Specific $\alpha_1$-Adrenergic Receptor Subtypes Modulate Catecholamine-Induced Increases and Decreases in Ventricular Automaticity

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Fifty percent of adult canine Purkinje fibers manifest a decrease in automaticity in response to $\alpha_1$-adrenergic stimulation with $10^{-10}$–$10^{-8}$ M norepinephrine (NE), and 50% manifest an increase. In contrast, most neonatal Purkinje fibers show an increase in automaticity in response to these concentrations of NE. We studied the modulation of NE effects, using the subtype selective $\alpha_1$-adrenergic antagonists chloroethylclonidine (CEC) and WB 4101. CEC selectively antagonized the decrease in automaticity such that, in both age groups, all Purkinje fibers showed NE-induced increases in automaticity. In Purkinje fibers from dogs treated with pertussis toxin, NE no longer induced a CEC-sensitive decrease in automaticity. In contrast, WB 4101 selectively antagonized the NE-induced increase in automaticity in both age groups. In the presence of WB 4101, NE decreased automaticity uniformly in adult Purkinje fibers and tended to induce no change in automaticity in neonatal Purkinje fibers. In the presence of prazosin ($10^{-6}$ M) or combined CEC ($10^{-7}$ M) and WB 4101 ($10^{-7}$ M), no $\alpha_1$-agonist-induced increase or decrease in rate was observed. Pretreatment of membranes from newborn and adult dog and rat ventricles with CEC resulted in a selective and irreversible inactivation of 25% of specific binding sites labeled with $[^{125}\text{I}]$IBE2254. In cultured neonatal rat ventricular myocytes, exposure to CEC resulted in a 35% decrease in the density of specific binding sites labeled with $[^{125}\text{I}]$IBE2254 but did not influence $\alpha_1$-adrenergic stimulation of inositol phosphate accumulation. In contrast, WB 4101 inactivated NE-stimulated inositol phosphate accumulation. Our results suggest that 1) at least two distinct $\alpha_1$-adrenergic receptor subtypes are present in neonatal and adult cardiac tissue, 2) the CEC-sensitive subtype is linked to a decrease in automaticity via a pertussis toxin–sensitive substrate, 3) the WB 4101–sensitive subtype is linked to an increase in automaticity (possibly via a mechanism related to phosphoinositide breakdown), and 4) although CEC- and WB 4101–sensitive $\alpha_1$-adrenergic receptor subtypes are present in the neonate, only the WB 4101–sensitive subtype is expressed functionally to induce effects on ventricular automaticity. (Circulation Research 1990;67:1535–1551)

Adrenergic amines have complex effects on automaticity in the ventricular conducting system: low concentrations decrease or increase automaticity via an $\alpha_1$-adrenergic mechanism, and high concentrations increase automaticity via $\beta$-adrenergic stimulation.\textsuperscript{1–3} Moreover, the effects of $\alpha_1$-adrenergic agonists on Purkinje fiber automaticity change with development.\textsuperscript{1–4} $\alpha_1$-Adrenergic stimulation with phenylephrine or epinephrine induces a decrease in automaticity in about two thirds and an increase in the remaining one third of adult canine Purkinje fibers. In contrast, the majority of neonatal canine Purkinje fibers respond to these agonists with an increase in automaticity.

Studies of rat hearts in vitro\textsuperscript{5} and in tissue culture\textsuperscript{6} as well as studies of the canine heart\textsuperscript{2} have demonstrated the dependence of the negative chronotropic response on the development of sympathetic innervation and the functional acquisition of a pertussis toxin–sensitive 41 kDa GTP regulatory protein. The decrease in automaticity itself appears to result from stimulation of the Na-K$^+$ pump current via a pathway transduced by this pertussis toxin–sensitive G protein.\textsuperscript{7,8}
The effector system responsible for the increase in automaticity is not yet known, but recent findings have suggested two potential mechanisms: 1) \( \alpha_1 \)-Adrenergic receptor stimulation decreases potassium conductance \((g_K)\). Although a decrease in \(g_K\) may increase automaticity in some instances, the effect of \( \alpha_1 \)-agonists to inhibit \(g_K\) is dependent on a pertussis toxin–sensitive G protein, whereas the \( \alpha_1 \)-adrenergic positive chronotropic response can occur when this protein is not functionally present.\(^2\) Thus, it is unlikely that \( \alpha_1 \)-adrenergic inhibition of \(g_K\) underlies the \( \alpha_1 \)-adrenergic positive chronotropic response. 2) \( \alpha_2 \)-Agonists also induce the hydrolysis of membrane phosphoinositides.\(^9,10\) This response is not inhibited by treatment with pertussis toxin and could link \( \alpha_1 \)-adrenoceptor stimulation to an increase in automaticity via the mobilization of intracellular calcium by inositol trisphosphate \((IP_3)\).

The aforementioned studies are consistent with a model in which different responses to \( \alpha_2 \)-adrenergic receptor stimulation arise principally from alterations in the characteristics of the coupling G proteins, which specifically link the \( \alpha_2 \)-adrenergic receptor to distinct effector mechanisms. However, it is possible that the developmental acquisition of a pertussis toxin–sensitive G protein is associated with a coordinate change in the properties of the myocardial \( \alpha_1 \)-adrenergic receptor per se. A recent study\(^11\) has provided evidence for at least two \( \alpha_2 \)-adrenergic receptor subtypes that activate discrete \( \alpha_2 \)-adrenergic response mechanisms and can be distinguished by their sensitivity to a para-substituted derivative of clonidine, chloroethylclonidine \((CEC)\), or the competitive \( \alpha_2 \)-antagonist \( WB 4101\).

The present study was designed 1) to test whether the predominance of an increase in automaticity in neonatal Purkinje fibers reflects the presence of only one pharmacologically distinct \( \alpha_1 \)-receptor subtype, 2) to test whether the \( \alpha_1 \)-adrenergic–mediated decrease and increase in automaticity in adult Purkinje fibers result from stimulation of distinct \( \alpha_1 \)-adrenergic receptor subtypes linked to different effector pathways, and 3) to further identify the molecular components of the transduction pathway that results in an increase or decrease in automaticity.

**Materials and Methods**

Forty-one adult mongrel dogs, weighing 12–20 kg, were anesthetized with 30 mg/kg i.v. pentobarbital sodium. Nineteen 1–7-day-old neonates (from four litters) and six 2-week-old dogs (from one litter) were anesthetized intraperitoneally or intravenously with 30 mg/kg pentobarbital. The chests were opened through a right lateral thoracotomy (adult dogs) or midline sternotomy (neonates and young dogs). Hearts were excised and rapidly immersed in cold Tyrode’s solution containing \((mmol/l)\) \( NaCl 131, NaHCO_3 18, NaH_2PO_4 1.8, MgCl_2 0.5, CaCl_2 2.7, dextrose 5.5, and KCl 2.7. Na_2EDTA \) was present in the Tyrode’s solution at a final concentration of \(5 \times 10^{-5} \) M. This concentration of EDTA does not affect action potential characteristics and spontaneous rate of Purkinje fibers.\(^1,12\)

In some experiments, we examined the effect of ADP-ribosylation and functional inactivation of the pertussis toxin–sensitive G proteins on the \( \alpha_1 \)-adrenergic pharmacological response. We used the method of Fleming et al\(^3\) and injected 2-week-old dogs intravenously with 30 \( \mu \)g/kg pertussis toxin. After 48–60 hours, the hearts were removed as described above.

