the last centrifugation were resuspended in ice-cold buffer B (20 mM Tris–HCl, 2 mM $MgCl_2$ in 154 mM NaCl, pH 7.4 at 30°) and the membranes were investigated directly. Protein determinations were performed by the method of Lowry *et al.* [14] using bovine serum albumin as the standard.

Preincubation with noradrenaline

When indicated, membranes were preincubated with noradrenaline (NA) and GTP and washed as follows.

Preincubation. Membranes were preincubated with increasing concentrations of NA (10^{-9}–10^{-4} M) for 20 min at 30° or with 10^{-7} M NA for increasing periods of time (0–45 min at 30°) with or without GTP. The preincubation was terminated by centrifugation for 1 min in an Eppendorf centrifuge (15,000 *g*) at 20°.

Washing. After removal of supernatant, the precipitated membranes were resuspended in 0.5 ml buffer B and centrifuged for 1 min. This washing step was repeated 3 times.

Binding of [^{125}I](−)pindolol

(−)Pindolol was iodinated and [^{125}I](−)pindolol (IPIN) was purified to a theoretical sp. act. (2200 Ci/mmole) as previously described [15]. Aliquots of membrane suspension (70–150 μg of protein) were incubated with IPIN (18 min at 30°) in the absence or presence of GTP (0.1 mM) in a final volume of 0.25 ml. The membranes and drugs were all diluted in buffer B. Binding assays were routinely carried out in polypropylene tubes (Sarstedt no. 55 538). The binding reaction was terminated by the addition of 10 ml buffer C (10 mM Tris–HCl, 2 mM $MgCl_2$ in 154 mM NaCl, pH 7.4) and the samples were immediately filtered through Whatman glass-fibre filters (GF/C). Each filter was washed with an additional 10 ml buffer C. The radioactivity remaining on the filters was determined in a Kontron gamma counter.

The specific binding of IPIN was defined as the amount of radioligand bound in the absence of competing drug minus the amount bound in the presence of 5×10^{-5} M (−)isoprenaline (IPR). This concentration of IPR is approximately 100 times its equilibrium dissociation constant (k_d) and with observed Hill coefficients (n_H) close to unity, it corresponds to occupancy of approximately 99% of the receptors [16, 17]. Specifically bound IPIN constituted 95% of the total binding, which itself was always less than 10% of the total amount of IPIN in the incubation mixture.

To determine the density of binding sites, B_{max}, the amount of specifically bound IPIN was determined at 9 concentrations of IPIN (20–400 pM). The data were analysed according to the method of Scatchard [18] to provide a value for the density of receptors and dissociation constants for IPIN. The concentration of IPIN was ~70 pM in all other experiments.

Catecholamine assay

Aliquots (1 ml) from the membrane suspension were transferred to small vials with HCl to obtain a pH between 3 and 4 and then put in a freezer (−70°) for later analysis of the NA content [19]. Catecholamines were isolated by adsorption onto aluminia and desorbed by elution with perchloric acid. The catecholamines were separated with ion-exchange liquid chromatography and electrochemically detected. The sensitivity of the method for NA is 0.1 nM.

Chemicals

(−)Isoprenaline hydrochloride and GTP were from Sigma (St Louis, MO), ^{125}I was obtained from Amersham International (Amersham, U.K.) and the (−)pindolol was a gift from Sandoz. All other chemicals were of the purest grade commercially available.

Statistics

Values in the text and Table 1 are presented as means ± S.D. The significance of the differences between the two groups was estimated by the non-parametric rank sum test and the significance of the differences (±GTP) in each group was estimated using the paired Student's *t*-test. The differences were considered to be significant when $P < 0.05$.

RESULTS

Specific binding of IPIN to the rat left ventricle membranes is a saturable process. A representative experiment is demonstrated in Fig. 1A. The Hill coefficient equals unity ($n_H = 1.02$, $r = 0.995$) and the Scatchard plot is linear ($r = 0.988$), which argues for the absence of co-operative interactions. The total number of binding sites determined by the Scatchard plot equals 10.9 fmoles/mg protein and the equilibrium dissociation constant for IPIN binding amounts to 90 pM. Addition of 0.1 mM GTP to the incubation medium causes a 16.5% increase in the number of receptors ($B_{max} = 12.7$ fmoles/mg protein). These new appearing sites show similar binding characteristics, since the Scatchard plot remains linear ($r = 0.997$), k_d equals 75 pM and the Hill coefficient is still close to unity ($n_H = 0.96$, $r = 0.998$).

The IPIN-saturation binding parameters are shown in Table 1. The values are the mean from 8 different membrane preparations—one from each animal. The average GTP-mediated increase in B_{max} equals 16.2%, and although the individual values vary between 4.0 and 34.6%, this effect is highly significant ($P < 0.01$).

