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Biphasic effect of pentobarbitone on chick myotube nicotinic receptor channel kinetics

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1 The modulatory action of pentobarbitone on chick myotube nicotinic acetylcholine receptor kinetics was studied by the patch clamp technique, particularly focussing on effects at low concentrations.

2 Open time sojourn distributions of foetal-type receptors recorded at room temperature (22-24°C) in cell-attached mode in the presence of 0.2 μM acetylcholine were well described by two exponentials, with fast and slow time constants of 0.53 ± 0.12 and 16.7 ± 2.2 ms (mean $\pm$ s.e.mean) respectively.

3 The duration of the slow open time constant was increased by low concentrations of pentobarbitone (up to 1 μM), and thereafter decreased with higher concentrations (10-50 μM) in a concentration-dependent manner.

4 Complementary evidence for the stimulatory effect of pentobarbitone on open time was obtained (i) by using a backfill technique where drug concentration at the patch gradually increases over time, and (ii) through use of perfused outside-out preparations where receptors in the same patch were successively exposed to acetylcholine in the absence and presence of pentobarbitone.

5 The dual action of pentobarbitone on channel kinetics probably indicates that an allosteric interaction mechanism is involved rather than simple steric channel blockade.

Keywords: Acetylcholine; nicotinic receptors; barbiturate effects; allosteric mechanism; general anaesthetic mechanisms; patch clamp; embryonic chick myotubes; biphasic effects; channel open time; burst duration

Introduction

Physical occlusion of the aqueous transmembrane pore of ion channels by drug molecules is a widely-described phenomenon, and the sequential model of channel blockade has often been useful in interpreting quantitatively the resulting concentration-dependent inhibition (Hille, 1992). Nevertheless, inconsistencies with this simple model have been known for some time (e.g. Neher, 1983), and in certain cases stimulatory effects have been observed at lower concentrations followed by the more usual inhibition at higher levels (Hu *et al.*, 1991; Le Dain *et al.*, 1991). These complexities have inevitably led to more detailed interaction models being proposed, such as those involving multiple modulatory binding sites and/or allosteric coupling effects between agonist and blocker molecules.

The nicotinic acetylcholine receptor has been extensively studied in this respect. This protein complex projects about 60 Å out from the cell plasma membrane and has an aqueous pore of diameter 20-25 Å from the extracellular mouth to the membrane level. The narrowest part of the pore, which is thought to include the gating region, lies within the membrane (Unwin, 1993). The high affinity binding site for non-competitive blockers of this receptor is located within the pore (Changeux *et al.*, 1984), but for steric occlusion to occur this site would have to be in the gating region as generally these molecules are not large enough to block the pore above the membrane. Johnson & Nuss (1994) have recently concluded from fluorescence resonance energy transfer studies that the binding site for these inhibitors is approximately 46 Å above the membrane level. This would mean that many blocking effects which previously have been interpreted as occlusion might more likely be due to allosteric enhancement of a pre-existing channel closing process. An allosteric interaction mechanism could also explain stimulatory effects, and even where stimulatory and inhibitory effects for a given drug occur in separate or overlapping concentration-ranges, might still be compatible with a single binding site in the pore. However, the details of these more complex processes in particular receptor systems remain to be elucidated.

In addition to these general considerations of ion channel modulation, particular interest in barbiturate effects on ion channels follows from recent advances made in the understanding of general anaesthetic mechanisms (Franks & Lieb, 1987). Traditionally, central nervous system (CNS) depressant drugs have been thought to act by perturbing the lipid bilayer of nerve membranes, but it is now apparent that many of their pharmacological effects may be caused by direct interaction with receptors and ion channels. The nicotinic receptor has served as a model for these interactions (Forman & Miller, 1989), and inhibitory effects due to a concentration-dependent decrease in single channel open time have generally been found (Gage & McKinnon, 1985; Jacobson *et al.*, 1991; Charlesworth & Richards, 1995). Less commonly in various *in vitro* studies there have been reports of biphasic CNS depressant effects (Roth *et al.*, 1986; Reynolds & Prasad, 1991), and the aim of the present study was to seek further evidence of such multiple drug effects, with a view ultimately of improving understanding of the underlying mechanisms.