**Microelectrode Techniques**

Purkinje fiber bundles were excised from the left and right ventricles, placed in a tissue bath, and superfused with Tyrode’s solution bubbled with 95% \( O_2 \)-5% \( CO_2 \) and warmed to 37\(^\circ\) C. The pH of the solution was 7.3, and its flow rate was 10–12 ml/min. The bath was connected to ground using a 3 M KCl bridge and an Ag/AgCl junction.

All fiber bundles were impaled with 3 M KCl–filled glass capillary microelectrodes with tip resistances of 10–20 M\( \Omega \). The electrodes were connected via 3 M KCl/Ag/AgCl interface to an amplifier having a high input impedance and capacity neutralization. The system was calibrated, and transmembrane potentials were displayed as previously described.\(^1\)

In experiments on automaticity, Purkinje fibers were permitted to beat spontaneously. In experiments on transmembrane action potential characteristics, Purkinje fibers were stimulated at a cycle length of 800 msec via Teflon-coated bipolar silver wire electrodes, using techniques described previously.\(^1\) Automatic rate, maximum diastolic potential, the amplitude and maximum upstroke velocity of phase 0, and the duration of the action potential to 50% and 90% repolarization were measured by methods previously described.\(^1\)

**Electrophysiological Protocols**

The preparations were impaled with microelectrodes, and transmembrane potentials and rhythm were observed for 1 hour, after which a regular rhythm (with variability of rate <10%) was usually demonstrable and control measurements were made. If the rhythm did not fall within these limits of variability, the fibers were discarded. After the stabilization period, fibers were superfused with norepinephrine \((1 \times 10^{-10} \text{ to } 1 \times 10^{-5} \text{ M})\). Action potential characteristics and spontaneous rate and rhythm were recorded after 15 minutes of superfusion at each concentration (preliminary studies showed that a steady-state response to norepinephrine was attained within 5 minutes after the onset of superfusion).

To study the effects of \( \alpha_1 \) and \( \beta \)-adrenergic blockade, prazosin \((1 \times 10^{-6} \text{ M})\) and/or propranolol \((2 \times 10^{-7} \text{ M})\) was added to the superfusate after the 1-hour control period. The fibers were superfused with the antagonist(s) for 30–40 minutes (time to steady state was 20–30 minutes), after which a second set of control readings was taken. Prior studies by others\(^14\) and by us\(^3\) have shown that these concentrations of the
antagonists have no significant effects on transmembrane resting and action potentials while inducing α-adrenergic and β-adrenergic blockade.

In studies of the effects of α₁-adrenergic receptor subtype blockade, a group of fibers was superfused with either CEC or WB 4101 (1×10⁻¹⁰ to 1×10⁻⁷ M), followed by a washout to determine the effects of each blocker on the automatic rates of spontaneously beating preparations and on transmembrane action potential characteristics of driven preparations. Superfusion periods were 15 minutes for each concentration (a steady-state effect was attained within 5–10 minutes.) In subsequent experiments, four different groups of fibers were studied. Norepinephrine (1×10⁻¹⁰ M and 1×10⁻⁹ M) was superfused in the presence of either 1) propranolol (2×10⁻⁷ M), 2) propranolol and CEC (1×10⁻⁷ M), 3) propranolol and WB 4101 (1×10⁻⁷ M), or 4) propranolol, CEC, and WB 4101. Each concentration of norepinephrine was superfused for 15 minutes. Antagonists were superfused for 20–30 minutes before addition of norepinephrine.

Data Analysis of Microelectrode Experiments

Action potential characteristics are reported only for those experiments in which the microelectrode impalement was maintained throughout the duration of the experiment. Similarly, automaticity is reported only for those experiments in which the control automatic rates showed a variance not greater than 10%. Data were expressed as mean±SEM. The statistical technique used was analysis of variance; Scheffe’s test was also used when the F value permitted. For Scheffe’s test, p<0.05 was considered significant.

Preparation of Membranes

Membranes were prepared from neonatal and adult ventricles and Purkinje fibers as described previously. Briefly, canine cardiac tissue was suspended in a buffer of sucrose (0.25 M) and histidine (0.03 M) at pH 7.4, homogenized at 4°C with a Teflon-coated pestle, and centrifuged at 1,500g for 10 minutes to remove nuclear debris and cellular organelles. The supernatant was centrifuged at 46,300g for 45 minutes, and the soft pellet was resuspended in sucrose-histidine buffer to give a final protein concentration of about 3 mg/ml.

Membranes also were prepared from irradiated myocyte cultures. Cultures were prepared from hearts of 2-day-old Wistar rats that were decapitated after CO₂ narcosis. The hearts were removed under sterile conditions, the atria were trimmed away, and the ventricular cells were isolated according to a trypsin dispersion protocol described previously. Isolated cells were pooled, centrifuged at 200g for 5 minutes, and resuspended in Dulbecco’s minimum essential medium (MEM) with 10% horse serum, 5×10⁻⁵ M hypoxanthine, and 12 mM NaHCO₃. The cells were preplated for 40 minutes at 37°C to decrease fibroblast contamination. The muscle cells were then resuspended in MEM supplemented with 10% horse serum and grown in 100-mm plates that had been precoated with fibronectin (25 ng/mm²) for 45 minutes. Although this culture technique effectively decreases fibroblast contamination, previous studies indicated that a small number of nonmuscle cells with proliferative capacity persist in the myocardial cell culture (e.g., see References 6 and 10). Therefore, in some experiments, the cultures were exposed to 30-Gy x-rays using a 300 kVp x-ray machine (Siemens Analytical X-Ray Instruments, Inc., Madison, Wis.) and a 0.2-mm copper filter at a target distance of 30 cm to provide a dose rate of 3.52 Gy/min. This protocol is not associated with any obvious injurious effects to myocytes but totally and selectively eliminates any residual fibroblast contamination of the cultures. Membranes were prepared from the cultures after 4 days. Cell-detaching medium containing (mM) NaCl 130, NaHCO₃ 16, KCl 3, NaH₂PO₄ 0.5, sucrose 10, and EDTA 1 was added to each plate. After 30 minutes, cells were removed by scraping with a rubber policeman and collected into the sucrose-histidine buffer. Membranes were prepared as described above.

Studies of α₁-Adrenergic Receptors

α₁-Adrenergic receptors were characterized using α-[β-4-hydroxyphenylethylaminomethyl]tetracaine (BE2254), which was radioiodinated to a theoretical specific activity of 2,200 Ci/mmol and purified according to methods previously published. To identify total and CEC-sensitive α₁-receptors, cardiac membranes were incubated in the presence or absence of 1×10⁻¹⁰ M CEC for 10 minutes at 37°C immediately before performing the binding assays. Under these conditions, CEC irreversibly modifies a subset of α₁-receptor sites that are rendered unavailable for subsequent identification by [¹²⁵I]BE2254. In a preliminary experiment, we established that more prolonged preincubation with CEC (up to 30 minutes) did not increase the proportion of receptors modified by CEC. Furthermore, although others have reported that α₁-receptors are maximally inactivated by CEC in hypotonic medium, we have observed a similar loss of binding sites in membranes exposed to CEC in either sucrose-histidine buffer or 10 mM NaHEPES buffer under our experimental conditions (data not shown), and thus, in these studies inactivation by CEC was performed in sucrose-histidine buffer.

