Differential Rate Responses to Nicotine in Rat Heart: Evidence for Two Classes of Nicotinic Receptors

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ABSTRACT
Nicotinic acetylcholine receptors are pentameric, typically being composed of two or more different subunits. To investigate which receptor subtypes are active in the heart, we initiated a series of experiments using an isolated perfused rat heart (Langendorff) preparation. Nicotine administration (100 μM) caused a brief decrease (−7 ± 2%) followed by a much larger increase (17 ± 5%) in heart rate that slowly returned to baseline within 10 to 15 min. The nicotine-induced decrease in heart rate could be abolished by an α7-specific antagonist, α-bungarotoxin (100 nM). In contrast, the nicotine-induced increase in heart rate persisted in the presence of α-bungarotoxin. These results suggest that the nicotinic acetylcholine receptors (nAChRs) that mediate the initial decrease in heart rate probably contain α7 subunits, whereas those that mediate the increase in heart rate probably do not contain α7 subunits. To investigate which subunits may contribute to the nicotine-induced increase in heart rate, we repeated our experiments with cytisine, an agonist at nAChRs that contain β4 subunits. The cytisine results were similar to those obtained with nicotine, thereby suggesting that the nAChRs on sympathetic nerve terminals in the heart probably contain β4 subunits. Thus, the results of this study show that pharmacologically distinct nAChRs are responsible for the differential effects of nicotine on heart rate. More specifically, our results suggest that α7 subunits participate in the initial nicotine-induced heart rate decrease, whereas β4 subunits help to mediate the subsequent nicotine-induced rise in heart rate.

Nicotine can stimulate nicotinic acetylcholine receptors (nAChRs) in the central and peripheral nervous systems to influence autonomic control of cardiac function (Robertson et al., 1988; Benowitz and Gourlay, 1997). Multiple neuronal nicotinic receptor subunits (α2–10 and β2–4) have been identified and different combinations of these subunits can associate to produce functional nAChR subtypes with distinct pharmacological and biophysical properties (Lindstrom et al., 1991, 1995, 1996; Sargent, 1993). It is thought that in most cases, two α subunits and three β subunits associate to form a functional pentameric nAChR structure, although some subtypes (e.g., α7) can form homomeric nAChRs (Cooper et al., 1991; Anand et al., 1993a, b). It is not clear, however, which subtype(s) participates in the regulation of cardiovascular function.

Within the heart, there is evidence that multiple nAChR subtypes may be operative (Kottergod, 1953; Yuan et al., 1993). For example, work with isolated rabbit auricle preparations demonstrated that nicotine can both decrease and increase heart rate (Kottergod, 1953). The initial decrease in heart rate is probably mediated by parasympathetic neurons because it could be blocked with the muscarinic antagonist atropine. Subsequent work showed that the increased heart rate response to nicotine could be blocked by either sympathetic
thectomy (Marano et al., 1999) or pretreatment with β-adrenergic antagonists such as propranolol (Ardell, 1994). In the isolated guinea pig heart and human atria, nicotine has been shown to stimulate the release of norepinephrine from sympathetic nerve terminals (Westfall and Brasted, 1972; Kruger et al., 1995).

In addition to the extrinsic nerve fibers that innervate the heart, a complex organization of intrinsic cardiac neurons exists (Ardell, 1994). Recently, Poth et al. (1997) showed that intrinsic cardiac neurons isolated from neonatal rat atria express a heterogeneous array of mRNAs coding for multiple nAChR subunits. Electrophysiological and pharmacological data indicate that functional α7 nAChRs exist in these neurons because nicotine-induced currents can be suppressed by the α7-selective antagonist α-bungarotoxin (α-BTX) (Cuevas and Berg, 1998). In addition, Bibevski et al. (2000) recently showed that α7 and other nAChR subtypes are expressed in canine cardiac parasympathetic neurons and that ganglionic transmission in these neurons can be partially blocked by α-BTX. Thus, α7 nAChRs represent a candidate subtype of nAChRs that could play a role in the local regulation of heart rate.

