The narcotic antagonist naltrexone has a biphasic effect on the nicotinic acetylcholine receptor

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It is known that narcotic antagonists interact with many cholinergic pathways but less in known about specific mechanisms. Using neonatal rat myoballs to study single channel behaviour of the acetylcholinegated nicotinic receptor, it was found that micromolar concentrations of naltrexone had no effect on channel conductance but caused open channel blockade by increasing the flickering from the open to the closed state in a concentration-dependent manner. At micromolar concentrations of naltrexone, the frequency of channel opening was decreased and bursts were grouped in clusters, whereas at nanomolar levels the frequency of opening was increased. The sequential model for ion-channel blockade cannot explain these effects, and an alternative allosteric mechanism of action is proposed.

Narcotic antagonist *Naltrexone* *Nicotinic receptor* *Electrophysiology* *Single channel recording* *Channel activation* *Channel blockade*

1. INTRODUCTION

The narcotic antagonists naloxone and naltrexone have potent and stereoselective effects on opiate receptors, and in addition, they may also interact with other receptors and neurotransmitter pathways [1,2]. In cholinergic systems, a variety of in vitro, in vivo and behavioural experiments have demonstrated that both muscarinic and nicotinic pathways can be affected by narcotic agonists and antagonists [3–9]. Although some of these interactions may not be at the postsynaptic receptor, there is electrophysiological evidence that naltrexone has a direct effect on the nicotinic receptor of the motor endplate [10,11]. Oliveira and Albuquerque [10] used a two microelectrode voltage clamp to record endplate currents (EPCs) and miniature endplate currents at the frog neuromuscular junction and suggested that naltrexone interacts with both the open and closed form of the receptor. Similarly, Argentieri and McArdle [11] studied EPCs of rat muscle and concluded that naltrexone blocks open channels in a manner similar to that of many local anesthetics.

The aim of the present study was to gain detailed information on the reaction between an opiate agent and the nicotinic receptor using the method of single channel recording [12]. With this technique it is possible to study the interaction of various agonists and antagonists on individual receptor molecules, thus allowing closer scrutiny and testing of possible mechanisms.

2. MATERIALS AND METHODS

General details of the experimental protocol and data analysis have been previously described [13]. Briefly, nicotinic receptors on cultured rat myoballs were studied in Hanks solution at 10°C (pH 7.2). Cells were viewed with Hoffman interference optics on an inverted microscope (Nikon), and mechanical vibrations were minimized with a pneumatically isolated table (Kinetic Systems).

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Patch pipettes (2 MΩ) were made from aluminosilicate glass (A-M Systems), fire-polished, and used without silicone coating. When seals were greater than 2-5 GΩ, single channel data under voltage clamp (List Electronic, L/M-EPC-5) were collected on a 4 channel FM tape recorder (Racal) at 1 kHz bandwidth for subsequent analysis. Acetylcholine (ACh) concentration used to activate the receptors was 0.2 μM and all solutions contained 0.3 μM tetrodotoxin (Sigma) to inhibit sodium channels. Recording modes were inside-out, and perfused outside-out using a microchamber [14]. With outside-out patches, Ca^{2+} was not added to the Hanks solution in the pipette. FM tape recordings of data were digitized at 5 kHz and analyzed using an automated computer procedure [13] which generated histograms of current amplitude and estimated open, shut and burst lifetimes; for the purposes of this study, bursts were defined as a sequence of openings separated by closures of not greater than 8 ms. Data passing through the 50% point of channel conductance were taken to indicate a change of state for channels opening and closing. (−)-Naltrexone was supplied by Endo Laboratories.

3. RESULTS

Naltrexone (0.2–30 μM) had no effect on single channel conductance (linear regression of conductance (γ) on naltrexone concentration from 0–10 μM gave an intercept of 21.4 ± 0.51 pS (SE) and a slope of -0.094 ± 0.091 for $n = 11$). However, in a concentration-dependent manner it both decreased mean open channel lifetime (fig.1) and caused openings to be grouped into clusters separated by silent periods of several seconds duration (fig.2). The decrease in mean channel open time was brought about by increased flickering during the open state, similar to that seen with the local anesthetic agents QX222 and QX314 [15]. This effect of decreased open lifetime through increased flickering was reflected in the number of openings per burst, with an average 2-fold increase for 30 μM naltrexone compared to control. Frequency histograms of closed times also illustrated this behaviour; fig.3 shows data from one inside-out patch where in the presence of ACh alone, flickering was minimal with 9% of closed times being less than 8 ms, while in another patch with 5 μM