For equilibrium binding experiments, aliquots of myocardial membranes (30–50 μg) were incubated at 37°C with [¹²⁵I]BE2254 (5–400 pM) in the presence or absence of 1×10⁻⁶ M prazosin in a final volume of 1 ml. The assay buffer contained 0.15 M NaCl, 0.01 M KCl, 2 mg/ml dextrose, 1 mg/ml bovine serum albumin, and 0.01 M Tris, pH 7.4. Binding was terminated after 30 minutes by rapid vacuum filtration of the entire assay volume over glass fiber filters (model A/E, Gelman Sciences Inc., Ann Arbor, Mich.), followed by one wash with 10 ml of 0.05 M Tris. Radioactivity trapped by the
filters was detected in an autogamma scintillation spectrophotometer (Packard Instrument Co., Inc., Meriden, Conn.). All experiments were performed in triplicate. Equilibrium binding data were analyzed by the method of Scatchard.\textsuperscript{17}

**GTP Regulatory Proteins**

Pertussis toxin catalyzes the covalent incorporation of ADP-ribose from NAD into a family of GTP regulatory proteins (G proteins). Assays of the pertussis toxin--sensitive G protein measure the pertussis toxin--catalyzed incorporation of [\textsuperscript{32}P]ADP-ribose from [\textsuperscript{32}P]NAD into an appropriate molecular weight protein, as previously described.\textsuperscript{2} Lubrol (0.1%) was included in the reaction buffer to ensure complete ADP-riboylation of the substrate present in the membrane preparation. The concentration of the pertussis toxin--sensitive G protein in the membrane preparation was calculated from the number of counts in this band in the polyacrylamide gel, the specific activity of the [\textsuperscript{32}P]NAD, and the protein concentration.

**Measurement of Phosphoinositide Turnover in Cultured Neonatal Rat Ventricular Myocytes and Cardiac Membranes**

Inositol phospholipid turnover was assessed by measuring the accumulation of [\textsuperscript{3}H]inositol phosphates according to methods described previously.\textsuperscript{10} Briefly, membrane phosphoinositides were labeled to isotopic steady state by incubating cultures in the presence of [\textsuperscript{3}H]myoinositol (3–4 Ci/ml; specific activity, 16.5 Ci/mmmol; NEN-Du Pont, Wilmington, Del.) for 4 days. On the fourth day, unincorporated isotope was removed by washing the monolayer five times with HEPES-buffered saline of the following composition (mM): NaCl 118, KCl 4.7, CaCl\textsubscript{2} 3, MgCl\textsubscript{2} 1.2, KH\textsubscript{2}PO\textsubscript{4} 1.2, EDTA 0.5, glucose 10, and HEPES 25 (pH 7.4). Cultures were preincubated in the absence or presence of 2.5×10\textsuperscript{-5} M CEC for 30 minutes at room temperature. For experiments designed to examine inositol phosphate accumulation in intact myocytes, cultures were grown in multiwell plates (6×35 mm). Protocols were initiated by the addition to each 35-mm well of 1 ml fresh incubation buffer containing 10 mM LiCl and the indicated norepinephrine concentration in the absence or continued presence of CEC. After incubation for 30 minutes at room temperature, the reactions were terminated by rapid aspiration of the incubation buffer and addition of 1.5 ml acidified chloroform: methanol:6 M HCl (500:1:1000:3). The lipids were extracted for 30 minutes at room temperature. Chloroform (0.5 ml) and H\textsubscript{2}O (1 ml) were then added. The mixture was vortexed vigorously and centrifuged at 1,000g for 2 minutes to separate the phases. Inositol phosphates in the aqueous phase were isolated by Dowex anion exchange chromatography as described previously.\textsuperscript{10}

In other experiments, myocyte membranes were prepared from [\textsuperscript{3}H]inositol-labeled myocytes grown in 100-mm plates. Ten plates were washed extensively to remove unincorporated label, as described above. Five plates were preincubated with 2.5×10\textsuperscript{-4} M CEC for 30 minutes at 37°C. One milliliter ice-cold homogenization buffer containing (M) sucrose 0.25, EGTA 1×10\textsuperscript{-3}, CEC 2.5×10\textsuperscript{-5}, and Tris 0.02 (pH 7.6) was added to each plate. The cells were removed by scraping with a rubber policeman, pooled, homogenized, and centrifuged at 1,000g for 15 minutes. The remaining five plates were treated in an identical fashion except that CEC was omitted from the preincubation and homogenization buffers. For each preparation, the small pellet was discarded, and the supernatant was transferred to a fresh polypropylene tube and centrifuged at 43,000g for 1 hour. Each pellet was resuspended in 1.25 ml incubation buffer containing (M) sucrose 0.13, KCl 0.026, MgCl\textsubscript{2} 5×10\textsuperscript{-3}, EGTA 1×10\textsuperscript{-3}, LiCl 0.01, ATP 1×10\textsuperscript{-4}, and Tris 0.05 (pH 7.0), with or without CEC 2.5×10\textsuperscript{-5}. Incubations were initiated by the addition of 100 l myocyte membranes to 10 l incubation buffer containing appropriate test agents. The incubations were carried out for 5 minutes at 37°C. The assay was stopped by the addition of 1.5 ml acidified chloroform: methanol:6 M HCl, and inositol phosphates in the aqueous phase were separated as described above.

**Materials**

We purchased norepinephrine and propranolol from Sigma Chemical Co., St. Louis, CEC and WB 4101 from Research Biochemical Inc., Natick, Mass., and pertussis toxin from List, Campbell, Calif. Prazosin was a generous gift from Pfizer, Groton, Conn. BE2254 was a generous gift from Drs. E. Hofferber and W. Hansen, Beiersdorf AG, Hamburg, FRG.

**Results**

**Effects of Norepinephrine on Automaticity of Spontaneously Beating Adult Canine Purkinje Fibers**

The spontaneous rates and maximum diastolic potentials of adult canine Purkinje fibers recorded before norepinephrine superfusion are presented in Table 1. As we have previously reported,\textsuperscript{1} all changes in automaticity that occurred in this study were paralleled by changes in the slope of phase 4 depolarization. Hence, only data for rate are reported here. The fibers were grouped as “monophasic” or “biphasic” based on their responses to norepinephrine (10\textsuperscript{-10}–10\textsuperscript{-8} M). This was done because the effect of \(\alpha\)-adrenergic stimulation on automaticity of spontaneously beating canine Purkinje fibers distinguishes two populations of fibers with distinct concentration–response relations, referred to as biphasic and monophasic (Figure 1).\textsuperscript{1} Biphasic indicates a decrease in automaticity at low agonist concentrations and an increase at high concentrations (previously shown by us to be \(\alpha_1\)– and \(\beta\)-adrenergic, respectively).\textsuperscript{1,3} Monophasic indicates an \(\alpha_1\)-adrenergic–induced increase in automaticity at low agonist concentrations and a \(\beta\)-adrenergic–induced increase at
high concentrations. The subset of fibers exhibiting a biphasic response accounted for 50% of the total preparations studied. The remaining 50% of fibers exhibited a monophasic response. Statistical comparison of these two populations to one another (biphasic versus monophasic) showed that values obtained between $10^{-10}$ and $10^{-7}$ M norepinephrine differed significantly ($p<0.05$). At norepinephrine concentrations above $10^{-7}$ M, however, the rates attained in the two populations were not different.

The effect of propranolol ($2 \times 10^{-7}$ M) on the response of adult Purkinje fibers to norepinephrine was studied in sixteen preparations (Figure 1). In the presence of $\beta$-blockade, both monophasic and biphasic responses still were present, with the biphasic response occurring in 50% of the preparations. The norepinephrine concentration–response curve in propranolol-superfused fibers was shifted downward and to the right compared with the curve in the presence of norepinephrine alone (Figure 1A). Note that the negative chronotropic response occurring at low doses of norepinephrine was not antagonized by the $\beta$-blocker.