The purpose of the present study was to determine whether the α7 and/or other subunits of nAChRs are capable

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; α-BTX, α-bungarotoxin; ANOVA, analysis of variance.
of locally regulating heart rate. To accomplish this, we have used an isolated perfused rat heart model to evaluate the direct actions of nicotine and related drugs on heart rate. Our results show that nicotine has differential influences on heart rate that can be pharmacologically distinguished, thereby suggesting that different nAChR subtypes mediate nicotine’s actions in the heart.

Materials and Methods

Drugs and Chemicals. All drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Each drug was prepared as either a 10 or 100 mM stock solution by dissolving it in purified deionized water and storing it in small aliquots at −80°C. Immediately before use, the drug(s) was thawed and diluted in Tyrode’s solution that was freshly prepared on the day each experiment was performed.

Animals. Mature female Sprague-Dawley rats, weighing 200 to 250 g each, were obtained from Taconic Farms (Germantown, NY). All of the experiments were conducted in strict concordance with the guidelines provided by the Georgetown University Animal Care and Use Committee. The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and given 1000 U of heparin (i.p.) to prevent clotting. The hearts were removed rapidly via midsternal thoracotomy and placed in ice-cold Tyrode’s solution (115 mM NaCl, 4.7 mM KCl, 2 mM CaCl2, 0.7 mM MgCl2, 1 mM NaH2PO4, 27.9 mM NaNHCO3, 20 mM glucose, and 0.04% purified bovine albumin), and the aortic root was cannulated for retrograde perfusion by the method of Langendorff (Langendorff, 1895). The hearts were then mounted in a Langendorff-perfusion apparatus. Perfusion (nonrecirculating) was at a constant flow rate of 5 to 7 ml/min with oxygenated

perfusion buffer and the initial response time (this is due to the time it takes for the perfusion buffer to travel from the perfusion buffer reservoir to the heart). Error bars have been taken out for better clarity of the graphs (see Fig. 2A for error bars and statistical analysis).

Results

To investigate the direct effects of nicotine on heart rate, we used spontaneously beating, retrogradely perfused, isolated rat heart preparations. Administration of a single dose of 100 μM nicotine induced an initial brief decrease followed by a much larger increase in heart rate (Fig. 1A, representative experiment; Figs. 1B and 2A, average results). Adding 100 μM nicotine to the perfusion buffer produced a small decrease (−7 ± 2%, n = 7) in heart rate that although not significantly different from control (p > 0.05 for zero time point versus 2-min time point; Fig. 2A), was consistently observed ~2 min after nicotine administration (note that if the Student’s t test is used to compare the control mean at the zero time point versus the 2-min time point then the means were found to be significantly different, p < 0.05). The volume of buffer in the perfusion tubing and glassware accounted for a lag time for nicotine exposure of approximately 2 min; therefore, the initial decrease in heart rate occurred essentially immediately upon exposure of the heart to nicotine and typically only lasted for a few beats. This decrease in heart rate was quickly followed by an increase, with peak responses (+17 ± 5%, n = 7, p < 0.01; Fig. 2A) occurring approximately 5 min after adding nicotine to the perfusion
buffer. These results suggest that nicotine can elicit a biphasic heart rate response consisting of a brief initial decrease followed by a greater and more sustained increase.

A second administration of 100 μM nicotine after a 25-min washout period with Tyrode’s buffer solution resulted in a greatly attenuated heart rate increase. However, the preceding decrease in heart rate after the second administration of nicotine was similar to that observed after the initial application of nicotine (Fig. 1A). These results suggest that the nicotine-induced decrease and subsequent increase in heart rate have different desensitization characteristics and, therefore, may be mediated by different subtypes of nAChRs.