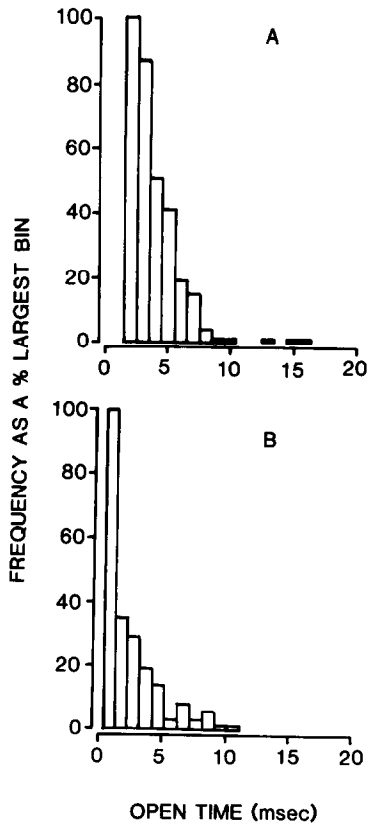

Fig.1. Frequency histograms showing the number of open channel events falling within incremental 1 ms bins. Data from two inside-out patches with a transmembrane potential (V_H) of −40 mV (pipette held at +40 mV with respect to bath). (A) Control in the presence of 0.2 μM ACh, 249 open channels counted, mean open channel lifetime (τ_{open}) = 8.3 ms estimated by log-linear regression, r = 0.96. Excess number of brief openings in the first 1.4 ms excluded. (B) 0.2 μM ACh in the presence of 10 μM naltrexone, 177 channels counted, τ_{open} = 4.9 ms, r = 0.95, data in the first 0.2 ms excluded.

naltrexone present, such closures represented 33% of the total. Naltrexone at concentrations above 5 μM also had a time-dependent effect on decreasing the frequency of channel opening, generally taking several minutes to achieve equilibrium. The time taken to reach equilibrium decreased with increasing concentration.

In contrast to these blocking effects seen most clearly at naltrexone concentrations in the micromolar range, it was noticed that 0.2 μM naltrexone increased the frequency of channel opening, and that this effect took some minutes to reach a max-

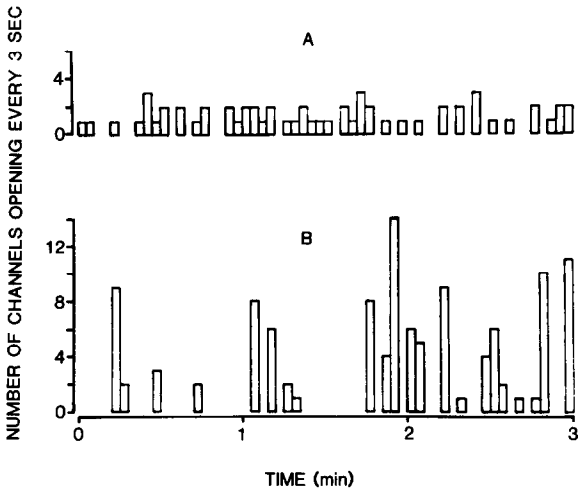

Fig.2. Frequency of channel opening as a function of time for two inside-out patches, $V_H = -60$ mV. (A) Control, 0.2 μM ACh, total of 63 openings showing relatively regular behaviour. (B) 0.2 μM ACh in the presence of 30 μM naltrexone, total of 116 openings, illustrating grouping into clusters separated by quiescent periods of several seconds duration.

