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Acute Toxicity Screening of Novel AChE Inhibitors Using Neuronal Networks on Microelectrode Arrays

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Abstract

Spontaneously active neuronal networks grown from embryonic murine frontal cortex on substrate integrated electrode arrays with 64 recording sites were used to assess acute neurobiological and toxic effects of a series of seven symmetrical, bifunctional alkylene-linked bis-thiocarbonate compounds designed to possess anticholinesterase activity. Acute functional neurotoxicity in the absence of cytotoxicity was defined as total collapse of spontaneous activity. All of the compounds were characterized as mixed inhibitors of AChE, with K_i's in the 10^{-7}–10^{-6} M range. The neuronal network assays revealed high repeatability for each compound, but surprisingly diverse effects among these closely related compounds. Six of the seven compounds produced changes in network activity at concentrations of 10–350 μM. Three of the compounds were excitatory, two were biphasic (excitatory at lower concentrations, inhibitory at higher), and one was solely inhibitory. Two of the inhibitory compounds produced irreversible inhibition of activity. Responses of cortical cultures to eserine were compared to the effects produced by the test compounds, with only one of seven providing a close match to the eserine profile. Matching of response patterns allows the classification of new drugs according to their response similarity to well-characterized agents. Spontaneously active neuronal networks reflect the interactions of multiple neurotransmitter and receptor systems, and can reveal unexpected side effects due to secondary binding. Utilizing such networks holds the promise of greater research efficiency through a more rapid recognition of physiological tissue responses. © *2001 Elsevier Science Inc. All rights reserved.*

Keywords: Substrate-integrated electrode arrays; AChE; Extracellular multichannel recording; Drug screening; Bio-sensors; Alzheimer

INTRODUCTION

Unexpected secondary binding by compounds designed to be specific for certain receptors or an enzyme is a problem that affects every drug development effort. A reliable method for screening novel compounds for unwanted or toxic side effects could significantly increase the efficiency of the drug development process and minimize the use of experimental animals. We have used cultured neuronal networks grown from embryonic murine frontal cortex to assess the effects on spontaneous activity of seven symmetrical, bifunctional alkylene-linked bis-thiocarbonate compounds designed and biochemically verified to interact with both the peripheral and catalytic sites of AChE. Our purpose was to explore the relative efficacies of these compounds in modulating spontaneous activity, and to utilize cultured neuronal networks as rapid screening platforms for determining general neurophysiological and toxic effects of novel compounds.

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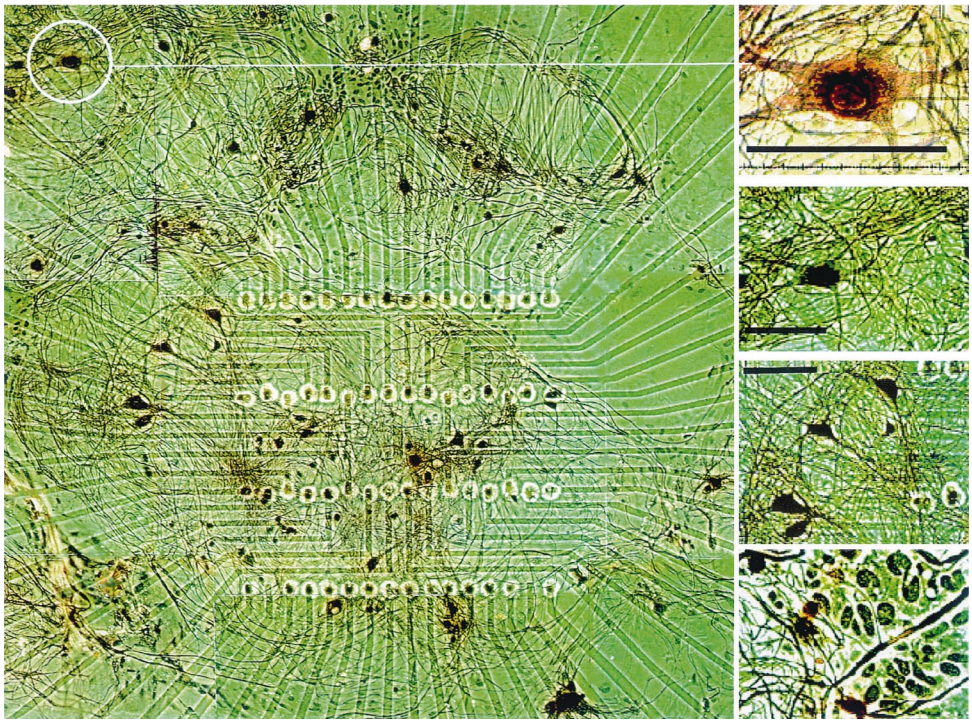

Fig. 1. Bodian-stained neuronal network grown on a 64-electrode array. Transparent indium–tin oxide conductors are 8 μM wide in the 0.8 mm^2 recording matrix. Right panels: higher magnification view of single neurons and neurites within the 56 days old network.