A monophasic response occurred in the remaining 50% of the preparations superfused with propranolol. Propranolol again shifted the concentration–response curve downward and to the right, but only at agonist concentrations greater than $10^{-7}$ M.

For both groups of propranolol-superfused fibers (i.e., those showing either monophasic or biphasic responses), the peak increase in rate seen at $10^{-5}$ M did not differ significantly from that observed in the presence of agonist alone. This presumably reflects the fact that there is a 50-fold excess of agonist over antagonist in these experiments. Higher concentrations of propranolol were not used because of their direct membrane effects. Hence, Figure 1 demonstrates for norepinephrine what we previously have shown for epinephrine and phenylephrine; that is, at high concentrations (i.e., $>10^{-7}$ M) the effect of agonist to increase automaticity is attenuated, although not completely blocked, by the $\beta$-adrenergic antagonist propranolol ($2 \times 10^{-5}$ M). We previ-

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**TABLE I. Control Automaticity and Maximum Diastolic Potential of Canine Purkinje Fibers**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fibers with subsequent biphasic response</th>
<th>Fibers with subsequent monophasic response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$</td>
<td>Rate (beats/min)</td>
</tr>
<tr>
<td>Adult</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5</td>
<td>20±5</td>
</tr>
<tr>
<td>PROP ($2 \times 10^{-7}$ M)</td>
<td>8</td>
<td>26±4</td>
</tr>
<tr>
<td>Neonate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3</td>
<td>31±4</td>
</tr>
<tr>
<td>PROP ($2 \times 10^{-7}$ M)</td>
<td>4</td>
<td>38±5</td>
</tr>
<tr>
<td>PROP+WB 4101 ($1 \times 10^{-7}$ M)</td>
<td>3</td>
<td>25±3</td>
</tr>
</tbody>
</table>

Values were recorded before norepinephrine superfusion and are expressed as mean±SEM. The treatment column includes fibers that were not superfused with antagonist before norepinephrine, as well as fibers superfused with various antagonists and combinations of antagonists before norepinephrine. Where antagonists are used, the rate and maximum diastolic potential (MDP) are those in the presence of antagonist and before norepinephrine. PROP, propranolol; $n$ = number of experiments. See text for discussion of monophasic and biphasic responses.

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**FIGURE 1.** Graphs showing response of adult canine Purkinje fibers to norepinephrine (NE) in the absence or presence of $2 \times 10^{-7}$ M propranolol (PROP). Data are presented as mean±SEM of change in beats per minute from control, which is expressed as zero. Panel A: Biphasic concentration–response curves. Comparison of the two curves (NE in the absence [○] and presence [Δ] of PROP) shows a significant difference ($\ast p<0.05$) between the curves at $10^{-7}$ and $10^{-6}$ M. The curve for NE alone differs significantly from control at $10^{-6}$ M, $10^{-5}$ M, and $10^{-4}$ through $10^{-3}$ M. The NE+PROP curve differs significantly from control at $10^{-5}$ M, $10^{-4}$ M, $10^{-3}$ M, and $10^{-2}$ M. Panel B: Monophasic concentration–response curves. Comparison of the two curves (NE in the absence [●] and presence [▲] of PROP) shows a significant difference ($\ast p<0.05$) between the curves at $10^{-7}$ M and $10^{-6}$ M. All values for both curves differ significantly from control. Control values for spontaneous rates of these experimental groups are shown in Table 1.
ously have shown that the β-adrenergic response is further attenuated, in both adult and neonatal fibers, by higher concentrations of the β-adrenergic blocker nadolol, which has fewer direct effects than propranolol.\(^{18}\)

It is to be emphasized that the focus of the present study is on the effects of low agonist concentrations (i.e., \(<10^{-7}\) M) to increase or decrease automaticity. These actions were not attenuated by a β-adrenergic antagonist and were consistent with an α-adrenergic response. In all subsequent experiments, to minimize any β-adrenergic actions of norepinephrine, propranolol was added to the superfusate.

**Effects of CEC and WB 4101 on Transmembrane Potentials of Driven Adult Canine Purkinje Fibers**

In the control setting for the Purkinje fibers subsequently superfused with CEC, maximum diastolic potential was \(-97±3.8\) mV, maximum upstroke velocity (\(V_{\text{max}}\)) was \(832±27\) V/sec, and overshoot was \(29±2.1\) mV. None of these values was changed significantly by \(10^{-10}\) to \(10^{-5}\) M CEC; that is, at \(10^{-5}\) M, the values, respectively, were \(-95±2.7\) mV, \(836±22\) V/sec, and \(31±3.5\) mV \((p>0.05)\). In the control setting for fibers subsequently superfused with WB 4101, maximum diastolic potential was \(-95±2.2\) mV, \(V_{\text{max}}\) was \(643±27\) V/sec, and overshoot was \(30±2.8\) mV. WB 4101 (\(10^{-10}\) to \(10^{-5}\) M) induced no significant changes; that is, the respective values at \(10^{-5}\) M were \(-99±4.0\) mV, \(643±42\) V/sec, and \(28±3.7\) mV \((p>0.05)\). CEC at \(10^{-6}\) M and \(10^{-5}\) M significantly prolonged action potential duration at 50% and 90% repolarization (Figure 2, upper panels). In contrast, \(10^{-5}\) M WB 4101 significantly shortened action potential duration at 50% and 90% repolarization (Figure 2, upper panels). As anticipated, the effects of the competitive antagonist WB 4101 on action potential duration were reversible; those of CEC, an alkylating agent, were not.

**Effects of CEC and WB 4101 on Automaticity of Spontaneously Beating Adult Canine Purkinje Fibers**

At an extracellular K\(^+\) concentration of 2.7 mM, CEC induced a decrease in rate at all concentrations studied (Figure 3, right panel). The peak decrease occurred at \(10^{-7}\) M, and higher concentrations caused a less pronounced negative chronotropic response. In these preparations, the control rate was \(30±7\) beats/min. To investigate the ability of CEC to alter automaticity in preparations having lower spontaneous rates, we also performed experiments at an extracellular K\(^+\) concentration of 4.0 mM (Figure 3, left panel). Here, the control rate was \(18±4\) beats/min, and CEC again decreased automaticity.

Because an early report suggested that CEC might possess some α-agonist activity,\(^{19}\) we studied the interaction of prazosin and CEC. Prazosin blocked the effects of CEC on spontaneous rate at both extracellular K\(^+\) concentrations (Figure 3). This result is consistent with a partial agonist effect of CEC.

In contrast to CEC, WB 4101 induced no significant change in spontaneous rate in seven fibers, (control, \(20±4\) beats/min; \(10^{-6}\) M WB 4101, \(22±5\) beats/min; \(p>0.05\)).

**Effects of CEC, WB 4101, and Prazosin on the Chronotropic Response to Norepinephrine**

In these experiments, we investigated the ability of CEC and WB 4101 to modify the slowing or acceleration of rate occurring at low norepinephrine concentrations. Figure 4 shows the effects of CEC and WB 4101 on changes in spontaneous rate induced by norepinephrine (\(10^{-10}\) M and \(10^{-9}\) M). As in Figure 1, low concentrations of agonist caused either an increase (eight of 14 preparations) or a decrease (six of 14 preparations) in automaticity of propranolol superfused canine Purkinje fibers (Figure 4A). We performed the identical experimental protocol in the presence of CEC (\(10^{-7}\) M), and all 12 fibers showed an increase in rate (Figure 4B). In contrast, in the presence of WB 4101 (\(10^{-7}\) M), a decrease in rate was seen in all 12 fibers studied (Figure 4C). Therefore, these results show that CEC and WB 4101 each antagonizes a distinct α-adrenergic response. CEC abolished the negative, whereas WB 4101 blocked the positive chronotropic response induced by low norepinephrine concentrations.

