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High Efficiency Coupling Between Beta-adrenergic Receptors and Cardiac Contractility: Direct Evidence for "Spare" Beta-adrenergic Receptors*

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SUMMARY

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The relationship between the concentration of β-adrenergic receptors and the activation of heart muscle by isoproterenol was investigated using the irreversible β-adrenergic receptor antagonist NHNP-NBE. The interaction of NHNP-NBE with β-receptors was characterized in isolated membranes, whole cells and intact cardiac muscle, using [^{125}I]-iodohydroxybenzylpindolol (IHYP). Formation of the NHNP-NBE-β-receptor complex was found to be irreversible and dependent on incubation time, temperature and ligand concentration. Occupation of β-receptors by adrenergic ligands prior to NHNP-NBE exposure protected the receptors from inactivation. These data are consistent with a covalent modification of β-receptors by NHNP-NBE at a site in or near the adrenergic ligand binding site. Incubation of NHNP-NBE with intact cardiac muscle produced a dose dependent β-receptor inactivation that survived tissue homogenization and membrane isolation. NHNP-NBE dramatically affects the concentrations at which isoproterenol produces positive inotropic responses in cat papillary muscles. The ED_{50} for isoproterenol under control conditions averaged 9.8 nM. The ED_{50} of isoproterenol increased to 22; 70; 500 and 5623 nM subsequent to a 10 min treatment of the muscles with 0.1; 1.0; 10 and 100 μM NHNP-NBE respectively. The same maximum inotropic response was achieved with isoproterenol following each concentration of NHNP-NBE. Ten micromolar isoproterenol subsequent to 100 μM NHNP-NBE increased the papillary muscle concentration of cyclic AMP to the same extent as 10 μM isoproterenol alone. Although the maximum cyclic AMP response was essentially identical, the time course for cyclic AMP production was substantially slower following irreversible β-receptor blockade. The ED_{50} for isoproterenol induced increases in cyclic AMP concentrations was 15 nM in the absence of NHNP-NBE and 600 nM following 100 μM NHNP-NBE. In contrast to the cardiac data, NHNP-NBE stoichiometrically inhibited IHYP binding and isoproterenol induced cyclic AMP formation in cultured human lung (VA_2) cells. These data indicate a high efficiency coupling between the isoproterenol-β-adrenergic receptor interaction, cyclic AMP formation and increased cardiac contractility, an efficiency not apparent with isoproterenol-induced cyclic AMP formation in cultured cells.

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INTRODUCTION

In 1956 Stephenson introduced the concept of "spare receptors" and drug efficacy to explain differing maximal responses obtained with a series of cholinergic agonists on guinea pig ileum contraction (1). Around the same time, Furchgott (2) and Nickerson (3), using irreversible α-adrenergic receptor antagonists, provided evidence for the existence of more α-receptors than were required for complete tissue activation in certain systems. Similar evidence for β-adrenergic receptors has been lacking.

Although cardiac muscle is thought to function as a syncytium with the ability to transmit waves of depolarization and force to adjacent cells via tight junctions and intercalated discs, it has been a general premise that catecholamine effects in heart muscle result from the interaction of the amines with β-adrenergic receptors in an "occupancy"-related manner; occupation of 50% of the receptors theoretically results in 50% of the maximum contractile response (4).

Results of experiments using catecholamines immobilized on glass beads (4) and soluble amino acid polymers (5, 6) indicated that full cardiac contractile responses could be elicited by the catecholamines directly contacting as little as 0.01% of the cells involved in the contractile response (4). Due to the apparent ability of cardiac muscle to propagate the inotropic responses to locally applied catecholamines (4–6), these data imply the existence of an "excess" of β-adrenergic receptors in the tissue in a three-dimensional sense. However the responses to immobilized catecholamines do not provide information concerning the number or percentage of receptors on an individual cell that needed to be occupied to achieve an inotropic response.

The absence of information concerning β-receptor-contractile response stoichiometry has been due in part to the lack of an irreversible β-receptor antagonist. The report by Atlas *et al.* on the irreversible blocker NHNP-NBE (7),[1] appears to have overcome this deficit.

In this present report, I have characterized the nature of the blockade of β-adrenergic receptors by NHNP-NBE, studying its effects on membrane β-receptor binding, cardiac muscle and cultured cell cyclic AMP responses to catecholamines, and catecholamine-induced cardiac inotropic responses. The data indicate that the receptor blockade by NHNP-NBE is irreversible, and that although cultured cell cyclic AMP responses to isoproterenol are inhibited stoichiometrically with β-adrenergic receptors, in intact heart muscle, cyclic AMP and contractile responses to catecholamines are not inhibited, as measured stoichiometrically. Full inotropic and cyclic AMP responses are elicited with isoproterenol when more than 90% of the cardiac β-adrenergic receptors are irreversibly inactivated.

MATERIALS AND METHODS

Papillary muscles approximately 1 mm in diameter or less were quickly dissected from right ventricles of cats and established for isometric contraction as previously described (5). Cat cardiac membranes were prepared by homogenization of minced hearts in four volumes of 20 mM HEPES, 2 mM $MgCl_2$, 1 mM EDTA, pH 8.0, (homogenization buffer) in a Sorvall Omnimixer at maximum speed for two 30 sec periods. The suspension was further homogenized using a motor-driven Teflon pestle homogenizer (Tri-instrument model 41K), three strokes at speed 4. The crude homogenate was filtered through one layer of gauze then centrifuged at 900 × g for 15 min. The supernatant was centrifuged at 48,000 × g using a Sorvall SS34 rotor. The pellet was resuspended in homogenization buffer containing 10% sucrose and layered on top of sucrose gradients containing 10 ml each of 40%, 30%, and 20% sucrose in the same buffer. Gradients were centrifuged for 80 min at 27,000 rpm in a SW-27 rotor at 2°. The bands at the 20/30 and 30/40 sucrose interfaces were collected and centrifuged at

[1] The abbreviations used are: NHNP-NBE, N-[2-hydroxy-3-(1-naphthoxy)-propyl]-N-bromoacetylethylenediamine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanosulfonic acid; IHYP, [^{125}I]iodohydroxybenzylpindolol; VA_2 cells, an SV40 transformed clone of human lung (WI-38).