The networks were grown on thin-film multielectrode arrays (MEAs) constructed with transparent indium–tin oxide (ITO) conductors allowing simultaneous monitoring of multiple neuronal spike trains and visual observation of neuronal somata and processes (Fig. 1). Such networks are spontaneously active in culture and are sensitive to pharmacological manipulation of the medium. This sensitivity provides an effective platform for rapidly assaying effects induced by known and novel neuroactive substances (Gross et al., 1995, 1997a,b; Morefield et al., 2000). Compounds may acutely modulate the activity of spontaneously active networks via several pathways. Among them are receptor activation and deactivation, metabolic disturbances, modification of membrane characteristics, alteration of vesicular dynamics, and changes in ionic potentials. These effects are reliably reported by changes in the temporal dynamics of neuron-to-neuron communication as monitored non-invasively with extracellular recording.

It is evident from the literature that the cholinergic system in the CNS has a wide range of physiological influences. Although single cell data provides important insights into these domains, sequential analysis of single cell responses cannot give an accurate picture of population response dynamics. These population responses are relatively easy to obtain with networks cultured on microelectrode arrays, at the cost however, of disrupting the original circuitry. Nevertheless, the reformed circuits in culture are pharmacologically histiotypic, and demonstrate changes in spike patterns characteristic of the parent tissue (Gross et al., 1997a,b; Morefield et al., 2000).

METHODS

Techniques used to fabricate and prepare microelectrode arrays (MEAs) have been previously described (Gross, 1979; Gross et al., 1985; Gross and Kowalski, 1991). The hydrophobic surfaces of the MEAs were activated via flaming through a stainless steel mask, followed by application of poly-D-lysine and laminin. Cortical tissues were harvested from embryonic 15 to 18 day gestational age Hsd:ICR mice. The tissue was dissociated enzymatically (papain) and mechanically, seeded on the prepared areas of the MEAs, and maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 5% horse serum and 5% fetal calf serum in a 90% air/10% CO_2 atmosphere. No antibiotics/antimitotics were used. Cultures were ''fed'' twice a week with DMEM containing 5% horse serum. Cultures maintained under these conditions can remain

spontaneously active and pharmacologically responsive for more than 6 months (Gross, 1994).

The recording medium consisted of a 50/50 mixture of fresh DMEM and conditioned DMEM (conditioned by culturing dissociated cortical tissue in flasks), supplemented with 5% horse serum. Osmolarity was adjusted to 320 mOsmole. Eserine, curare and atropine were purchased from Sigma, St Louis, MO, and dissolved in sterile water. The test compounds were dissolved in 0.8% saline. All drugs were bath applied (bath volume 1.0–1.5 ml). Stock concentrations were prepared so that total volume added never exceeded 1% of the total bath volume. The details of the synthesis and the AChE (purified from calf forebrain) inhibition analyses of the test compounds have been submitted for publication elsewhere (Drug Development Research).

MEAs were placed in stainless steel recording chambers (Gross and Schwalm, 1994), and maintained at 37°C on an inverted microscope stage. The pH was stabilized at 7.4 by passing a stream of humidified 10% CO_2 (flow rate of 10–15 ml/min) in air through a cap featuring a heated ITO window to prevent condensation and allow continued observation. Neuronal activity was recorded with a two-stage, 64 channel amplifier system (Plexon Inc., Dallas), and digitized simultaneously via a Dell 300 MHz computer (spike analysis) and a Masscomp 5700 computer (burst analysis). Total system gain used was normally 12 K. The neuronal activity was discriminated with a template-matching algorithm (Plexon Inc.) in real time to provide single unit spike rate data; whole channel (multiple-units/channel) data was analyzed using custom programs for burst recognition and analysis. The 16 cultures used in this study had an average electrode yield of 48%, and a mean signal to noise ratio of 3.3:1 (including 1.5:1 SNRs). Maximum SNRs often exceeded 10:1. Cultures were randomly assigned to experimental groups, with the only requirement being they were at least 21 d.i.v. and exhibited significant spontaneous activity. No influence of age on mean levels of spontaneous activity was observed.