To determine whether the heart could still respond to a chronotropic stimulus after nicotine exposure, we challenged the heart with the β-adrenergic receptor agonist isoproterenol. Perfusion with 0.1 μM isoproterenol consistently elicited an increase in heart rate of about 75 beats/min from the baseline rate, which was usually greater than the initial nicotine-induced heart rate increase (Fig. 1A). Thus, the hearts were fully capable of responding to β-adrenergic stimulation despite the fact that they were apparently desensitized to the stimulating effects of nicotine.

Dose-response curves were generated by perfusing the heart with increasing concentrations of nicotine (0.1–100 μM). No change in heart rate was elicited at concentrations of nicotine ≤0.1 μM (data not shown). Notably, however, when the heart was exposed to 0.1 μM nicotine, subsequent exposure (within 15 min) to higher concentrations of nicotine (1–100 μM) failed to elicit any changes in heart rate (data not shown). Thus, subthreshold concentrations of nicotine seem capable of desensitizing the heart to nicotine stimulation. If, however, the heart was not exposed to subthreshold concent-
trations of nicotine (<1 μM), before the addition of higher nicotine doses (1–100 μM), then we began to observe heart rate changes. As shown in Fig. 1B, the decreased heart rate responses to nicotine were similar at 1 to 100 μM nicotine, whereas the increased heart rate response was clearly dose-dependent in this range of nicotine concentrations. Thus, these results show that nicotine’s action was clearly more potent at decreasing heart rate than it was at increasing heart rate in this preparation, despite the difference in the magnitude of the responses.

To investigate which nAChR subunits may be involved in mediating the increased heart rate response to nicotine, we challenged the rat heart with cytisine, which is a full agonist at β4-containing nAChRs but only a partial agonist at β2-containing nAChRs (Luetje and Patrick, 1991; Papke and Heinemann, 1994). As shown in Fig. 2B, cytisine produced an increased heart rate response that was similar to and slightly more robust (+30 ± 6%, n = 5, p < 0.01) than that produced by nicotine (compare with Fig. 2A) at equivalent concentrations (100 μM). In contrast to nicotine, however, cytisine failed to consistently produce the initial decreased heart rate response. Moreover, once the heart was initially stimulated with cytisine, a subsequent application of 100 μM nicotine produced a highly attenuated response (data not shown, but similar to that shown in Fig. 1A). Because cytisine is thought to act as a full agonist at nAChRs containing β4 subunits, our results suggest that the nAChRs that mediate the nicotine-induced increase in heart rate contain β4 subunits.

Because previous studies showed that intrinsic cardiac ganglia in rat hearts express functional α7 nAChRs (Cuevas and Berg, 1998), we sought to determine whether the α7 nAChR was mediating some of the direct cardiac rate responses observed after nicotine administration. To test this hypothesis, we administered 100 nM α-BTX, a selective α7 nAChR antagonist, 5 min before the addition of nicotine, and then continuously perfused the heart with 100 μM nicotine in the presence of 100 nM α-BTX for an additional 20 min. The presence of α-BTX abolished the decreased heart rate response to nicotine (Fig. 2C), whereas the increased heart rate response was still present (+17 ± 4%, n = 4, p < 0.05), although somewhat attenuated compared with that observed in the absence of α-BTX (compare Fig. 2, A and C). These results suggest that the nicotine-induced decrease in heart rate is probably mediated by nAChRs containing α7 subunits.

Because α7 nAChRs seem to be responsible, at least in part, for mediating the nicotine-induced decrease in heart rate, we hypothesized that the α7 nAChRs influence the release of acetylcholine from parasympathetic neurons innervating the heart. If true, then the muscarinic acetylcholine receptor antagonist atropine should prevent the initial nicotine-induced decrease in heart rate. As predicted, when we perfused the heart with nicotine in the presence of 1 μM atropine, the decreased heart rate response was no longer observed (Fig. 2D). Surprisingly, the degree of increase in heart rate was also attenuated in the presence of atropine (compare Fig. 2, A and D). The effects of atropine on heart rate were comparable with the changes in heart rate after administration of α-BTX with nicotine in that the initial decrease in heart rate was absent while the increased heart rate persisted, although at an attenuated level.