45,000 rpm (100,000 × *g*) for 60 min in a 50 Ti rotor. This pellet was resuspended in the homogenization buffer at a protein concentration of 20–30 mg/ml and snap frozen in liquid N_2. Membrane suspensions were stored at −85° until used. This procedure yielded a 4–5-fold purification of receptors with 23% recovery. Membranes from cultured cells were obtained by Dounce homogenization and differential centrifugation as described (8, 9).[2]

Membrane β-receptor assay. The IHYP binding assay was performed in polypropylene tubes by the method of Maguire *et al.* (8) with slight modifications. Each tube contained assay buffer (50 mM Hepes, pH 8.0, 5 mM magnesium sulfate), approximately 32–93 pM IHYP (56,000–160,000 cpm) and other compounds, as specified, in a final volume of 500 μl. The reaction was initiated by addition of the membrane preparation (50–150 μg membrane protein). The reaction mixture was incubated for 30 min at 30°, then diluted with 1.25 ml of filtration buffer (20 mM potassium phosphate buffer pH 8.0, 1 mM $MgSO_4$ containing 0.1 mM *dl*-propranolol) maintained at 37°, and immediately filtered through a 24 mm Gelman Type A/E glass fiber filter under low vacuum. As soon as the dilute reaction mixture was completely filtered, the filter was washed at the same flow rate with 25 ml of the filtration buffer without *dl*-propranolol, at 37°. The filter was then dried by application of a higher vacuum for a few seconds and counted in a Beckman 4000 Gamma counter at 72% efficiency. Specific binding is defined as the difference in IHYP binding in the presence and absence of 10 μM *l*-propranolol.

VA_2 cells (an SV40 transformed clone of human lung [WI-38]) were grown in an atmosphere of 10% CO_2 in Minimum Essential Medium supplemented with 10% fetal calf serum. For membrane preparations or for cyclic AMP studies, the cells were grown for two days past confluency.

Cyclic AMP was determined on trichloroacetic acid extracts of muscles or cells using the radioimmunoassay of Steiner *et al.* (10), as supplied by Schwartz Mann or New England Nuclear. Protein was determined by the fluorometric method described (11) using bovine serum albumin as a standard. NHNP-NBE was synthesized and characterized as described.[3] Purity was assessed by thin layer chromatography.[3] One peak was found on each of three solvent systems.[3] NHNP-NBE at the appropriate concentrations was dissolved in Krebs solution (papillary muscles), phosphate buffered saline (cultured cells) or assay buffer (membranes) immediately prior to use.

RESULTS

Characterization of NHNP-NBE interaction with β-receptors. Incubation of NHNP-NBE with membrane bound β-adrenergic receptors results in complete abolishment of IHYP specific binding. For example, IHYP (40 pM) binding to 150 μg of cat heart membranes was 18,474 ± 560 cpm ($n = 3$). In the presence of 10 μM *l*-propranolol, the binding averaged 9621 ± 514 cpm ($n = 3$) yielding specific binding of 8853 ± 760 cpm or 17.2 fmol/mg protein. Preincubation of membranes for 10 min with 100 μM NHNP-NBE resulted in IHYP binding of only 7380 ± 813 ($n = 3$ cpm), a level below that obtained with propranolol. This IHYP binding obtained in the presence of NHNP-NBE was not displaceable by either isoproterenol or propranolol, indicating that NHNP-NBE displaced primarily β-receptor specific IHYP binding.

In order to characterize the interaction between NHNP-NBE and β-receptors, a series of washout experiments were performed. *l*-isoproterenol (10 μM), *l*-propranolol (10 μM) and NHNP-NBE (100 μM) were preincubated with cat heart membranes as described in Table 1. Aliquots were removed from each incubation and the extent of receptor occupation by each ligand ascertained with IHYP binding. The remaining membrane ligand complexes were then subjected to six 30 min incubations at 30°

[2] Fraser, C. M. and Venter, J. C. β-Receptor turnover rates in cultures cells: Regulation by glucocorticoids, submitted for publication.

[3] Venter, J. C., Strauss, W. L., Triggle, D. and Soiefer, A. Purification of the mammalian lung β-adrenergic receptor, submitted for publication.

TABLE 1

Receptor recoveries subsequent to adrenergic ligand treatment and extensive washing

Cat cardiac membranes (90 μg/assay) were incubated with the indicated adrenergic ligand for 10 min at 30°. Membranes were then centrifuged at 48,000 × *g* for 20 min at 0°. Membranes were then resuspended in 1 ml HEPES buffer and either assayed immediately for IHYP binding (control) or incubated at 30° for 30 min, followed by centrifugation. This cycle of incubation and centrifugation was repeated five times over a six hour period. Subsequent to the final wash, the membranes were resuspended in 100 μl assay buffer and the IHYP specific binding determined using 180,000 cpm (73.5 pM) IHYP per assay. The experiment was repeated three times in triplicate. Values are reported as the IHYP specific binding ± standard deviations ($n = 9$) for each point for no drug, propranolol and NHNP-NBE; $n = 3$ for isoproterenol.