It is noteworthy that these membranes still contain measurable amounts of NA (8.5 nM S.D. 2.07, $N = 8$) under radioligand-binding conditions. To test the possible relationship between the presence of endogenous catecholamines and the GTP-mediated increase in the number of β-adrenergic receptors, similar IPIN-saturation binding experiments were

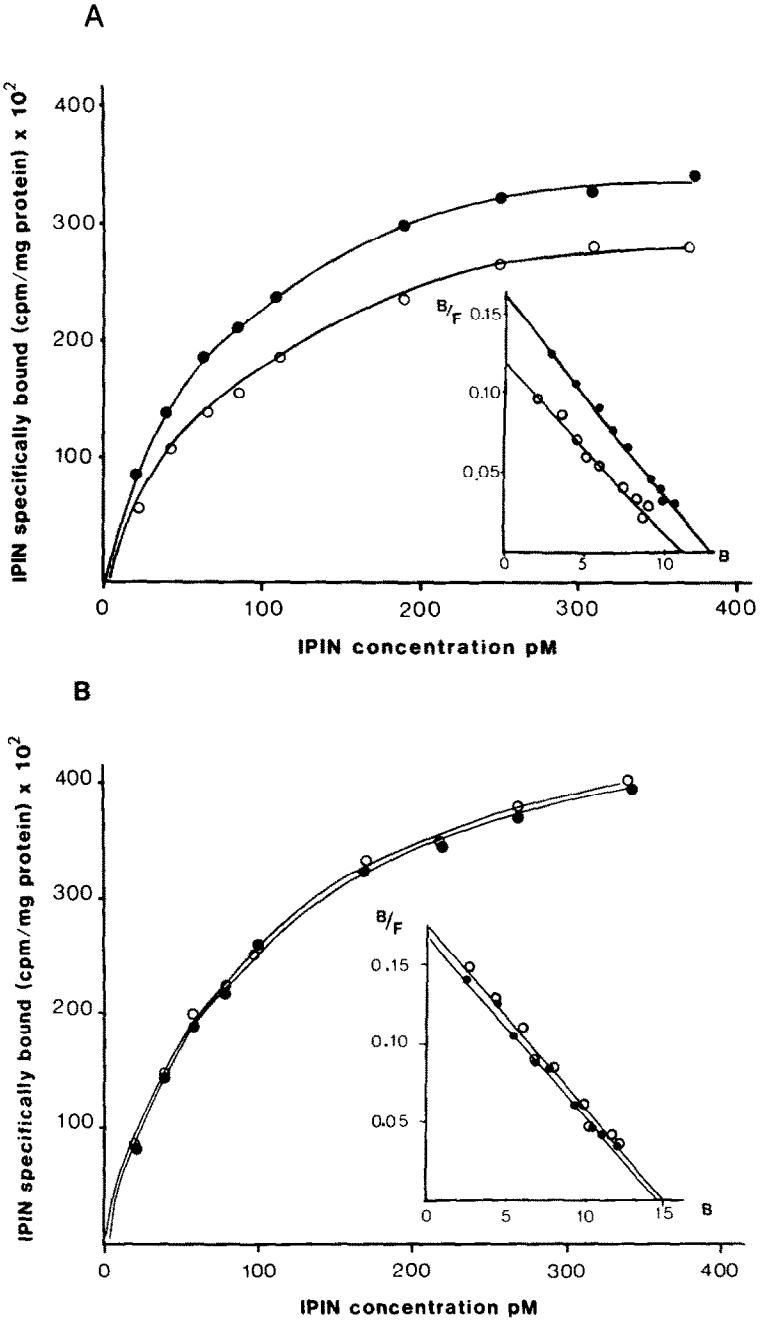

Fig. 1. The effect of GTP on IPIN binding to left ventricle membranes from control and reserpinized rats. IPIN saturation binding (20–370 pM) either in the absence (○) or presence (●) of 0.1 mM GTP. (A) Membranes from control rat. (B) Membranes from reserpine-treated rat. Inserts: Scatchard plots of the saturation-binding data. In the figures B is the specific binding in fmoles/mg protein and B/F is the ratio of bound to free radioligand. Mean data obtained in 8 similar experiments for each group are in the text.

performed using membranes from reserpine-treated animals. In that material NA could no longer be detected by the method used.

As depicted in Table 1, GTP no longer has any effect on IPIN binding to membranes from reserpinized animals. The Scatchard plots of the typical example shown in Fig. 1B are almost superimposable. The density of binding sites (B_{max}) equals 14.8 and 14.5 fmoles/mg protein and k_d values are 83.9 and 86.0 pM in the absence and presence of GTP, respectively. Here again the linearity of the Scatchard plots ($r = 0.997$ and 0.996) and the values of the Hill coefficients ($n_H = 1.05$, $r = 0.998$ and $n_H = 1.06$, $r = 0.997$) indicate that binding occurs at one single site.