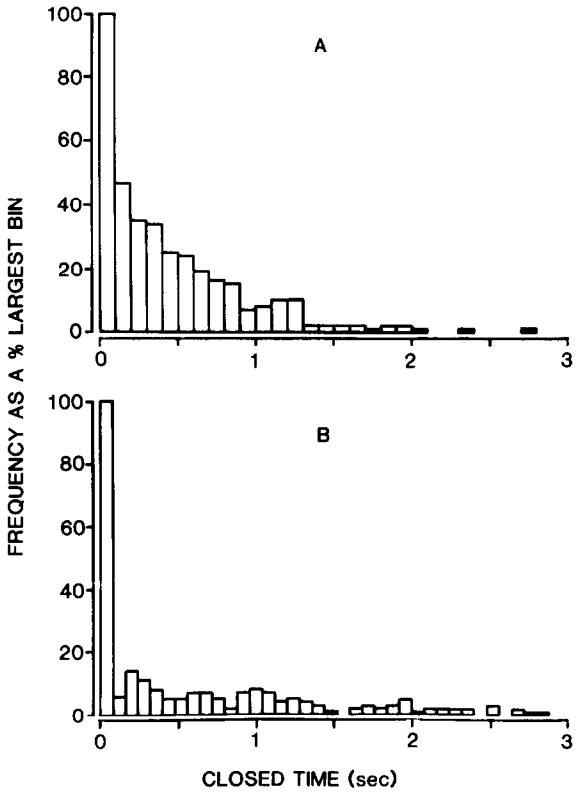

Fig.3. Closed time histograms in the absence (A) and presence (B) of 5 μM naltrexone. Separate inside-out patches, ACh concentration in both was 0.2 μM, $V_H = -60$ mV. In (A) 9% of closed times were less than 8 ms while in (B) such closures were 33% of the total.

imum following exposure and seal formation. This stimulatory effect was confirmed in a separate perfused outside-out preparation where the same patch was sequentially exposed to 0.2 μM ACh, 0.25 μM naltrexone and then the two agents together. Fig.4 shows that 0.25 μM naltrexone had no intrinsic agonist activity at the nicotinic receptor, whereas when combined with 0.2 μM ACh there was an increase in channel opening activity compared to ACh alone, manifesting in many instances as double and triple concurrent openings. Mean open lifetime was reduced from 7.3 to 5.2 ms and average openings/burst increased from 1.4 to 2.0 for ACh alone and ACh plus 0.25 μM naltrexone, respectively. The frequency of channel opening in the presence of both agents immediately following exposure was 4 times greater than in the control situation, and this ratio increased to 6-fold within 2 min. In this recording mode, as with the inside-out patch experiments, there was no effect of naltrexone on single channel conductance.

4. DISCUSSION

This study reports various effects of naltrexone on normal ACh activation of the nicotinic receptor, namely: (i) a concentration-dependent increase in flickering of open channels to the closed state, leading to a progressive decrease in mean open channel lifetime and increased number of openings per burst; (ii) a time- and concentration-dependent decrease in frequency of channel opening, with burst of openings grouped into clusters; and (iii) at nanomolar concentrations, an increase in the frequency of channel opening. Considering the literature documenting non-opiate effects of the narcotic antagonists, it is not surprising that naltrexone interacts directly with the nicotinic receptor. However, the potency, with demonstrable effects even in the nanomolar concentration range, was unexpected. This phenomenon reinforces the statements of others [1,2] that demonstration of change in some biological response following administration of narcotic antagonists should be