Adrenergic ligand	[^{125}I]IHYP β-receptor specific binding		β-receptor recovery
	Control	After 6 washes	
	(fmol/mg protein)		*(%)*
l-propranolol (10 μM)	0	12.3 ± 1.1	95
NHNP-NBE (100 μM)	0	0	0
l-isoproterenol (10 μM)	5.7 ± 1.7	11.5 ± 1.5	89
None[a]	21.2 ± 1.3	12.95 ± 0.78	100[a]

[a] The membrane incubation/washing procedure reduced the control IHYP specific binding by 39%. For comparative purposes this value is considered 100% receptor recovery.

each, followed by high speed centrifugation to wash the membranes free of reversible ligands. Subsequent to the sequential incubation-centrifugation steps that consumed a period of six hours, the degree of specific IHYP binding again was assessed. As summarized in Table 1, 95% of the β-receptors previously occupied by *l*-propranolol and 89% of the receptors previously occupied by *l*-isoproterenol were recovered by the incubation/washing process. In contrast β-receptor specific IHYP binding was not recoverable from membranes pretreated with NHNP-NBE (Table 1). These experiments were repeated using membranes isolated from cultured VA_2 cells with similar results. Propranolol and isoproterenol, competitive binders of the receptors for NHNP-NBE, did not replace NHNP-NBE. These data indicated that the NHNP-NBE interaction with cardiac and VA_2 cell β-receptors is irreversible and consistent with a covalent attachment of the ligand.

The covalent modification of β-adrenergic receptors by NHNP-NBE should display a time, temperature and concentration dependence. NHNP-NBE at a concentration of 100 μM irreversibly inactivates 100% of the membrane β-receptors in less than 60 sec. At a concentration of one and 10 μM the receptor inhibition is only 70 and 80% complete in 10 min. After 10 min, receptor inhibition proceeds at a much slower rate with 90 and 95% of the receptors being irreversibly inhibited after 60 min. Extensive washes had no effect on the data indicating that reversible binding of NHNP-NBE to the receptors does not exist. These data are illustrated in Figure 1.

Varying the reaction temperature from 2° to 37° had little apparent effect on the receptor inhibition with 100 μM NHNP-NBE. Complete (100%) receptor block occurred at all temperatures within two minutes. However, at lower concentrations β-receptor inactivation displayed temperature dependence. NHNP-NBE binding to β-receptors increased progressively as the incubation temperature was increased from 10 to 37°.

NHNP-NBE interacts with β-adrenergic receptors in a dose-dependent manner (Fig. 2). Consistent with the above findings, the dose response curve was affected by the duration of the incubation of the NHNP-NBE with membranes (Fig. 2).

The time, temperature and dose-dependence of NHNP-NBE association with β-receptors together with the irreversibility of the interaction is consistent with the covalent modification of the β-receptors by NHNP-NBE.

Receptor protection. One of the assumptions involving the use of irreversible "affinity" ligands is that the ligand covalently modifies a chemical residue in or near the "specific binding site." One test of this assumption involves the prevention of covalent modification by prior occupation of the specific site in question. In the case of the β-adrenergic receptor, the receptor occu-

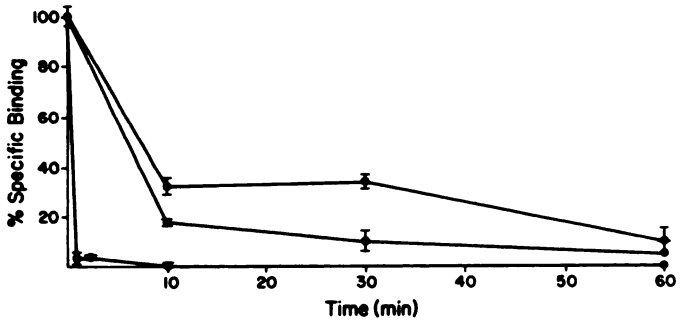

FIG. 1. *Time curve of NHNP-NBE inhibition of IHYP specific binding in cat cardiac membranes*

Cat heart membranes (150 μg/assay) were incubated i) with IHYP (55 pM) in the presence and absence of 10 μM propranolol to determine 100% specific binding; ii) or with NHNP-NBE; (1 μM top curve; 10 μM middle curve; and 100 μM bottom curve) for the times indicated in the figure. Subsequent to NHNP-NBE incubation, membranes were centrifuged at 48,000 × *g* for 20 min, resuspended in assay buffer and IHYP specific binding assessed. Error bars represent standard deviations for two experiments performed in triplicate.

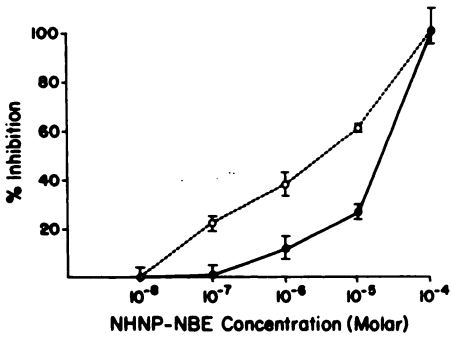

FIG. 2. *Log dose response curves for NHNP-NBE inhibition of IHYP specific binding in cat heart membranes*

Membranes (150 μg/assay) were incubated with NHNP-NBE at the indicated concentrations for 10 min (filled circles) and 60 min (unfilled circles); followed by centrifugation at 48,000 × *g*. Membranes were resuspended in assay buffer and assayed for IHYP specific binding (with and without *l*-propranolol) using 65 pM IHYP (10 min) and 58 pM IHYP (60 min) curves. Each point represents the mean of six determinations (two experiments performed in triplicate). Error bars represent the standard deviations.

pation by an adrenergic ligand should "protect" the receptor from NHNP-NBE inactivation.