The effects of the test compounds were interpreted in terms of two network activity variables, burst rate and spike rate. These variables are easily quantified, and changes in the mean values associated with experimental manipulations relative to a reference period provide reliable information on the functional state of the neuronal networks. The simultaneous monitoring of spike and burst rates allows detection of activity increases, decreases, and spike organization into bursts on a single unit basis, while cross-channel averaging provides information on network activity coordination. These relatively simple analysis techniques allow quantification of the data and easily visualized graphical representations of network responses to pharmacological compounds.

All analyses were done with binned data, with a bin size of 60s. Single unit activity was averaged across channels yielding mean network spike and burst rates. These quantities were graphed as a function of time, providing a picture of the temporal evolution of activity and allowing a determination of stationary activity states (variations in burst and spike rates of $< 15\%$). An experimental episode mean was obtained from the values comprising the stationary state. Control periods were always 30 min, whereas experimental episodes ranged from 20–30 min because of exclusion of the transition states. Within single experiments, significance of changes induced by drug application was tested with a paired Student's t-test, $P < 0.05$. For pooled data (Figs. 2d and 3), significance was tested with a one-way ANOVA followed by a post hoc Dunnett's test, $P < 0.05$.

RESULTS

The responses of frontal cortex cultures to a series of cholinergic agonists, antagonists, and the well-characterized AChE inhibitor eserine, were determined in order to demonstrate that these cultures were suitable assay vehicles for the series of novel AChE blockers. Fig. 2a illustrates the results of applying curare (15 μM), an antagonist at nicotinic receptors containing the α7-subunit. An increase in both bursting and spiking occurs within 7 min, which persists until the end of the experiment. Panel 2b shows the response to an application of the non-specific muscarinic antagonist, atropine. Both burst and spike rates are depressed below reference period levels (28 and 32%, respectively), lasting until the end of the recording. Under conditions of spontaneous activity, antagonists are effective in revealing functional cholinergic synapses because they interfere with an ongoing synaptic process.

Fig. 2c shows a network where 30 μM bicuculline, the $GABA_A$ antagonist, was applied at the beginning of recording, followed by application of 30 μM carbachol at 190 min. The network response shows a near doubling of burst and spike rates that persist for the duration of the experiment. The bicuculline–carbachol effect could be antagonized by application of 20 μM atropine (not shown). The major potentiation of activity is reminiscent of the well-known effects of