Methods

Experimental techniques have been previously described (Le Dain *et al.*, 1991). Briefly, thigh muscles of 10-11 day old chick embryos were dissected free and incubated in divalent cation-free phosphate buffer containing 0.25% trypsin. Dissociated cells were collected by centrifugation, placed on gelatin coated cover-slips in a nutrient medium, and then incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 7-10 days to produce the foetal form of the receptor (used in cell-attached experiments), or 12-14 days for the adult form (used in outside-out experiments). Myotubes were then bathed in a simple physiological recording solution (in mM: NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10 at pH 7.4)

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and single ACh channel currents were recorded at room temperature (22–24°C). For a steady-state experiments in cell-attached mode, patch pipettes contained ACh (0.2 μM) and various concentrations of sodium pentobarbitone (0, 0.5, 1, 10, 25 and 50 μM). For the backfill experiments (Auerbach, 1991), pipettes were filled through the tip to a height of 2–4 mm with buffer containing ACh (0.2 μM), and then backfilled with ACh and pentobarbitone (10–50 μM). In both these experimental protocols, cells were routinely hyperpolarized by 20 mV to improve signal to noise ratio. Two perfused outside-out experiments were conducted where ACh (0.2–0.4 μM) alone or in the presence of pentobarbitone (0.5–1 μM) was perfused sequentially onto channels in the same patch. In these experiments the pipette contained an intracellular solution (in mM: KCl 140, $MgCl_2$ 2, HEPES 10, EGTA 10 at pH 7.4) and the cell-free membrane potential was held at −50 mV.

Experimental data were routinely filtered at 3 kHz unless otherwise indicated, stored on a Racal FM tape recorder, digitized offline at 20 kHz and then analysed as described (Le Dain *et al.*, 1991). Sections of data sometimes contained more than a single channel opening at any one time; these concurrent events were removed from subsequent analysis and usually did not represent more than 1–2% of total openings given the low agonist concentrations used. All cell-attached data sets which were analysed contained a minimum of 500 openings. Exponential distributions were fitted to individual open and closed time sojourns by maximum likelihood, with the number of exponentials indicated when there was effectively no further increase in likelihood. The critical closed time for burst analysis was set at 20 ms following examination of an equal proportion misclassification approach (Colquhoun & Sigworth, 1995). Acetylcholine and α-bungarotoxin were supplied by Sigma, sodium pentobarbitone by Drug Houses of Australia, and all other chemicals were AR grade.

Results

Single channel inward currents were induced by ACh (0.2 μM) in membrane patches recorded in cell-attached mode (see Figure 1 inset). These currents were not seen in the absence of ACh or when α-bungarotoxin (200 nM) was added to the pipette. Pentobarbitone (10 μM) alone had no agonist activity, and as reported elsewhere (Jacobson *et al.*, 1991), it had no effect on ACh-induced single channel current amplitude.

Under the present recording conditions, the distributions of single-channel open and closed sojourns in the absence and presence of pentobarbitone were best fitted by two and three exponential components respectively (see Table 1). The slow open time constant was increased significantly compared to control at pentobarbitone concentrations of 0.5 and 1 μM, and thereafter decreased in a regular concentration-dependent manner as observed in earlier studies (see Figure 1). The other open and closed exponential parameters shown in Table 1 generally exhibited a simpler, and in some cases less marked, monophasic dependence on pentobarbitone concentration; the exception was the slow closed time constant which in cell-attached mode was mainly determined by the number of receptors in different patches. Because these data were subject to limited time resolution (at least 100 μs), very fast closed events may have been missed and as a consequence analysis of burst durations incorporating apparent openings could be a more robust way of viewing the data. The burst time constants in Table 1 showed a similar trend to the open time analysis, suggesting that the stimulatory effect on channel activity at lower pentobarbitone concentrations seemed predominantly due to an increase in open time rather than the number of openings per burst.