In isometrically contracting cat papillary muscles incubation of the muscles with 10 μM *l*-isoproterenol prior to and during the exposure to 10 μM NHNP-NBE resulted in receptor protection from the effects of NHNP-NBE. The ED_{50} of *l*-isoproterenol on the muscles remained 10 nM both before and after NHNP-NBE treatment. The exposure of muscles to 10 μM NHNP-NBE in the absence of isoproterenol produced a rightward shift in the log dose response curve to isoproterenol. The isoproterenol ED_{50} was shifted from a control value of 10 nM to 500 nM after a 10 min exposure to NHNP-NBE. IHYP binding in membranes isolated from intact heart muscle treated with 10 μM NHNP-NBE for 10 min showed a loss of 71% of IHYP specific binding; muscles treated with 10 μM NHNP-NBE in the presence of 10 μM *l*-isoproterenol showed a loss of only 11% IHYP specific binding. When similar experiments were performed on isolated cat heart membrane β-receptors using *l*-propranolol as the protection agent, propranolol (10 μM) was able to protect 67% of the β-receptors from irreversible inactivation by NHNP-NBE (100 μM). These data, which are summarized in Table 2, suggest that NHNP-NBE interacts covalently with a residue in or extremely close to the adrenergic ligand binding site, and that prior occupation of the receptor can prevent covalent modification of the receptor by NHNP-NBE.

Irreversible β-receptor inactivation in intact cardiac muscle. Due to the nature of the cardiac inotropic responses to isoproterenol subsequent to NHNP-NBE treatment (see below), it became important to demonstrate a dose-related receptor occupation by NHNP-NBE in cardiac muscle. Intact cat cardiac muscle was treated with increasing concentrations of NHNP-NBE from 0.1 μM to 100 μM, for 10 min. The heart

TABLE 2

Protection from NHNP-NBE induced β-receptor inactivation by prior receptor occupation with adrenergic ligands

Preparation	Protective agent	Assay	Control	+ NHNP-NBE	NHNP-NBE after protection agent	β-receptor protection
						(%)
1) Contracting papillary muscles[a]	10 μM *l*-isoproterenol against 10 μM NHNP-NBE	Inotropic response ED_{50}'s	10 nM	500 nM	10 nM	100
2) Cat cardiac muscle[b]	10 μM *l*-isoproterenol against 10 μM NHNP-NBE	IHYP binding	2.5[d]	0.73[d]	2.2[d]	89
3) Cat heart membranes[c]	10 μM *l*-propranolol against 100 μM NHNP-NBE	IHYP binding	11.9[e]	0[e]	8.0[e]	67

[a] Three isometrically contracting papillary muscles that had a control dose-response relationship ascertained to isoproterenol were treated for 10 min with 10 μM isoproterenol prior to the addition of 10 μM NHNP-NBE to the muscle bath solution containing isoproterenol. Subsequent to a 10 min incubation the muscle baths were washed six times, and a second isoproterenol dose response relationship obtained upon muscle stabilization (30 min). The ED_{50} remained 10 μM before and after the NHNP-NBE treatment.

[b] Experiments were performed with cardiac cell membranes isolated and IHYP binding assessed as described in Figure 3. 10 μM NHNP-NBE produced a 71% reduction in IHYP specific binding in nonprotected cardiac muscle.

[c] Experiments were performed as outlined in Table 1 assessing the degree of recovery of IHYP specific binding with membranes treated with propranolol alone, NHNP-NBE subsequent to and in the continued presence of 10 μM propranolol and NHNP-NBE alone.

[d] fmol/assay.

[e] fmol/mg protein.

muscle was then extensively washed, homogenized, and a membrane fraction isolated. The concentration of remaining unoccupied β-receptors was determined by IHYP binding. As illustrated in Figure 3, NHNP-NBE produced a dose-dependent inactivation of β-receptors in the intact muscle. This receptor inactivation occurred despite extensive washing, muscle homogenization, and membrane isolation procedures. The inset of Figure 3 illustrates the dose-dependent inhibition of IHYP specific binding by NHNP-NBE.

NHNP-NBE effects on isoproterenol-induced cardiac positive inotropic responses. NHNP-NBE dramatically affects the concentrations at which *l*-isoproterenol produces positive inotropic responses in isolated cat papillary muscles. As illustrated in Figure 4, increasing the concentration of NHNP-NBE from 0.1 μM to 100 μM produced a progressive rightward shift in the log dose response curve to *l*-isoproterenol. However, importantly, there was no reduction in the maximum inotropic response to isoproterenol with any of the concentrations of NHNP-NBE (Fig. 4). An example of the actual polygraph tracings is presented in Figure 5. Unlike propranolol, which produces a nonspecific inhibition of muscle contraction at concentrations above 1 μM, NHNP-NBE displayed little or no effects on control contractile tension at concentrations up to 100 μM. At 100 μM NHNP-NBE has no effect on paired electrical stimulation, force-frequency responses, or length-tension relationships. The inotropic response to 10 mM Ca^{2+} is identical prior and subsequent to NHNP-NBE, with a maximum inotropic response achieved in each case. There was some evidence for partial agonist activity of NHNP-NBE: Addition of 1 μM to 100 μM NHNP-NBE to the muscle bath produced slight (~10%) transient increase in the force and velocity of muscle contraction (not shown).