Our data on control rats are compatible with the

Table 1. IPIN B_{max} and k_d values in left ventricle membranes from control (N = 8) and reserpine treated (N = 8) rats: effects of GTP. Membranes were prepared separately from each animal. IPIN-saturation binding experiments were performed and analysed as described in the legend for Fig. 1. Values represent mean ± S.D.

Membrane	N	GTP (0.1 mM)	IPIN-binding parameters k_d (pM)	B_{max} (fmoles/mg protein)	% change
Control rats	8	−	116 (±36.7)	19.9 (±5.76)	
		+	128 (±49.9)	23.2 (±6.99)	+16.2 (±9.63)
Reserpinized rats	8	−	80.5 (±6.16)	17.2 (±6.90)	
		+	89.7 (±6.78)	17.1 (±7.29)	−0.7 (±5.99)

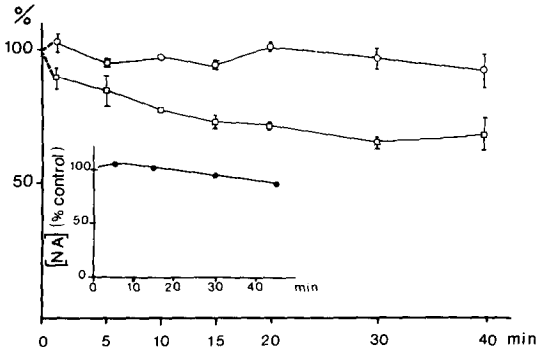

Fig. 2. NA-mediated decrease in IPIN binding: time dependence. Membranes from reserpinized animals were preincubated with buffer alone (○) or 10^{-7} M NA (□) for increasing periods of time (abscissa) at 37°, after which the preincubation medium was removed by 3-fold washing of the membranes and the specific binding of ~70 pM IPIN measured. Control corresponds to zero-time preincubation with buffer alone. Values are means of 3 experiments and bars represent S.D. Insert: Effect of the preincubation time on the NA concentration. Membranes were incubated with NA for the indicated periods of time, after which the remaining NA concentration was analysed.

earlier described ability of GTP to prevent and reverse tight agonist binding to frog erythrocyte β-adrenergic receptors [9]. This hypothesis is confirmed by the demonstration that NA causes a time- and concentration-dependent decrease in the number of receptor sites in membranes from reserpinized rats. For this purpose membranes were preincubated with 10^{-7} M NA for increasing periods of time (Fig. 2), or for 20 min with increasing concentrations of NA (Fig. 3), after which the free agonist was removed by repeated washing of the membranes and IPIN binding measured. As shown in Figs 2 and 3, there is indeed a time- and NA-concentration-dependent decrease in the IPIN-binding activity. Preincubation of the membranes for the same time periods with buffer only has no significant effect on IPIN binding. Scatchard analysis from saturation-binding experiments reveals that the NA-mediated decrease is due to the loss of the number of binding sites and not to a change in the IPIN affinity (data not shown).

It is of interest to note that both the kinetic- and the NA-concentration-dependency curves appear to level off at a plateau corresponding to 60–65% of the original IPIN binding. In the insert of Fig. 2, it

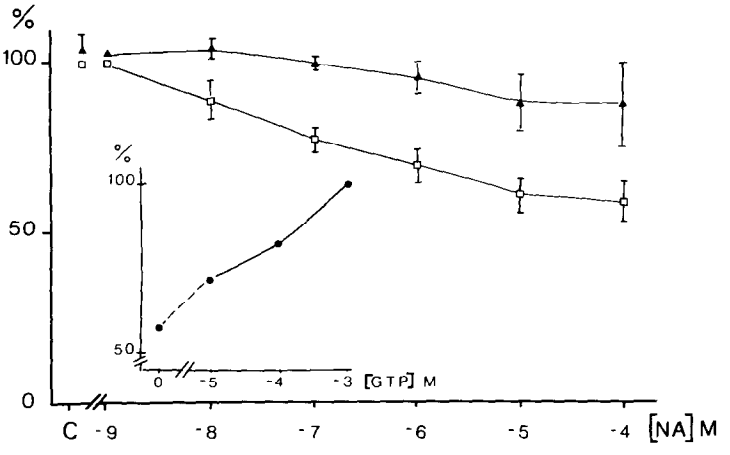

Fig. 3. NA-mediated decrease (%) in IPIN-binding sites: NA-concentration dependence. Membranes from reserpinized animals were preincubated with increasing concentrations of NA (abscissa) either in the absence (□) or in presence (▲) of 0.1 mM GTP for 20 min at 30°. Membranes were washed and IPIN binding measured as described in the legend for Fig. 2. Control binding (C) corresponds to membranes incubated with buffer only. Values are means of 3–4 experiments and bars represent S.D. Insert: GTP-mediated receptor protection: concentration dependence. Membranes were preincubated with 10^{-5} M NA either in the absence of presence of increasing concentrations of GTP (abscissa).