Given that cell-attached data will always incorporate some variation in recording conditions across patches and cells (e.g. receptor number and cell membrane potential), we sought to confirm the steady-state finding of a stimulatory effect on long

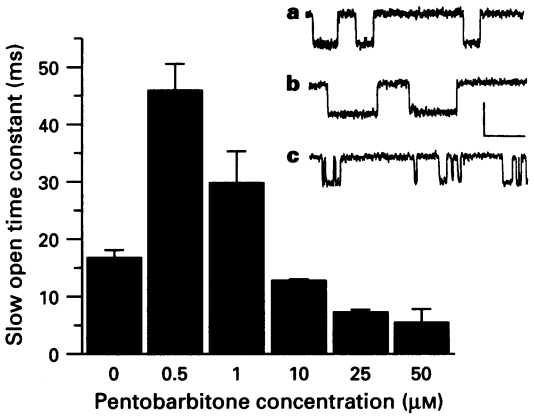

Figure 1 Effect of pentobarbitone on the slow open time constant estimated from cell-attached nicotinic receptor recordings. Data taken from Table 1. Inset shows representative channel openings (downward deflections) under control conditions (a) and in the presence of 0.5 (b) and 50 μM (c) pentobarbitone. Calibration: −4 pA and 100 ms.

Table 1 Effect of pentobarbitone on nicotinic receptor single channel kinetic parameters

No. of Expts	*Pentobarbitone concentration* (μM)	*Open time constants* (ms) *Fast*	*Slow*	*Closed time constants* (ms) *Fast*	*Intermediate*	*Slow*	*Burst time constants*† (ms) *Fast*	*Slow*
8	0	0.53±0.12 (34%)	16.7±2.15 (66%)	0.52±0.21 (14%)	129±60.9 (26%)	720±166 (60%)	0.47±0.10 (24%)	19.9±2.38 (76%)
2	0.5	0.78±0.33 (44%)	51.3±2.07* (56%)	1.58±1.43 (17%)	284±273 (44%)	2735±1578 (39%)	0.65±0.34 (35%)	62.6±0.45* (65%)
4	1	0.42±0.08 (24%)	30.1±6.34* (76%)	1.21±0.53 (15%)	48.7±25.3 (24%)	786±257 (61%)	0.35±0.03 (22%)	41.7±9.68* (78%)
3	10	0.77±0.47 (25%)	12.6±0.74 (75%)	2.41±0.39 (29%)	26.5±9.16 (16%)	905±155 (55%)	0.52±0.26 (26%)	25.9±4.68 (74%)
2	25	0.37±0.02 (16%)	7.36±0.33 (84%)	1.08±0.93 (18%)	5.70±1.87 (41%)	473±78.5 (41%)	0.32±0.02 (29%)	26.5±0.05 (71%)
3	50	1.66±0.98 (55%)	5.52±2.09 (45%)	1.12±0.73 (44%)	4.90±2.28 (36%)	1499±757 (20%)	0.80±0.21 (33%)	32.1±6.46 (67%)

Data recorded in cell-attached mode and presented as mean±s.e.mean; only experiments with more than 500 openings included; % indicates the relative proportion of different sojourn types; *represents a significant increase compared to control ($P<0.05$); †critical closed time set at 20 ms.

openings of the nicotinic receptor ion channel at low pentobarbitone concentrations through use of the backfill technique (Auerbach, 1991). With this approach the tip of the patch pipette is filled with agonist and the remainder backfilled with agonist plus a relatively high concentration of the modulatory drug. Channels in the patch are activated initially at the time of seal formation by agonist alone, and then under non-steady state conditions become exposed to gradually increasing concentrations of the drug. Figure 2a shows the results of two such experiments, where clearly a biphasic effect was again apparent. As expected when the diffusion distance for pentobarbitone was reduced from 4 to 2 mm, the peak arithmetic mean open time was less pronounced, with some shift to the left. Similar analysis of normal cell-attached data showed no such reproducible effect (see Figure 2b).