Cardiac β-receptor occupation by NHNP-NBE vs. inotropic responses to isoproterenol. The percentages of cardiac β-adrenergic receptors irreversibly inacti-

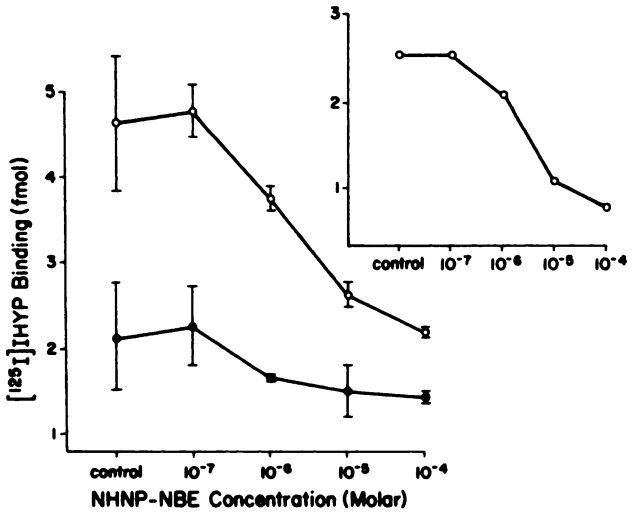

FIG. 3. *IHYP binding to cat heart membranes prepared from cat heart muscle treated with various concentrations of NHNP-NBE*

Freshly dissected cat cardiac muscle (2 g wet weight) minced into 1 mm diameter sections was incubated for 10 min at 30° with NHNP-NBE at the indicated concentrations subsequent to a 15 min equilibration in Krebs solution at 30°. Muscle fragments were washed four times with Krebs solution, then homogenized in 10 ml of 5 mM HEPES, 5 mM $MgSO_4$ at high speed for 2 min in a motor driven Teflon pestle homogenizer. Homogenates were then subjected to centrifugation at 800 × *g* for 10 min. The supernatant was then centrifuged at 48,000 × *g* for 20 min at 0°. The pellet was resuspended in assay buffer and assayed for IHYP specific binding. The figure denotes IHYP binding in the absence (unfilled circles) and presence (filled circles) of 10 μM *l*-propranolol. The error bars denote standard deviations for six determinations from two experiments performed in triplicate. The inset shows the IHYP specific binding with increasing concentrations of NHNP-NBE.

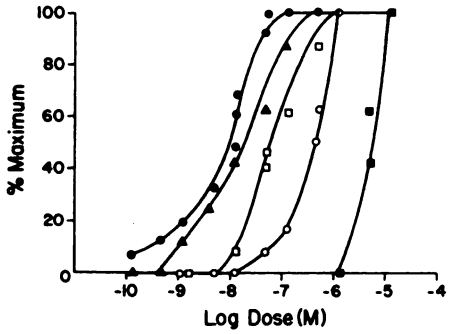

FIG. 4. *Log dose response curves for isoproterenol producing positive inotropic responses in cat papillary muscles in the presence of increasing concentrations of NHNP-NBE*

Cat papillary muscles (METHODS) were treated with 0.1 μM NHNP-NBE (▲); 1.0 μM (□); 10 μM (○) and 100 μM NHNP-NBE (■) for 10 min followed by 5 bath changes with fresh pre-warmed Krebs over a period of 30 min. *l*-isoproterenol dose response curves were performed over the ranges indicated in the figure. The control isoproterenol dose response curve is depicted by (●). Each set of experiments was performed sequentially on single papillary muscles. The peak (100%) response was the same for each dose response curve. Data points represent the mean of at least three experiments.

vated by various concentrations of NHNP-NBE are compared in Table 3 to the shift produced in the ED_{50} for isoproterenol-induced positive inotropic responses. The control ED_{50} for isoproterenol was 9.8 ± 2.3 nM (n = 7). With 0.1 μM, 1 μM, 10 μM, and 100 μM NHNP-NBE, the ED_{50}s for isoproterenol were 20 nM, 70 nM, 500 nM, and 5623 nM. However, with each concentration of NHNP-NBE, the same maximum contractile response to isoproterenol was obtained.

NHNP-NBE effects on isoproterenol-induced increases in cyclic AMP concentration. Cyclic AMP formation is a documented response to β-receptor stimulation in most tissues including heart (12). Previous studies from this laboratory have demonstrated that cyclic AMP does not appear to be involved in the propagation of cardiac inotropic responses to catecholamines (4–6), although it was proposed (4) that cyclic AMP could play a role in response initiation. With the above findings of maximal inotropic responses with less than 10% of the β-receptors accessible, it was of some interest to assess the cyclic