In contrast to the above, the non–subtype-selective blocker prazosin antagonized both the positive and
FIGURE 3. Graphs showing effects of chloroethylclonidine (CEC) in the absence (●) or presence (△) of 10^-6 M prazosin on spontaneous rate of adult canine Purkinje fibers. Left panel: Extracellular K⁺ concentration ([K⁺]o)=4 mM (control values: for ●, rate=18±4 beats/min; maximum diastolic potential=−88±0.7 mV; for △, rate=16±4 beats/min; maximum diastolic potential=−87±2.0 mV). Right panel: [K⁺]o=2.7 mM (control values: for ●, rate=30±7 beats/min; maximum diastolic potential=−97±3.8 mV; for △, rate=28±5 beats/min; maximum diastolic potential=−99±5.0 mV). Data are expressed as mean±SEM. *p<0.05 compared with control (C).

the negative chronotropic responses to agonist concentrations of 10^-7 M or less (Figure 5A). All data points for each experiment are shown in Figure 5A to demonstrate the absence of any change in rate at low agonist concentrations. One might predict that if CEC and WB 4101 each antagonized a subset of the receptors that, in sum, are blocked by prazosin, then the effect of a combination of CEC and WB 4101 would be equivalent to that of prazosin. That this, in fact, occurred is shown in Figure 5B; both the increase and decrease in automaticity at norepinephrine concentrations of 10^-7 M or less were abolished, and all values for automatic rate clustered about the line indicating no significant change (p>0.05). Hence, when fibers with the monophasic and biphasic responses were studied together, the failure to respond to norepinephrine in the presence of prazosin or combined CEC and WB 4101 truly reflected the absence of change rather than the averaging of positive and negative values. At norepinephrine concentrations of 10^-6 and 10^-5 M, there was an increase in automaticity in Figures 5A and 5B. However, as indicated above, this increase was β- rather than α-adrenergic.

Response of Neonatal Canine Purkinje Fibers to Norepinephrine

The experiments in adult canine Purkinje fibers indicated clearly that the decrease and the increase in automaticity could be attributed to two pharmacologically distinct α-receptor responses. The question that then arose was whether the increase in automaticity seen in most neonatal fibers might result from the preferential expression of a WB 4101-sensitive α₂-adrenergic receptor mechanism. For this reason, we studied neonatal fibers superfused with norepinephrine alone or with norepinephrine in the presence of propranolol, to block any β-adrenergic response.

Table 1B reports control spontaneous rates for neonatal Purkinje fibers. As for the adults, the fibers were grouped based on their biphasic or monophasic response to norepinephrine. We superfused 10 neonatal fibers with norepinephrine alone (Figure 6). Whereas 50% of adult fibers showed a biphasic response to agonist, in the neonate only 28% were biphasic, and the remainder were monophasic. In the presence of propranolol, neonatal fibers showed responses similar to those of the adults. As shown in Figure 6A, the biphasic agonist curve was shifted downward and to the right of the curve for norepinephrine alone. Similarly, β-blockade shifted the monophasic agonist curve downward and to the right at high agonist concentrations (>10^-5 M), whereas the initial portion of the curve (agonist ≤10^-8 M) was not affected (Figure 6B).
We next studied the effects of WB 4101 and CEC on automaticity of propranolol-superfused neonatal Purkinje fibers. As for the adult fibers, we first determined the effects of CEC and WB 4101 on the transmembrane potentials of driven neonatal Purkinje fibers. As shown in Figure 2 (lower panel), 10^{-5} M CEC significantly prolonged neonatal action potential duration, whereas 10^{-6} and 10^{-5} M WB 4101 significantly accelerated repolarization. In the control setting for the fibers subsequently superfused with CEC, maximum diastolic potential was $-102 \pm 9.5$, $V_{\text{max}}$ was $525 \pm 95$ V/sec, and overshoot was $36 \pm 3.8$ mV. None of these values was changed significantly by $10^{-10}$-10^{-7} M CEC; that is, at 10^{-5} M, the values, respectively, were $-104 \pm 1$ mV, $525 \pm 68$ V/sec, and $36 \pm 2.4$ mV (all $p > 0.05$). For fibers subsequently superfused with WB 4101, control maximum diastolic potential was $-97 \pm 1.3$ mV, $V_{\text{max}}$ was $575 \pm 33$ V/sec, and overshoot was $31 \pm 4.2$ mV. At 10^{-5} M WB 4101, the respective values were $-98 \pm 2.5$ mV, $555 \pm 22$ V/sec, and $30 \pm 3.3$ mV (all $p > 0.05$). The results were entirely identical to those seen in adults, as was the reversibility of the effect of WB 4101 but not CEC.

The effects of WB 4101 on norepinephrine-dependent changes in automaticity are included in Figure 6. As shown in Figure 6A, three of 11 fibers exposed to norepinephrine (10^{-10}–10^{-7} M), propranolol, and WB 4101 exhibited a biphasic response. Eight fibers showed a monophasic response (Figure 6B) in which there was neither a decrease nor an increase in rate at 10^{-10}–10^{-7} M norepinephrine. Comparing these experiments with those in adults, an important difference emerges. In adults, in the presence of WB 4101, 10^{-10}–10^{-9} M norepinephrine induced a negative chronotropic response in 100% of 12 preparations (Figure 4). In contrast, in neonates, only a minority (three of 11 fibers, or 28%) now showed decreased automaticity at 10^{-10}–10^{-8} M norepinephrine (Figure 6A). In the remainder of neonatal fibers, the low norepinephrine concentration induced no change in automatic rate in the presence of WB 4101 (Figure 6B). This suggested to us that in the subset of fibers that showed no decrease in automaticity either the $\alpha_1$-receptor subtype capable of reducing automaticity (i.e., CEC sensitive) was absent and/or a mechanism distal to the receptor (e.g., the pertussis toxin-sensitive G protein) was functionally absent.

We then tested whether neonatal fibers like those in Figure 6A, which showed a negative chronotropic response to low norepinephrine concentrations had a functionally mature receptor-effector coupling mechanism. To do this, we studied the effects of CEC on automaticity of nine propranolol-superfused neonatal fibers. As shown in Figure 7, CEC had no effect on the response of those fibers that initially showed an increase in automaticity when stimulated with 10^{-10}–10^{-9} M norepinephrine ($n = 6$). In contrast, CEC antagonized and actually reversed the decrease in automaticity in three fibers that had shown a decrease in automaticity in the presence of the same norepinephrine concentrations. This result is entirely consistent with that seen in adult fibers in Figures 4A and 4B. Of importance in the neonatal fibers is that CEC alone induced no decrease in automatic effect (Figure 7, legend). This is in contrast to the result in adults and suggests no partial agonist action of CEC in the younger age group. Nonetheless, CEC blocked the decrease in automaticity in the neonates just as it had in the adults.