Finally, to assess whether the stimulatory effect of low pentobarbitone concentrations was a feature only of the foetal form of the chick nicotinic receptor, the adult form was studied in perfused outside-out preparations. In one experiment recorded at 1 kHz bandwidth and analysed using the standard intra-burst critical closed time of 20 ms, the burst durations were best fitted by two exponentials with control time constants of 1.87 ms (incorporating 30% of 126 events) and 5.66 ms (70%). Comparable analysis of bursts from the same patch in the presence of ACh and 1 μM pentobarbitone gave time constants of 2.48 ms (54% of 243 events) and 11.1 ms (46%). A similar increase in channel activation, independent of exponential fitting, was demonstrated in a further outside-out experiment recorded at 3 kHz. In this instance the arithmetic mean open time for all events longer than 2 ms under control conditions was 9.78 ± 0.30 ms (mean $\pm$ s.e.mean, $n = 697$), and in the presence of ACh and 0.5 μM pentobarbitone the corresponding result was 12.76 ± 0.62 ms ($n = 507$).

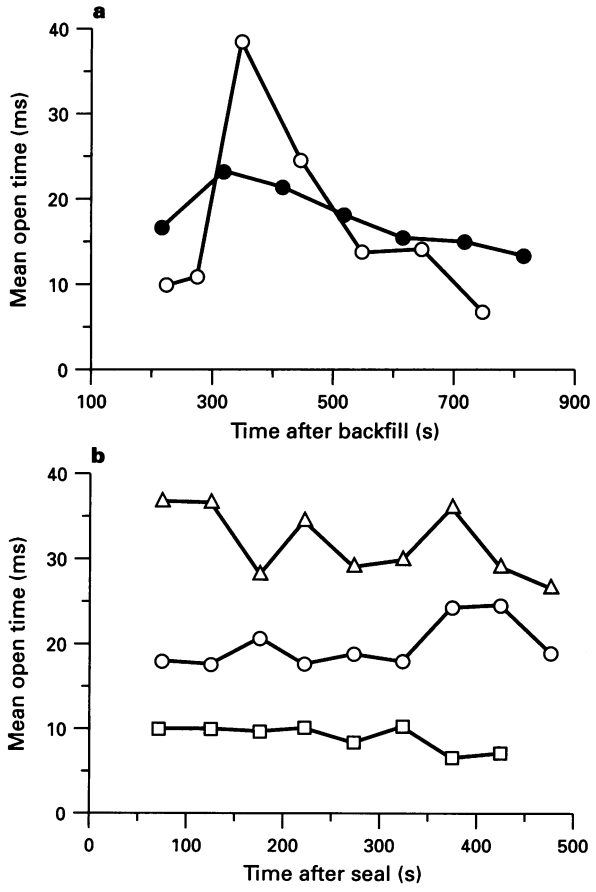

Figure 2 (a) Two backfill experiments showing time-dependent effects of gradually increasing concentrations of pentobarbitone on channel open time. ACh was tip filled to a height of 2 (●) and 4 (○) mm and then the pipette was backfilled with ACh and 25 μM pentobarbitone. Ordinate values are arithmetic mean open time for all openings longer than 2 ms in successive 50–100 s intervals following seal formation. (b) Comparable analysis of cell-attached data with ACh in the presence of 0 (○), 1(△) and 25 μM (□) pentobarbitone.

Discussion

Kinetic parameters for chick nicotinic receptor activity recorded under the present study conditions compared favourably to earlier studies. Two open time distributions were found, with time constants of 0.53 and 16.7 ms. Previous work includes a study on chick myotubes in cell-attached mode (Eusebi *et al.*, 1987) where a single open distribution time constant of 9.5 ms was reported for less than 500 events recorded at 2 kHz bandwidth, and others where two exponential distributions best described the data. The latter include time constant estimates of 0.7 and 17.9 ms in denervated rat muscle cells (Gage & McKinnon, 1985), 3 and 15 ms (filter bandwidth 250 Hz) for neuronal-like nicotinic receptors in bovine adrenal chromaffin cells (Jacobson *et al.*, 1991), and 0.41 and 12.2 ms for bursts in receptors from rat sympathetic neurones (Mathie *et al.*, 1991).

There were two main perspectives in this study, the first on understanding pharmacological modulation of ion channel behaviour and the second, possible implications regarding general anaesthetic mechanisms and interaction of CNS depressants with ion channels.