The nicotine-induced heart rate increase is probably medi-ated by stimulation of nAChRs on sympathetic nerve terminals because previous studies demonstrated that nicotine could elicit release of [3H]norepinephrine from sympathetic nerve terminals in the heart (Westfall and Brasted, 1972). To test this hypothesis in our isolated rat heart preparation, we performed the nicotine challenge in the presence of the ganglionic blocker hexamethonium (500 μM). Hexamethonium is an nAChR antagonist, but seems to be ineffective or less effective at blocking α7 subtypes of the nAChR (Bertrand et al., 1992). Consistent with this hypothesis, our results show that in the presence of hexamethonium, nicotine failed to stimulate an increase in heart rate (Fig. 2E). The decreased heart rate response, although small (−2 ± 1%), seemed to remain. In the absence of nicotine, hexamethonium had no apparent effect on heart rate in our preparations (data not shown). These results suggest that hexamethonium blocks the nAChRs that mediate the increased heart rate response.

To examine this hypothesis further, we used the β-adrenergic receptor blocker timolol. As predicted, the increased heart rate response was blocked by 10 μM timolol, but the initial decreased heart rate response to nicotine remained (Fig. 2F). Similar to the results obtained from the hexamethonium experiments (Fig. 2E), timolol abolished the nicotine-induced heart rate increase while allowing the decrease in heart rate to continue. Thus, these results suggest that nAChRs located on sympathetic nerve terminals probably mediate the increase in heart rate induced by nicotine.

The results of these various experiments are summarized in Fig. 3. Figure 3A compares the changes in heart rate that occur initially after the administration of nicotine, whereas Fig. 3B compares the changes in heart rate that occur during the peak response. As shown in Fig. 3A, the nicotine-induced heart rate decrease could be effectively blocked by either α-BTX or atropine (p < 0.05) but not by timolol or hexamethonium. These results suggest that the nAChRs mediating the initial decrease in heart rate contain α7 subunits (Fig. 3A). Conversely, timolol and hexamethonium were each able to block the nicotine-induced heart rate increase (p < 0.01), whereas atropine and α-BTX only partially blocked this response (Fig. 3B). The cytisine results were similar to those obtained with nicotine, thereby implicating involvement of β4 nAChR subunits for mediation of the nicotine-induced increase in heart rate. Thus, these results suggest that within the heart, β4-containing nAChRs are good candidates for mediating adrenergic neurotransmission, whereas α7-containing nAChRs are good candidates for mediating cholinergic neurotransmission.

**Discussion**

It is perhaps not surprising that different nAChR subtypes may control the opposing responses evoked by nicotine in the isolated rat heart preparation, especially when considering that rat intrinsic cardiac neurons express most of the nAChR subunits identified to date (Poth et al., 1997). Nevertheless, it seems that within the heart, there are at least two different subtypes of nAChRs that mediate heart rate in response to nicotine. The evidence for this conclusion is based upon the following facts: 1) nicotine elicits a biphasic heart rate response; 2) the decrease in heart rate after perfusion with nicotine occurs first and is much more sensitive to nicotine than is the subsequent increase in heart rate; 3) the nicotine-
A and B). To determine whether the mean at any of the indicated time points was significantly different from the control, we performed ANOVA and Dunnett’s post hoc test for multiple comparisons to a control condition. The mean percent change in heart rate at 5 min after the addition of nicotine was taken as the primary outcome measure (note that there was a 2-min lag time between the time when nicotine was added to the perfusion buffer and the time when that buffer reaches the heart region). The peak heart rate occurring 2 min after the addition of nicotine (top panel) and the subsequent increase in heart rate (bottom panel) were measured and recorded. The peak increase in heart rate was taken at 5 min after the addition of nicotine. The concentration of each drug was as follows: 100 μM nicotine, 100 μM atropine, 100 μM timolol, and 500 μM hexamethonium.