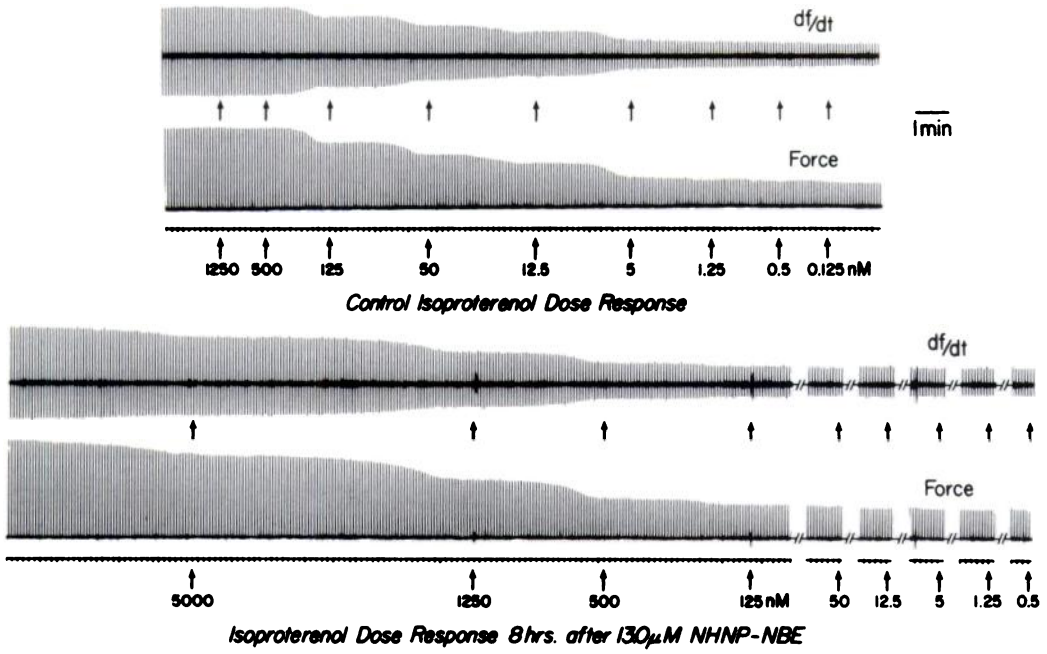

FIG. 5. *Sample polygraph tracing for isoproterenol dose response relationships before and after NHNP-NBE treatment*

Upper tracings illustrate the changes in force and df/dt in response to the indicated concentrations of *l*-isoproterenol. The lower set of tracings are an example of the isoproterenol dose response relationship 8 hr subsequent to a 10 min exposure to 13 μM NHNP-NBE demonstrating the persistence of the NHNP-NBE effect. The tracings are read from right to left.

TABLE 3

β-receptor occupation by NHNP-NBE vs. cardiac inotropic responses to isoproterenol

NHNP-NBE concentration[a]	% total β-receptors occupied[b]	Inotropic response ED_{50} *l*-isoproterenol	% control maximum response achieved
(μM)		(nM)[c]	
Control	0	9.8 ± 2.3 (n = 7)	100
0.1	0	22	100
1.0	43	70	100
10	69	500	100
100	90	5623	100

[a] Represents the final concentration of NHNP-NBE in cat papillary muscle baths for a 10 min incubation.

[b] Calculated from IHYP binding data.

[c] Determined from the midpoint of log dose response curves performed over 6 orders of magnitude of isoproterenol concentration.

nucleotide response under these same conditions. Control cyclic AMP concentrations were 3.42 ± 0.14, range (3.04–4.2) n = 8 pmol/mg protein. Isoproterenol increased the cardiac muscle concentration of cyclic AMP in a dose-related manner (Fig. 6). The ED_{50} for isoproterenol-induced changes in cyclic AMP concentration was 15 nM. With a concentration of 10 μM isoproterenol muscle cyclic AMP concentrations rose to 9.64 ± 0.77 pmol/mg protein; range (6.3–15), n = 10, a value significantly different from control $p \leq 0.001$ (two-tailed Student's t-test). NHNP-NBE (100 μM), in addition to effecting β-receptor binding and cardiac inotropic responses, dramatically affects the concentration at which isoproterenol produces cyclic AMP responses (Fig. 6). Isoproterenol (10 μM) increased cyclic AMP concentrations to 8.94 ± 0.90 pmol/mg protein, range (5.2–16), n = 12, a value significantly different from control ($p \leq 0.001$) (two-tailed Student's t-test); but not from the cyclic AMP concentration obtained with isoproterenol (10 μM) in the absence of irreversible β-receptor blocker ($p \leq 0.5$). NHNP-NBE shifted the ED_{50} for the isoproterenol-induced cyclic AMP response to 600 μM (Fig. 6).

In addition to the effect on the isoproter-

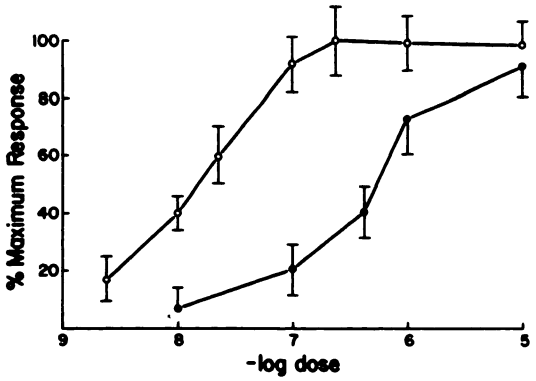

FIG. 6. *Log-dose response for l-isoproterenol induced cyclic AMP concentration increases in the presence and absence of an irreversible β-receptor blocker*

Isometrically contracting cat papillary muscles were treated with the indicated concentrations of *l*-isoproterenol for 180 sec prior to freeze clamping. The unfilled circles denote the control dose-response relationship. The filled circles denote the isoproterenol dose response relationship subsequent to muscle treatment with NHNP-NBE (100 μM) for 10 min, followed by four bath changes with fresh Krebs solution and muscle reequilibration. Frozen papillary muscles were rapidly homogenized in 500 μl of 6% trichloroacetic acid (TCA) at 0°. The homogenates were centrifuged for 5 min in a microfuge (12,000 × *g*). The supernatant was extracted three times with H_2O saturated ether; concentrated to dryness with a stream of nitrogen; redissolved in acetate buffer pH 4.0 and assayed for cyclic AMP using a radioimmunoassay. Protein was determined on the TCA pellets as described (METHODS). Each point represents the mean ± S.E.M. of at least three determinations.

enol dose response, NHNP-NBE also affected the time course of the cyclic AMP response (Fig. 7). In the absence of NHNP-NBE the isoproterenol-induced increase in cyclic AMP was half maximal in 8 sec (or a rate of 36.2 pmol cyclic AMP/min/mg muscle protein (Fig. 7). Following a 10 min treatment of papillary muscles with NHNP-NBE (100 μM), the rate of cyclic AMP formation was reduced to 4.6 pmoles cyclic AMP/min/mg muscle protein (Fig. 7).