Regarding the first point, it has been known for many years that the nicotinic receptor contains a number of non-competitive inhibitor binding sites, one of high affinity in the channel pore, and several others of lower affinity located probably on the exterior of the receptor-channel complex at the protein lipid interface (Changeux *et al.*, 1984). From the present study and others, it seems that pentobarbitone at concentrations of 10–500 μM is one of the many inhibitors known to cause channel blockade (Lambert *et al.*, 1983), with the simplest assumption being that it sterically occludes the pore in a manner compatible with the sequential blocking model. However, even in those studies at relatively high concentrations of barbiturate where only blockade was observed, it has been generally concluded that mechanisms more complicated than steric occlusion seem to apply (Gage & McKinnon, 1985; Jacobson *et al.*, 1991; Charlesworth & Richards, 1995). The stimulatory effects found at low pentobarbitone concentrations in the present study are also incompatible with a simple sequential model, which predicts only concentration-dependent inhibition. These data therefore support the earlier work in suggesting that another interaction mechanism must operate. A likely alternative, as suggested by Gage & McKinnon (1985), is that pentobarbitone binding to nicotinic receptors affects gating behaviour allosterically rather than physically.

Biphasic effects of drugs on ion channels have been previously reported in a variety of systems. For example, Gage *et al.* (1979) found that miniature endplate currents in toad sartorius muscles decayed faster in the presence of ether concentrations less than 20 mM, and slower when the concentration was greater than 40 mM. Hess *et al.* (1984) reported mixed effects of 5–10 μM nitrendipine on ventricular muscle calcium channels, with a predominantly antagonistic action nevertheless incorporating an increase in channel open time. Where these dual effects have been interpreted from a mechanistic viewpoint at the molecular level, it has seemed reasonable to infer (e.g. McDonald *et al.*, 1989) that at least two binding sites are involved, one excitatory and the other inhibitory, particularly if these two opposing effects occur in separate drug concentration-ranges. Nevertheless, we have been able to show theoretically that with a simple linear four state activation scheme for the nicotinic receptor incorporating an additional terminal closed state when drug is present, it is

possible to obtain biphasic effects in an allosteric model when only two or three of the transition rate constants change as a result of drug binding (Yeo & Madsen, unpublished). In our previous study on the interaction of naltrexone with nicotinic receptors (Le Dain *et al.*, 1991), allosterically-induced changes in three kinetic scheme rate constants all occurred over the same concentration range (0.1–5 μM), yet effects at 0.5–1 μM manifested as excitatory while those at 5 μM and greater were inhibitory and increasingly dominant. It was concluded that the excitatory effects of naltrexone were due to enhanced ACh binding, and there is a suggestion in data presented by Roth *et al.* (1989) that a similar enhancement of ACh binding to *Torpedo* nicotinic receptors might occur with the (+)-isomer of 10 μM pentobarbitone.

In terms of the observable consequences of the stimulatory effect of pentobarbitone on single channel events, the present results suggest mainly a prolongation of the slow open time with a smaller increase in the number of openings per burst. It must be acknowledged however, that with the finite time resolution involved in analysing these data, the increased duration of long openings could have resulted from an increased number of brief (i.e. < 100 μs), unobserved closures. Higher time resolution studies will be required to answer this question definitively.

On the second perspective concerning CNS depressant mechanisms, most studies on ion channels at the cellular and molecular level have reported monotonic concentration-dependent effects. However *in vivo*, progression to surgical anaesthesia following administration of pharmacological agents proceeds through a stimulatory phase, albeit brief for some agents, before resulting in more generalized CNS depression. The usual explanation for this phenomenon is that the excitatory phase is due to inhibition of inhibitory interneurones. In the present study, such a biphasic effect was seen for pentobarbitone in an *in vitro* system at the receptor/ion channel level. While in this instance the skeletal muscle form of the nicotinic receptor was used, similar dual effects have been reported in several neuronal preparations, e.g. the effect of ethanol on excitability of cultured cerebellar Purkinje neurones (Urrutia & Gruol, 1992), and on NMDA-activated currents in rat hippocampal pyramidal cells (Lima-Landman & Albuquerque, 1989) and GABA-activated currents in chick cerebral cortical neurones (Reynolds & Prasad, 1991). Together, these findings raise the possibility that in some cases the excitatory phase of general anaesthetics could have a receptor-dependent component that is separate from the interplay between excitatory and inhibitory neurones.

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