Fig. 3. Summary of the effects of various nACHR agonists and antagonists on the initial decrease (top) and subsequent increase (bottom) in heart rate. In each experiment, the antagonists were added to the perfusion buffer 5 min before the addition of 100 μM nicotine. The peak decrease in heart rate was taken as the average percentage of change in heart rate occurring 2 min after the addition of nicotine (note that there was a 2-min lag time between the time when nicotine was added to the perfusion buffer and the time when that buffer reaches the heart region). The average peak increase in heart rate was taken at 5 min after the addition of nicotine. The concentration of each drug was as follows: 100 μM nicotine (n = 7), 100 μM cytisine (n = 5), 100 μM α-BTX (n = 4), 1 μM atropine (n = 4), 10 μM timolol (n = 4), and 500 μM hexamethonium (n = 5). Statistical significance was assessed using one-way ANOVA and Dunnett’s post hoc test for multiple comparisons to determine whether the mean at any of the indicated time points was different from control (nicotine alone, represented by the first column in A and B). * p < 0.05; ** p < 0.01.

The initial nicotine-induced decrease in heart rate was apparently desensitized to the effects of nicotine, whereas the decreased heart rate response was relatively resistant to nicotine desensitization; 4) the nicotine-induced heart rate decrease was selectively blocked by either atropine or α-BTX but not by timolol or hexamethonium; 5) the nicotine-induced heart rate increase was selectively blocked by either timolol or hexamethonium but not by atropine or α-BTX; and 6) cytisine produced results similar to nicotine, thereby suggesting a role for nACHRs containing β4 subunits in the nicotine-induced heart rate increase. These findings suggest that the initial nicotine-induced decrease in heart rate is mediated by α7 nACHRs located on intrinsic cholinergic cardiac neurons, whereas the subsequent increase in heart rate is mediated by β4 nACHRs located on sympathetic nerve terminals and/or intrinsic cardiac adrenergic cells in the heart (Westfall and Brasted, 1972; Lloyd and Williams, 2000). This hypothesis is consistent with the cytisine data from the present study as well as previous work showing that α3β4 nACHRs respond to cytisine as a full agonist (Papke and Heinemann, 1994; Wong et al., 1995). Because previous studies have demonstrated that cytisine is only a partial agonist on nACHRs containing β2 subunits (Luetje and Patrick, 1991; Papke and Heinemann, 1994), it would seem less likely that β2 nACHRs are involved in the nicotine-induced sympathetic heart rate response. In addition, there is little evidence that α4 nACHR subunits may be functionally expressed in sympathetic nerve terminals, so this would also seem to be an unlikely candidate for participation herein. In contrast, α5 and α7 nACHR subtypes have been observed in chick sympathetic neurons (Yu and Role, 1998a,b). Either or both of these subtypes could be involved in the nicotine-induced heart rate increases observed in the present study, although participation of the α7 subtype does not seem to play a major role in this experimental paradigm because blockade of the α7 nACHR subtype with α-BTX did not significantly block the peak nicotine-induced heart rate increase (Figs. 2C and 3B). On the other hand, the α7 nACHR subtype is clearly implicated in mediating the nicotine-induced initial decrease in heart rate because α-BTX effectively reversed this effect. Although α7 nACHRs can function as homomers in vitro, it is unclear whether they associate with other nACHR subtypes in cardiac parasympathetic neurons, although there is some electrophysiological evidence suggesting that α7-containing nACHRs in cultured neonatal rat cardiac neurons have unique properties (Cuevas and Berg, 1998). Thus, although we have identified some of the nACHR subunits that are probably involved, much work still needs to be done to fully decipher the native constituencies of nACHRs in the nerves that mediate nicotinic responses in the heart.