β-receptor occupation by NHNP-NBE vs. cyclic AMP responses to isoproterenol in cultured cells. VA_2 cells provide a simple well-characterized system with regard to IHYP binding (8) and cyclic AMP production (13). Using intact VA_2 cells to measure cyclic AMP formation in response to isoproterenol and isolated VA_2 membranes to measure β-receptor concentrations with IHYP, the degree of the cyclic AMP response and β-receptor inhibition with various concentrations of NHNP-NBE were determined (Table 4). Control cyclic AMP concentrations in VA_2 cells were 24 ± 9 pmol/mg protein (n = 8). As with the cardiac contraction, NHNP-NBE appeared to have some partial agonist activity. At NHNP-NBE concentrations of 0.1 μM, 1 μM, 10 μM, and 100 μM, cyclic AMP concentrations were 88 ± 20, 58 ± 21, 51 ± 12, and 71 ± 16 pmol/mg respectively (n = 8) for each concentration values significantly different from the nontreated control ($p < 0.001$ Student's t-test). In the absence of NHNP-NBE, *l*-isoproterenol (10 μM) increased cyclic AMP concentrations to 1210 ± 53 pmol/mg. As can be seen in Figure 8, NHNP-NBE progressively reduced the isoproterenol response in a dose-related manner. At a concentration of 100 μM NHNP-NBE, the cyclic AMP concentration in response to isoproterenol was 90 ± 23 pmol/mg, a value not significantly different from its control of 71 ± 16 pmol/mg. Higher concentrations of isoproterenol did not increase the maximum cyclic AMP response in the absence or presence of NHNP-NBE.

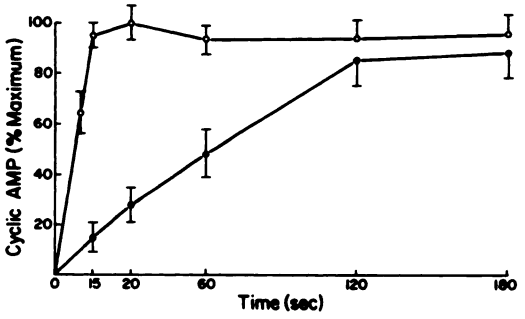

FIG. 7. *The effect of irreversible β-receptor blockade on the time course of increased cardiac muscle cyclic AMP concentrations in response to isoproterenol*

Isometrically contracting cat papillary muscles were treated with 10 μM isoproterenol for the times indicated and assayed for cyclic AMP as described in Figure 6. The unfilled circles denote the control cAMP time course to isoproterenol. The filled circles denote the cAMP time course to isoproterenol subsequent to NHNP-NBE (100 μM) treatment, as described in Figure 6. Each point represents the mean ± SEM of at least three determinations.

TABLE 4

Inhibition of isoproterenol-induced VA_2 cell cyclic AMP formation and IHYP binding by NHNP-NBE

NHNP-NBE concentration	Intact cell cyclic AMP response		Inhibition of [^{125}I]IHYP specific binding	
	Cyclic AMP[b]	Inhibition of cyclic AMP production	[^{125}I]-IHYP specific binding[c]	% β-receptors occupied by NHNP-NBE
(μM)	*(pmol/mg protein)*	*(%)*	*(fmol/mg protein)*	
Control[a]	1210 ± 53	0	5.4	0
0.1	609 ± 50	57	1.67	69
1.0	452 ± 70	68	1.15	79
10	115 ± 12	95	0.94	83
100	90 ± 23	99	0	100

[a] Control represents responses in the absence of NHNP-NBE.

[b] Cellular cyclic AMP production in response to 10 μM *l*-isoproterenol for 10 min at 37° (see Fig. 6 and text for details). $n = 8$ determinations for each point.

[c] Determined in isolated VA_2 membranes using IHYP as described in Fig. 8 and text.

DISCUSSION

NHNP-NBE was found to be an irreversible inhibitor of the β-adrenergic receptors in intact cat cardiac muscle and cultured human cells, as well as in the isolated membrane preparations. These data confirm and extend the report of Atlas *et al.* on the irreversible binding of NHNP-NBE to the β-receptor of turkey erythrocytes (7).

The availability of a covalent β-receptor antagonist make a number of experiments concerning β-adrenergic receptor coupling to cellular events feasible. By irreversible inhibition of existing β-adrenergic receptors in VA_2 cells, the synthesis and plasma membrane incorporation rate of new β-receptor molecules has been determined (14).[2] In the present study NHNP-NBE was utilized to irreversibly "inactivate" various percentages of β-adrenergic receptors to study the stoichiometry of coupling between β-receptors, cardiac contractility, and cyclic AMP production. In cardiac muscle, NHNP-NBE in increasing concentrations, irreversibly occupied an increasing percentage of β-adrenergic receptors, such that at a concentration of 100 μM NHNP-NBE, greater than 90% of the heart receptors were irreversibly inactivated (Table 3, Figure 3). The increasing occupation of β-receptors by NHNP-NBE produced a progressive rightward shift in the dose response curve of the heart muscle to *l*-isoproterenol (Figs. 4, 5, and Table 3). However, the maximum positive inotropic response to isoproterenol was not reduced with any of the concentrations of NHNP-NBE (Figs. 4, 5 and Table 3). These results indicate that isoproterenol can produce full (100%) inotropic responses with less than 10% of the total β-adrenergic receptors in an accessible form. Under these same conditions the cardiac muscle cyclic AMP response to isoproterenol was also undiminished, suggesting that the cardiac β-receptors can couple extremely efficiently to adenylate cyclase.