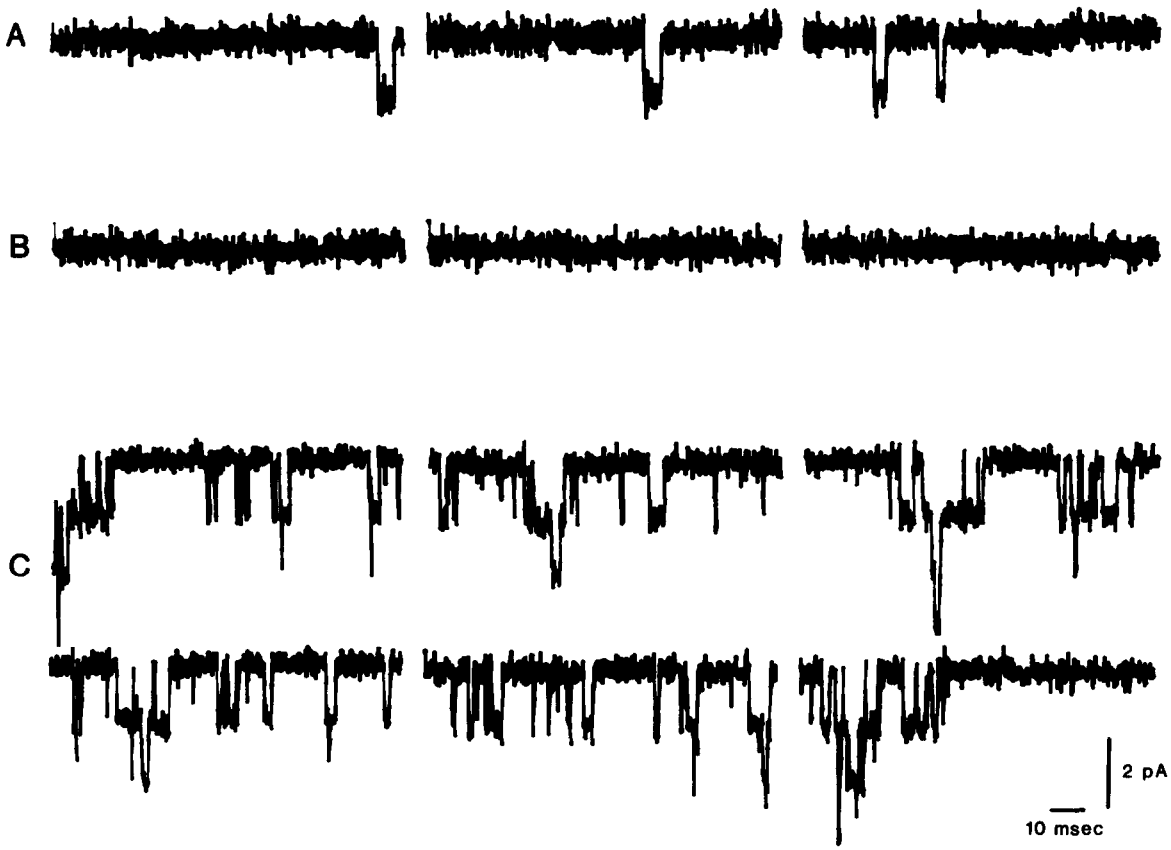

Fig.4. Single channel records from the same perfused patch showing the excitatory effect of naltrexone. Data were recorded at 1 kHz bandwidth, temperature 10°C, $V_H = -40$ mV. (A) 0.2 μM ACh; (B) 0.25 μM naltrexone; (C) 0.2 μM ACh plus 0.25 μM naltrexone. Downward deflections represent positive inward current.

regarded as necessary but not sufficient evidence for proof of an opioid pathway in the response.

At concentrations in the micromolar range, naltrexone has effects very similar to those agents which produce a non-competitive blockade of the open ion channel, and which have commonly been interpreted in terms of a sequential blocking model (e.g., procaine [16], atropine [17] and bupivacaine [18]). The general applicability of this model has been questioned [19–22] and here, a sequential block model would not directly explain the observed naltrexone closed channel blockade and clustering behaviour reminiscent of agonist-induced desensitization, nor would it predict the concentration-dependent biphasic effect, with activation at low levels followed by blockade at higher levels.

We favour an allosteric model to describe the multiple effects of naltrexone at the nicotinic receptor as follows:

$$2A + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} A_2R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R^* \rightleftharpoons A_2D_i$$

where A = ACh, R = receptor, A_2R^* = open form of the receptor, and A_2D_i = various desensitized forms of the receptor, and k_1, k_2, α, β = binding and isomerization rate constants. The observations and interpretations at low naltrexone concentrations (where clustering and presumably desensitization were minimal) are that firstly, naltrexone shortens channel open time and so has the principal effect of increasing α, and secondly, the average number of openings per burst is increased, which implies that the ratio β/k_2 is increased [23]. It is possible that nanomolar concentrations of naltrexone affect only the isomerization rate constants α and β in the scheme above, increasing both. Alternatively, naltrexone could

stabilize the biliganded but closed species (A_2R), since this would also increase openings and openings per burst by shifting the binding equilibrium to the right. Higher concentrations of naltrexone would seem to shift the equilibrium between open channels (A_2R) and the various inactivated forms (A_2D_i) in favour of the latter, while continuing to also increase α. The potency of the effect on openings per burst suggests a specific interaction between naltrexone and the receptor where only a single molecule of naltrexone may be bound. The further graded change in open channel lifetime and bursting behaviour with increasing naltrexone concentration suggests additional lower affinity sites which may be located elsewhere on the receptor, or could involve the lipid membrane phase [24]. The finding that many of the effects of naltrexone did not reach equilibrium for some minutes suggests restricted site access such as would occur if they were located near or in the lipid phase.

A number of studies provide supporting evidence that the interaction of naltrexone with the nicotinic receptor, at least at lower concentrations, is probably specific. Recent sequencing of the four subunit forms of the receptor has shown that the hydrophobic and putative ACh binding sites in the α subunits are highly conserved in the other three forms [25], and hence one might expect at least three non-agonist recognition sites where drug molecules could bind. It has also been suggested that there is a high affinity allosteric site for the noncompetitive channel blockers, probably located within the ion channel itself [24]. Furthermore, from experiments on the nicotinic receptor of a clonal muscle cell line it has been found that dibucaine, QX314 and histrionicotoxin act by similar allosteric mechanisms in binding to at least two sites per receptor molecule, causing increased agonist affinity and desensitization [26].

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