Despite a previous report to the contrary (Westfall and Saunders, 1977), the isolated perfused rat heart preparation seems to be a good model in which to address these questions. Most of the early work examining the actions of nicotine in the heart was performed with isolated rabbit or guinea pig hearts. One study compared the ability of isolated guinea pig and rat hearts to secrete [3H]norepinephrine in response to perfusion with nicotine, and found that the rat hearts responded either weakly or not at all compared with the guinea pig hearts (Westfall and Saunders, 1977). In contrast, the results from our study show that the isolated rat heart can respond to nicotine, producing heart rate responses very similar to those observed in previous studies with nicotine in the rabbit and guinea pig. Possible explanations for the apparent discrepancy between our results and those of Westfall and Saunders (1977) could be related to the different endpoints measured in the respective studies. Perhaps, it is more
difficult to load the rat heart with [3H]norepinephrine, or there might be enhanced metabolism of this radiolabeled catecholamine in the rat compared with its fate in the guinea pig heart. Interestingly, however, the dose-response curves for the increased release of [3H]norepinephrine from the guinea pig heart and the increased rate in the isolated rat heart were remarkably similar, suggesting that the cardiac effects of nicotine are similar in these two species. Because much of the work on nAChRs has been performed in rats, it may be advantageous to use the rat heart model for the purpose of evaluating nAChR subtype functions in cardiac neurons, and the data presented herein indicate that this is possible.

Indeed, the isolated perfused rat heart model has been used successfully to study parasympathetic function (Hoover and Neely, 1997), and several studies using a variety of models have suggested that α7 nAChRs are present on cardiac parasympathetic neurons (Sargent and Garrett, 1995; Poth et al., 1997; Cuevas and Berg, 1998; Bibeveski et al., 2000). Our results (e.g., dose-response data shown in Fig. 2) seem to be consistent with those of Cuevas and Berg (1998) who showed that α7 nAChRs in rat intrinsic cardiac neurons have slow desensitization properties compared with those observed for homomeric α7 nAChRs expressed in heterologous systems (Couturier et al., 1990; Gopalakrishnan et al., 1995). These results suggest that either α7 subunits interact with other nAChR subunits to form heteromeric nAChRs in these neurons or that the functional activity of homomeric α7 nAChRs is altered to somehow reflect the apparent resistance to desensitization observed in our study as well as that of Cuevas and Berg (1998). Interestingly, however, these α7-containing nAChRs seem to retain little sensitivity to blockade by hexamethonium, a property shared with homomeric α7 nAChRs (Bertrand et al., 1992). In our system, nicotine induced a robust and sustained decrease in heart rate in the presence of hexamethonium. One possible explanation for this result would be that hexamethonium primarily blocks non-α7 nAChRs. In support of this hypothesis, the β-adrenergic antagonist timolol produced a response similar but less pronounced than that produced by hexamethonium on the nicotine-induced heart rate responses in the isolated perfused rat heart. Certainly, the intrinsic cardiac nervous system is complex and with many nAChR subunits expressed in these neurons, a great challenge lies ahead in trying to discriminate the specific contributions of these nAChRs in physiological settings.

Previous studies have shown that central and carotid body sensory systems have an important influence on nicotine-induced heart rate changes in vivo when clinically relevant concentrations of nicotine are administered i.v. (Geberb, 1969; Murphy et al., 1994). Thus, although the isolated perfused rat heart model is valuable for evaluating the actions of nicotine in the heart itself, it may not necessarily provide clinically relevant information regarding the use of tobacco and nicotine. Nevertheless, high concentrations of nicotine may be able to activate the nicotinic receptors in cardiac neurons, and this study provides new information about the specificity and capability of nAChR subtype responses in the heart itself. Thus, this study and others like it help to identify the physiological components of the neural regulatory system that controls heart rate. Other nAChRs may also be active in cardiac neurons and could conceivably regulate contractility, coronary flow, and/or other cardiac functions not evaluated in the present study. Clearly, this is an important area of investigation that needs further attention.

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References


Sargent PB and Garrett EN (1995) The characterization of α7-bungarotoxin receptors


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