Previous data from this laboratory have demonstrated the ability of cardiac muscle to propagate an inotropic response

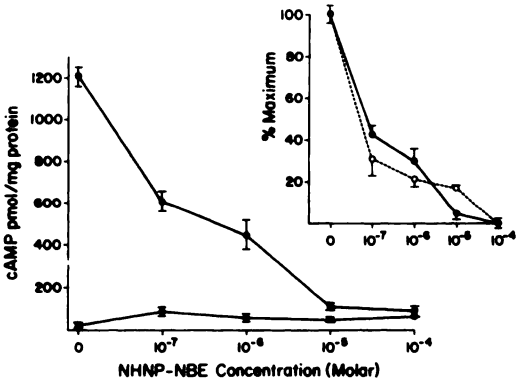

FIG. 8. *The inhibition of isoproterenol induced cyclic AMP formation in cultured cells by increasing concentrations of NHNP-NBE*

VA_2 cells grown to confluency on 60 mm culture dishes were assayed for cyclic AMP in trichloroacetic acid (TCA) extracts, subsequent to treatment with 10 μM *l*-isoproterenol for 10 min (upper curve). Cells were preincubated for 10 min with the indicated concentrations of NHNP-NBE followed by three washes with phosphate-buffered saline. The cells were then subjected to isoproterenol for 10 min or no treatment, followed by TCA extraction. Cyclic AMP was determined by radioimmunoassay (11). Error bars represent standard errors from two experiments performed in quadruplicate. The lower curve represents "basal" levels of cyclic AMP (0 concentration) and cyclic AMP produced in response to NHNP-NBE. The inset illustrates the percent inhibition of the isoproterenol-induced cyclic AMP production by NHNP-NBE (●) and of IHYP specific binding to VA_2 membranes (○).

throughout the muscle from a discrete site of catecholamine application to the muscle (4–6). This propagation suggests a high degree of coupling between cardiac cells, and that the maximal activation of a limited number of cells by isoproterenol can induce a spread or propagation of the inotropic response throughout the muscle (4–6). The measurement of cyclic AMP concentrations in cardiac muscle treated with immobilized catecholamines indicated that cyclic AMP may not be involved in the cell-to-cell propagation of the inotropic response (4–6). However, based upon cyclic AMP responses in cultured heart cells, it was proposed (4) that cyclic AMP could be involved in the response initiation. The data I have presented here suggest that only a small percentage (<10%) of the β-receptors in cardiac cells are required (in the presence of saturating concentrations of isoproterenol) to produce maximal cyclic AMP and inotropic responses. These data are consistent with a role for cyclic AMP in inotropic response initiation under these conditions, although as shown in Figure 7, the rate of activation of adenylate cyclase under receptor limiting conditions is substantially slower than control conditions. It is not presently known to what extent propagated responses occur with 90% of the β-receptors inactivated by NHNP-NBE. Activation of the propagation response may require the majority of receptors on only a few cells or less receptors on a majority of cells (6, 15). Experiments with glass bead and polymeric immobilized catecholamines under various degrees of NHNP-NBE inhibition may resolve this issue.

In contrast to the data obtained on cardiac contraction and concentrations of β-adrenergic receptors required for full muscle and cyclic AMP stimulation, the experiments on cyclic AMP formation in cultured cells (Fig. 8, Table 4) suggest a direct stoichiometric coupling between β-receptors and cyclic AMP production. The inhibition of IHYP binding to VA_2 membranes by NHNP-NBE paralleled the inhibition of cyclic AMP formation by saturating concentrations of isoproterenol (Fig. 8).

This apparent stoichiometry found in cultured VA_2 cells and S49 cells (16) between β-receptor number and cyclic AMP production argues against a single β-receptor simultaneously activating multiple units of adenylate cyclase. In a recent study it was suggested that NHNP-NBE affects the time course of adenylate cyclase activation by β agonists but that the same maximal enzyme activity was eventually obtained when guanylyl imidodiphosphate was included in the reaction mixture to irreversibly activate adenylate cyclase once stimulated by the β-receptor (17). The NHNP-NBE induced reduction in β-receptor binding produced a proportional decrease in the rate of enzyme activation (17). Tolkovsky and Levitski concluded from these and other data that collisions between β-receptors and adenylate cyclase resulted in enzyme activation (17). The VA_2 cell experiments in this present report are consistent with a collision coupling mechanism for adenylate cyclase activation, however the apparent stoichiometry between β-receptor number and cyclic AMP production argues that the half-life of the activated enzyme is no greater than the half-life of the agonist-receptor-catalytic unit interaction. Therefore a single receptor could not activate multiple catalytic units. The cardiac model also argues in agreement with Tolkovsky and Levitski (17) that single β-receptors cannot *simultaneously* activate multiple units of adenylate cyclase, but that with time single receptors can activate multiple units of the enzyme (Fig. 7). The difference between the VA_2 cell system and the cardiac muscle system, which allows for "spare receptors" to be expressed, may reside in the mode of coupling of β-receptors to adenylate cyclase and/or in the half-life of the receptor activated catalytic unit of adenylate cyclase. In contrast to the VA_2 system, the half-life of the cardiac cyclase may be prolonged upon receptor dissociation, therefore permitting the receptor to activate more than one catalytic unit.

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