is shown that there is only a moderate degradation of NA for the preincubation time periods tested. Accordingly the partial nature of the NA effect appears to be related to the fact that only some of the receptors can be involved in tight agonist binding rather than to NA degradation. Simultaneous presence of 0.1 mM GTP in the preincubation phase effectively prevents the NA-mediated decrease in the number of receptors (Fig. 3). This protection is complete for NA concentrations up to 10^{-6} M. The incomplete protection at higher NA concentrations (10^{-5} M) can be overcome when the GTP concentration is raised from 10^{-4} to 10^{-3} M (Fig. 3, insert).

DISCUSSION

Myocardial membrane preparations from reserpinized rats contain no detectable amounts of endogenous catecholamines. Using this material, we show here that preincubation with NA causes maximally a 35–40% decrease in the number of β-adrenergic binding sites. This observation appears to be related to the formation of a slow dissociating agonist-receptor complex, a phenomenon which has already been shown to occur with the β_2-adrenergic receptors in frog erythrocyte membranes [9]. The results on both heart and frog erythrocyte membranes show two striking similarities. (1) The slow dissociation agonist-receptor complex formation is prevented by GTP. This indicates a functional coupling between these receptors and N_S. (2) Only some of the receptors undergo such tight agonist binding. This agrees with other observations that only a part of the receptor population can undergo functional coupling to N_S [20].

Membrane preparations from non-reserpinized rats, however, still contain appreciable amounts of NA. Since the membrane preparation includes several centrifugation/washing steps, the remaining NA is probably associated with the membrane, trapped and/or present in secretory vesicles [21]. The presence of the noradrenaline is associated with spontaneous formation of high-affinity agonist complexes, as is demonstrated by the ability of GTP to cause an increase in the number of IPIN-binding sites. There is an important individual variation in the amount of slowly dissociating complexes (4–35%) in control membranes despite rather similar NA levels. Part of the NA-receptor complexes might have been formed during the membrane preparation and the variability in tight NA binding can then be attributed to differences between the individual preparations, especially since the NA concentration can be expected to be higher during the early stages.

Pseudo non-competitive binding, for β-receptors commonly referred to as tight agonist binding [6] of residual NA has also been demonstrated to occur for α_2-receptors in the brain [22] and have been proposed to occur for β_1-receptors in the parotid glands [23].

In the rat myocardial membrane, addition of GTP did not affect the receptor-binding affinity of IPIN. This is in contrast to an earlier report by Wolfe and Harden [24], showing a significant decrease in the k_d-value of IPIN after addition of GTP in L6 cells, which apparently contain β_2-receptors. This may reflect a difference between the β_1- and β_2-receptors, which requires further investigation.

Tight agonist binding might give rise to a non-negligible underestimate of the actual number of β-adrenoceptors in heart membrane preparations. An easy way to overcome this problem is to perform radioligand-saturation binding experiments in the presence of GTP or related guanine nucleotides. This experimental procedure might be particularly useful when investigating the effect of normal and pathophysiological regulation, as well as drug administration, on the number of β-adrenoceptors in the heart. In this context the great majority of reported modulations in the number of receptors are based on experiments performed in the absence of GTP on crude membrane preparations similar to the one described in this study. Whereas tight agonist binding probably does not affect the validity of the conclusions based on substantial variations in the number of receptors, this phenomenon needs to be taken into consideration in the case of small or no variations.

Reports dealing with the action of reserpine upon the β-adrenergic receptor concentration belong to this category. The radioligand-binding data, reported by several groups [25–27], indicate that there is no difference in the number of cardiac β-adrenergic receptors between reserpine-treated and control rats or guinea-pigs. In contrast, several other authors have reported a moderate, less than 50% increase in the number of β-adrenergic receptors in hearts from reserpinized guinea pigs [28, 29]. Whereas it cannot be excluded that differences in the dose of reserpine, as well as the time schedule of administration, might also be responsible for the conflicting results in the literature, our data indicate that, at least under certain conditions, catecholamine depletion by acute reserpine treatment is not accompanied by changes in the number of β-adrenergic receptors in the heart.

In conclusion, the present study demonstrates that, as is the case in frog erythrocyte membranes, noradrenaline can undergo tight binding to β-adrenoceptors in rat heart membranes. Rat membrane preparations contain endogeneous noradrenaline which, via tight agonist binding, is responsible for masking part of the receptor population.

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