**Effects of Pertussis Toxin on the Response of Canine Purkinje Fibers to Norepinephrine**

We hypothesized that the negative chronotropic response blocked by CEC in neonatal fibers (Figure 7) depended on a pertussis toxin-sensitive G protein.
If this is the case, it should be abolished by pretreatment with pertussis toxin, which ADP-ribosylates and functionally inactivates the G protein, thereby un-
The Ontogeny of α1-Adrenergic Receptor Subtypes in Ventricular Myocardium

CEC selectively and irreversibly interacts with a subpopulation of pharmacologically distinct α1-adrenergic receptor binding sites.8 This property of CEC was exploited to determine whether the canine heart contains distinct α1-adrenergic receptor subtypes and whether the proportion of receptors that is distinguishable by CEC changes during development in the context of the maturational change in the α1-adrenergic chronotropic response. Because of the limited mass of Purkinje tissue in young animals, the experiments were done using ventricular myocardium. Saturation binding assays with [125I]IBE2254 revealed the presence of a single class of high affinity and limited capacity α1-adrenergic receptor binding sites in membranes derived from newborn and adult canine ventricle (Figure 9). α1-Adrenergic receptors were identified easily in the 3-day-old canine heart, and their density increased progressively during the first month of life from 50 to 220 fmol/mg protein. At some point thereafter, the density of specific binding sites identified by [125I]IBE2254 in canine ventricular myocardium decreased dramatically so that the level of α1-adrenergic receptor expression measured in the adult canine heart was only one tenth of that measured in the 1-month-old heart. There was considerable variability in the proportion of CEC-sensitive α-receptors from one preparation to the next, and no consistent pattern of developmental change emerged.

Similar experiments were performed on membranes derived from newborn and adult rat hearts (Figure 10). These studies were extended to the rat because, as in the dog, development is associated with the coordinate expression of an inhibitory α1-adrenergic chronotropic response and the functional acquisition of a pertussis toxin–sensitive G protein.5,6 In agreement with results reported previously,20 the density of α1-receptors in the newborn rat heart was approximately 50% greater than in the adult heart (binding site density: newborn, 274±76 fmol/mg, n=4; adult, 184±33 fmol/mg, n=6). Pretreatment of membranes from hearts at either developmental stage with CEC resulted in an approximately 25% decrease in the density of specific binding sites subsequently identified by [125I]IBE2254 (binding site density in CEC-pretreated membranes: newborn, 203±69 fmol/mg; adult, 145±32 fmol/mg; p<0.05 compared with corresponding control membrane preparations). The effect of CEC was dose depen-

Figure 8. Left panel: Pertussis toxin–dependent [32P]ADP-ribosylation of Purkinje fiber membranes from control dogs (C) and from dogs injected with pertussis toxin (PT). Right panel: Graph showing interaction of antagonists and norepinephrine (NE) on Purkinje fibers from pertussis toxin–treated dogs. Fibers were superfused with 2×10−7 M propranolol and 1×10−7 M WB 4101. Each set of identical symbols represents a concentration–response curve for a single preparation. The solid line represents the mean of nine experiments performed. Control values were as follows: rate=30±3 beats/min; maximum diastolic potential=−101±1.5 mV.
Figure 9. Graphs showing α₁-adrenergic receptors in canine ventricular myocardium. Ventricular myocardial membranes from newborn (aged 3–27 days) and adult dogs were prepared, and saturation binding of [¹²⁵I]IBE2254 in control and chloroethylclonidine (CEC)-pretreated membranes was performed. Results of representative equilibrium binding experiments demonstrating α₁-receptor characteristics in the 3-day, 6-day, 27-day, and adult dog ventricle are depicted in panel A, and the density of specific binding sites identified by [¹²⁵I]IBE2254 in seven young and six adult ventricular myocardial preparations is summarized in panel B. The affinity of [¹²⁵I]IBE2254 for the α₁-adrenergic receptor in the young and the adult canine heart was similar (Kᵦ: young, 130±16 pm; adult, 108±30 pm). In each case, pretreatment with CEC had no effect on the Kᵦ for [¹²⁵I]IBE2254 (Kᵦ: young, 111±16 pm; adult, 82±3 PM). An increase in the total number of specific binding sites identified by [¹²⁵I]IBE2254 (r=0.92, p<0.05) as well as the number of sites not sensitive to inactivation by CEC (r=0.84, p<0.05) was evident in the first month of life. In contrast, although the proportion of sites sensitive to CEC varied in individual preparations, no relation between age and the percent of receptors sensitive to CEC was evident (r=0.16, p=NS). The density of total and CEC-insensitive specific [¹²⁵I]IBE2254 binding sites in the adult was considerably reduced (23±3 fmol/mg total; 13±4 fmol/mg after pretreatment with CEC).

dent. Inactivation was detectable at 0.1 M CEC and was maximal at approximately 10 M CEC; little further inactivation was achieved even when the dose of CEC was increased 50-fold. Furthermore, there was no recovery of specific [¹²⁵I]IBE2254 binding sites after extensive washing of the membrane preparation, consistent with an irreversible effect of CEC to modify α₁-adrenergic receptor binding sites.

The membrane preparations used in these studies were derived from the whole ventricle and, therefore, surely contained elements other than myocardial cells, including vascular smooth muscle, sympathetic neurons, and connective tissue. We considered the possibility that the myocardial cell contains only one α₁-adrenergic receptor subtype but contamination with membranes from these other sources might lead to the detection of another α₁-adrenergic receptor subtype. Therefore, the next set of receptor binding experiments examined the characteristics of α₁-adrenergic receptor binding in membranes prepared from neonatal rat ventricular myocyte cultures that had been irradiated according to a protocol that completely and selectively eliminates contaminating fibroblasts (Figure 11). In this pure myocyte membrane preparation, a short exposure to CEC resulted in a 35% decrease in the density of specific binding sites subsequently identified by [¹²⁵I]IBE2254, indicating that receptor subtype heterogeneity exists at the level of the ventricular myocardial cell.

Effect of CEC and WB 4101 on the α₁-Adrenergic Inositol Phospholipid Response

Cultured neonatal rat ventricular myocytes invariably show an increase in automaticity on superfusion with α₁-agonists⁰ and thus provide a useful model to study the mechanisms underlying the α₁-adrenergic
positive chronotropic response expressed in the newborn heart. We previously demonstrated that these cultures also possess a vigorous α₁-adrenergic inositol phospholipid response. This response results from the receptor-dependent activation of phospholipase C to hydrolyze membrane-bound inositol phospholipid, thereby forming two intracellular second messengers, IP₃ and diacylglycerol. In many tissues, the actions of IP₃ to rapidly mobilize intracellular calcium and diacylglycerol to activate protein kinase C have been implicated in the regulation of an array of cellular responses. With respect to the heart, recent studies suggest that the biological actions of diacylglycerol may result in an increase in automatic rate.

On the basis of pharmacological data that indicate that the α₁-adrenergic positive chronotropic response is inhibited by WB 4101 but not by CEC, we postulated that WB 4101 (but not CEC) also would inhibit inositol phosphate accumulation in cardiac cultures. Although results from another laboratory indicate that in some tissues α₁-adrenergic inositol phospholipid hydrolysis is preferentially activated by the α₁-adrenergic receptor subtype identified by CEC and argue against this formulation, the subtype specificity of the α₁-adrenergic inositol phospholipid response in the heart has not previously been defined. Accordingly, we examined whether CEC or WB 4101 modulates α₁-adrenergic-dependent inositol phosphate accumulation in cardiac cells. For studies examining the effect of CEC, neonatal rat ventricular myocyte monolayers were preincubated for 30 minutes in the absence or presence of 2.5 × 10⁻³ M CEC. Basal and norepinephrine-stimulated inositol phosphate accumulation over 30 minutes in the absence or continued presence of CEC was then measured (Figure 12). CEC did not alter basal levels of inositol monophosphate nor attenuate the maximal effect of norepinephrine to enhance inositol monophosphate accumulation. Furthermore, the concentration-response relation for norepinephrine-dependent inositol monophosphate formation was identical in control and CEC-pretreated myocyte cultures. Basal levels of inositol bisphosphate and IP₃ as well as their responses to norepinephrine were similarly unaffected by CEC.

The next series of experiments examined the effect of CEC to modulate IP₃ accumulation in myocardial membranes. These experiments were important since phospholipase C is calcium dependent, and one could argue that the bulk of the increase in inositol phosphate in intact myocytes in response to norepinephrine occurs as an indirect consequence of a rise in cytosolic calcium via α₁-adrenergic receptor–dependent activation of calcium influx. Such a mechanism might not be inhibited by CEC and might obscure a small effect of a CEC sensitive α₁-adrenergic receptor to directly stimulate inositol phospholipid hydrolysis. To resolve this issue, norepineph-

**Figure 10.** Graphs showing α₁-adrenergic receptors in rat ventricular myocardium. Ventricular myocardial membranes from 2–3-day-old or adult rats were prepared, and saturation binding of [¹²⁵I]IBE2254 in control and chloroethylclonidine (CEC)-pretreated membranes was performed according to "Materials and Methods." Results of a single experiment on a newborn (top panel) and an adult (bottom panel) rat heart are presented. The data are representative of four similar experiments on the newborn and six similar experiments on the adult rat heart. The affinities of [¹²⁵I]IBE2254 for α₁-adrenergic receptors in newborn and adult rat heart preparations was not significantly different (154 ± 26 and 99 ± 24 pM, respectively).

**Figure 11.** Graphs showing α₁-adrenergic receptors in irradiated rat myocyte cultures. Membranes were prepared from irradiated neonatal rat ventricular myocardial cell cultures and specific [¹²⁵I]IBE2254 binding after preincubation with (■) or without (○) CEC was determined according to "Materials and Methods." The results are representative of two separate experiments performed on separate myocardial cell cultures.
rino-dependent IP₃ formation was examined in membranes prepared from intact myocytes that had been preincubated with or without CEC (Figure 13). Similar to results reported previously,¹⁰ norepinephrine stimulated the formation of IP₃ in myocyte membranes. This response was dependent on the presence of GTPγS, a nonhydrolyzable analogue of GTP. Pretreatment with CEC did not modify norepinephrine-dependent IP₃ accumulation, indicating that phosphoinositide hydrolysis in the heart is stimulated by a CEC-insensitive α₁-adrenergic receptor subtype.

In contrast, WB 4101 had a marked effect on norepinephrine-dependent inositol phosphate accumulation (Figure 14). The inhibitory effects of WB 4101 were half maximal at approximately 2×10⁻⁹ M (Figure 14). In the presence of 10⁻⁷ M WB 4101, only a small response to a high concentration of norepinephrine (10⁻⁵ M) remained.

**Discussion**

This study demonstrates that qualitatively and quantitatively different automatic responses to α-adrenergic stimulation are attributable to different α₁-adrenergic receptor subtypes, which discriminate in their interaction with intracellular response mechanisms. This is a major increment in our knowledge because, to date, the assumption made by us and others²³,²⁴ was that only one type of α₁-receptor existed. Within such a framework, the occurrence of a positive or negative chronotropic response was explicable based on the function of a pertussis toxin-sensitive 41 kDa GTP regulatory protein⁶; in its absence, α₁-stimulation increased automaticity; in its presence, automaticity decreased.

Previous studies have established that α₁-adrenergic agonists decrease automaticity through the stimulation of Na-K pump current (a pertussis toxin-sensitive, G protein-dependent response). The effector mechanism responsible for the α₁-agonist-induced increase in automaticity has been less well defined. However, recent studies demonstrate that phospholipase C²⁵ and thrombin²⁶,²⁷ stimulate phosphoinositide hydrolysis in ventricular myocytes via a mechanism that is independent of the α₁-receptor. Jones et al²⁸ have suggested that the thrombin response may occur by a proteolytic mechanism. The accumulation of IP₃, induced by phospholipase C or thrombin, is associated with a rise in intracellular Ca²⁺ concentration and an increase in automaticity. These results lend support to the hypothesis that the phosphoinositide signaling system is mechanistically involved in the positive chronotropic response. By analogy, α₁-adrenergic receptor-dependent stimulation of phosphoinositide metabolism also might increase automaticity. In support of this suggestion, we found that low nanomolar concentrations of WB 4101 inhibit α₁-adrenergic-dependent inositol phosphate accumulation; in preliminary studies, these low concentrations were also found to inhibit [³²P]IBE2254 binding to adult and neonatal rat ventricular myocardial membranes (unpublished observation, S.F. Steinberg). The α₁-adrenergic increase in automaticity is also inhibited by WB 4101. These results are consistent with but not proof of the hypothesis that inositol phospholipid hydrolysis is mechanistically involved in the α₁-adrenergic positive chronotropic response.²⁵ The precise mechanism whereby intermediates generated via the breakdown of membrane phosphoinositides might in-
crease automaticity still is uncertain and is under investigation in our laboratory.

Our study confirms previous reports\(^1,1,18\) that the effects of high concentrations of catecholamine to increase beating rate are attenuated by \(\beta\)-adrenergic antagonists such as propranolol or nadolol. The positive chronotropic effect of high concentrations of norepinephrine in both neonatal and adult fibers is explained by the \(\alpha\)-adrenergic actions of catecholamines on the pacemaker current and phase 4 depolarization.\(^29,30\) However, the primary focus of this report is on the actions of lower concentrations of norepinephrine \((<10^{-7} \text{ M})\), to induce either a negative or a positive chronotropic response in adult and neonatal canine Purkinje fibers.\(^1,4\) The slowing and acceleration of spontaneous rate are abolished by \(\alpha\)-but not by \(\beta\)-blockade; these findings are consistent with earlier studies demonstrating that phentola-

\(1^3\) and prazosin\(^3\) block the positive and negative chronotropic actions of epinephrine and phenylephrine. It was compelling to consider that these two distinct \(\alpha\)-adrenergic responses are determined by different receptor subtypes based on recent investigations supporting the existence of \(\alpha\)-adrenoceptor subclasses. Specifically, the actions of certain agonists and antagonists to modulate contraction of smooth muscle and to influence cellular calcium homeostasis differ in various tissues from various species.\(^22\) Although in each case \(\alpha\)-adrenergic receptor stimulation elevates cytosolic calcium ion concentration, in some tissues this occurs via IP\(_3\)-dependent mobilization of intracellular calcium stores, whereas in other tissues this results from enhanced influx of extracellular calcium.\(^31\) It is on the basis of these observations that the existence of distinct \(\alpha\)-adrenergic receptor subtypes that can be distinguished by their tissue distribution, radioligand binding characteristics, and effector response mechanisms has been proposed.\(^22\)

We used norepinephrine rather than epinephrine or phenylephrine as the agonist in these studies for two reasons. First, norepinephrine is the relevant neurotransmitter that stimulates the heart. Information describing its positive and negative chronotropic actions is essential if we are to understand the neurohumoral modulation of cardiac rhythm in vivo. Second, phenylephrine has been reported to bind
preferentially to the subtype of α₁-adrenergic receptors that are linked to calcium influx rather than IP₃-induced calcium mobilization. In contrast, norepinephrine appears to be equipotent at both α₁-adrenergic receptor subtypes. In fact, a previous study from our laboratory demonstrates a more vigorous myocardial cell inositol phosphate response to norepinephrine than to phenylephrine, in agreement with this observation. The fact that adult Purkinje fibers manifest a positive chronotropic response in about 50% of preparations and a negative response in the other 50% also suggests differences in subtype selectivity between this agonist and phenylephrine (with which about two thirds of preparations show a negative chronotropic response).3

To pharmacologically distinguish between two subtypes of α₁-adrenergic receptors in Purkinje fibers, we used CEC and WB 4101 at a concentration having no effect on the transmembrane action potential (i.e., 10⁻⁷ M). Although CEC is generally considered to be an α₁-antagonist, our results indicate that CEC has partial agonist activity, in agreement with earlier studies by LeClerc et al.9 Functioning as an agonist, CEC significantly attenuated the α₁-adrenergic decrease in automaticity. Moreover, CEC-treated Purkinje fibers invariably expressed an α₁-adrenergic positive chronotropic response. In contrast, WB 4101 antagonized the α₁-adrenergic increase in automaticity. Our electrophysiological experiments also suggest that at least two α₁-receptor subtypes are present in young as well as adult Purkinje fibers with the WB 4101-sensitive response being preferentially expressed in the young. This observation could be taken as evidence for a developmental change in receptor subtype specificity. However, our biochemical studies indicate that the proportion of α₁-receptors sensitive to CEC does not change during development. As evidence, CEC effectively decreased the density of specific binding sites subsequently identified by [¹²⁵I]IBE2254 in ventricular myocardium of neonatal and adult dogs. In the age range studied, approximately 25% of the specific α₁-adrenergic binding sites labeled by [¹²⁵I]IBE2254 were sensitive to CEC. A similar result was obtained in newborn and adult rat cardiac tissue, in which an analogous developmental change in the α₁-adrenergic chronotropic response from excitatory to inhibitory occurs.5 Moreover, the use of irradiated neonatal rat ventricular myocardial cell cultures established the existence of receptor heterogeneity (i.e., CEC-sensitive and CEC-insensitive receptors) within newborn cardiac myocytes freed from other contaminating cellular elements. Thus, it appears that the CEC-sensitive α₁-receptor subtype is present in the young heart but that the CEC-sensitive inhibitory chronotropic response is not expressed because the effector pathway is not functional. The failure to see a physiological response of the type that is blocked by CEC may depend on absence of the GTP regulatory protein that transduces the response (although other steps in the receptor-effective pathway may also play a role).

This interpretation is consistent with our previous studies in canine Purkinje fibers and rat ventricular myocardium that indicate that the functional competence of components of the receptor complex distal to the receptor itself are determinants of the α₁-adrenergic automatic response.

Although our results are consistent with an action of the CEC-sensitive α₁-adrenergic receptor to inhibit automaticity through stimulation of Na⁺-K⁺ pump current, one might suggest that the decrease in the transient outward current that has been attributed to α₁-adrenergic stimulation would prolong action potential duration and, in this way, might contribute to a decrease in automaticity. Against this suggestion are preliminary experiments demonstrating that the α₁-adrenergic prolongation of repolarization is present in all young and adult Purkinje fibers studied and is maximal at agonist concentrations of 10⁻⁵ M or more and seen only minimally at 10⁻⁸ M. Furthermore, whereas α₁-adrenergic inhibition of automaticity requires the functional presence of a pertussis toxin-sensitive G protein, α₁-adrenergic prolongation of repolarization does not.

Although the interpretation of α₁-adrenergic action in the heart that derives from the physiological and biochemical studies reported herein is internally consistent, these results do not conform to the classification of α₁-adrenergic receptor subtypes as originally described by Minneman and colleagues.1,12 Specifically, according to this classification, the receptors with high affinity for WB 4101 (α₁₁-receptors) are coupled to the influx of extracellular calcium, whereas the CEC-sensitive α₁₅₃-receptors are linked to inositol phospholipid turnover and the mobilization of intracellular calcium. This classification is based on detailed studies performed on several specific tissues (i.e., primarily liver, vas deferens, and spleen). The studies did not examine the properties of the cardiac α₁-adrenergic receptor in any detail. In this regard, it is noteworthy that the CEC insensitivity of the cardiac α₁-adrenergic inositol phospholipid response is consistent with an earlier report indicating that α₁₁-adrenergic-dependent inositol phosphate accumulation in the brain also is not inhibited by CEC.37 Furthermore, Han et al.38 have recently presented new data that are inconsistent with the selective coupling of the α₁₁-receptor subtype and inositol phosphate formation. Specifically, they demonstrated that renal tissue contains a distinct α₁₅₃-adrenergic receptor that stimulates inositol phosphate accumulation and is blocked by WB 4101.38 The pharmacological properties of this renal α₁₅₃-receptor are quite similar to the cardiac α₁₁-receptor that is also inhibited by low nanomolar concentrations of WB 4101. Further evidence that the CEC-sensitive receptor does not stimulate phosphoinositide metabolism in the heart has been presented in preliminary fashion by others (also, P. Simpson, personal communication). Therefore, it appears that further complexity in the classification of the α₁-adrenergic receptor, due to potential differences in α₁-adrenergic receptor
subtype density, the efficiency of coupling to effector mechanisms, or further heterogeneity of the \( \alpha_1 \)-adrenergic receptor itself must be considered.

Molecular biological studies have also provided convincing evidence for the heterogeneity of the \( \alpha_1 \)-adrenergic response. Cottecha and colleagues\textsuperscript{40,41} have cloned an \( \alpha_1 \)-adrenergic receptor from a hamster smooth muscle cell line that has relatively low affinity for WB 4101 and stimulates phosphoinositide hydrolysis via a pertussis toxin–sensitive G protein. These pharmacological properties are characteristic of the \( \alpha_{1C} \)-receptor. However, the pharmacological properties of another \( \alpha_1 \)-adrenergic receptor subtype recently cloned from bovine brain are unique. This receptor resembles the \( \alpha_{1A} \)-adrenergic receptor in that it binds with WB 4101 with high affinity but, unexpectedly, is partially inactivated by CEC.\textsuperscript{42} Current uncertainties regarding \( \alpha_1 \)-adrenergic receptor linkages are reminiscent of the confusion regarding the classification of muscarinic cholineric receptor subtypes, which was resolved only through the application of molecular cloning techniques. By analogy, a complete understanding of \( \alpha_1 \)-adrenergic receptor subtypes and their linkages to cellular response mechanisms will depend on the availability of clones for distinct \( \alpha_1 \)-adrenergic receptor subtypes and their expression in cells lacking endogenous \( \alpha_1 \)-adrenergic receptors.

In summary, our results from physiological studies, radioligand binding studies, and studies of receptor-mediated second messenger demonstrate that at least two distinct \( \alpha_1 \)-adrenergic receptor response mechanisms are present in both neonatal and adult cardiac tissue. We propose that the CEC-sensitive \( \alpha_1 \)-receptor subtype is linked to a decrease in automaticity via a pertussis toxin–sensitive G protein and activation of the Na`\textsuperscript{+}–K`\textsuperscript{+} pump current, whereas the WB 4101–sensitive \( \alpha_1 \)-receptor subtype induces an increase in automaticity possibly through an intracellular metabolite generated via the breakdown of membrane phosphoinositides. Moreover, although the CEC-sensitive \( \alpha_1 \)-adrenergic receptor subtype is present at an early developmental stage, it is functionally expressed only as age increases. These observations are important to our understanding of receptor–effector coupling in the modulation of rhythm, and they further suggest that specific blockers of the WB 4101–sensitive \( \alpha_1 \)-adrenergic receptor subtype may be useful in the suppression of automatic tachyarrhythmias.

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**KEY WORDS** • α-adrenergic receptors • Purkinje fibers • ventricular automaticity • catecholamines
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Circ Res. 1990;67:1535-1551
doi: 10.1161/01.RES.67.6.1535

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