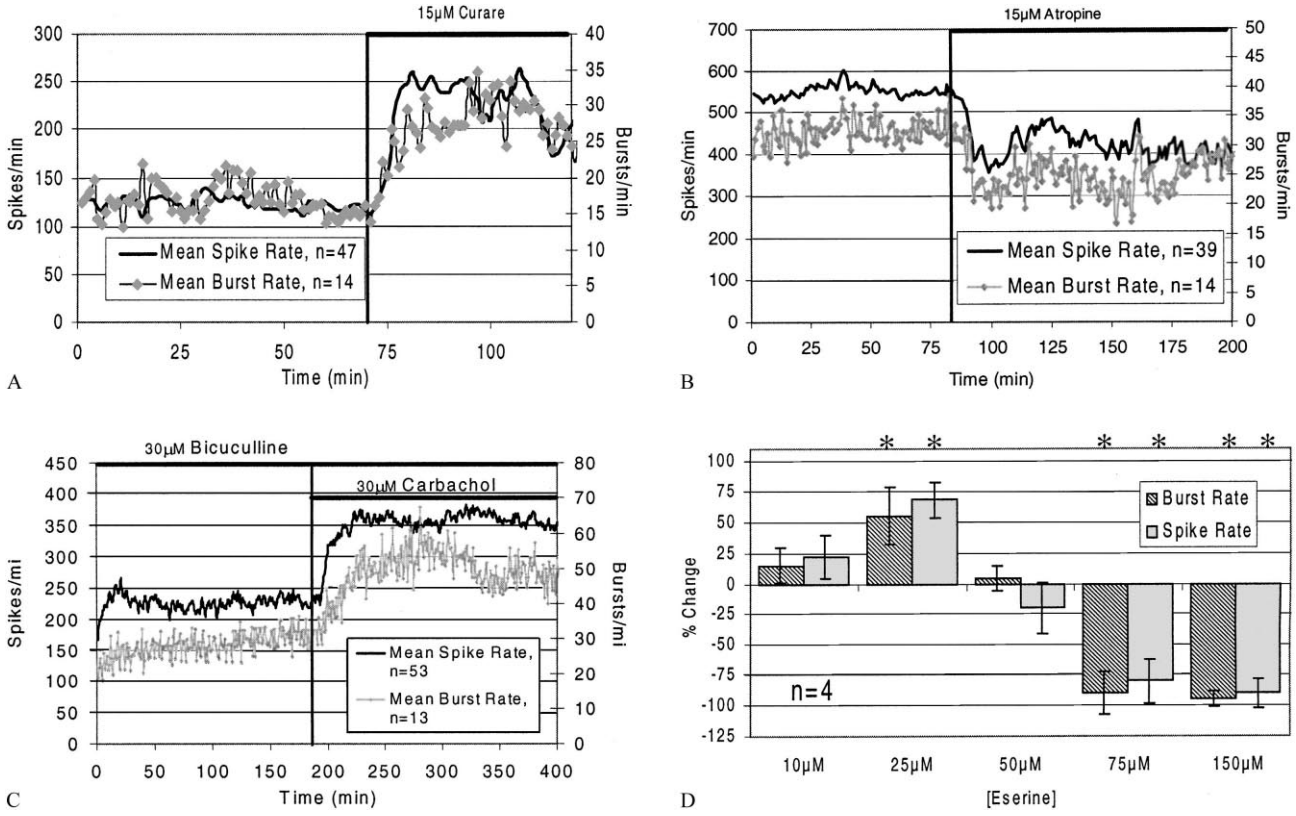

Fig. 2. Response of frontal cortex cultures to well characterized agents affecting cholinergic neurotransmission. (A) Application of the nicotinic receptor antagonist curare (15 μM) induced an increase in burst and spike rates. (B) The non-specific muscarinic receptor antagonist atropine (15 μM) produced an inhibition of bursting and spiking. (C) Application of carbachol 30 min after bicuculline produced large increases in the burst and spike rate activity. This effect persisted for the duration of the experiment. (D) Mean responses of four experiments to titration of the acetylcholinesterase inhibitor eserine (10–150 μM). At low concentrations, eserine produced an increase in both burst and spike rates. The excitation was reversed by increasing concentrations, resulting in an average 95% suppression of burst rates and an average 90% inhibition of spike rates at 150 μM. The effects produced by eserine were fully reversible by washing. Here, '∗' indicates significant difference from control period ($P < 0.05$, one way ANOVA with Dunnett's post hoc test).

carbachol in producing epileptiform bursting in hippocampal preparations (Williams and Kauer, 1997).

Panel 2d summarizes the results from four experiments where eserine, a well characterized inhibitor of AChE, was titrated from 10 to 150 μM. The mean response is biphasic, with eserine increasing both bursting and spiking at 10 and 25 μM, and inhibiting activity at concentrations above 50 μM. The inhibition of activity by eserine was reversible by washing (not shown). Together, these data indicate that the frontal cortex cultures used possess both curare-sensitive nicotinic and atropine-sensitive muscarinic cholinergic receptors. Additionally, functional cholinergic synapses mediate a tonic inhibition of activity through nicotinic receptors concomitant with a tonic excitation of activity through muscarinic receptors.

Averaged cortical network responses to eserine (Fig. 2d, $n = 4$) were used to define a response profile to this known AChE inhibitor. The main features of this profile include an initial excitation at lower concentrations (10–40 μM), followed by a suppression of activity at concentrations beyond 40 μM, with almost total inhibition of bursting and spiking at 150 μM. The effects were reversible with one or two complete medium exchanges. This profile served as a reference for the novel AChE inhibitors.

For testing the seven novel AChE inhibitors, which were identified as pure with TLC, ^{1}H NMR, and FTIR spectrophotometry, we used 16 murine frontal cortex cultures. The median age of the networks was 27 days in vitro, with a minimum of 21 and a maximum of 41 days. Cultures stabilize 3 weeks after seeding and do not change their pharmacological responses for up to 4–6 months (Gross et al., 1997a). Table 1 lists the structure and AChE inhibition constant of each compound.

The network activity changes induced by the novel compounds were grouped into two categories: 'excitatory', and 'inhibitory'. A compound was classified 'excitatory' or 'inhibitory' based on the lowest used

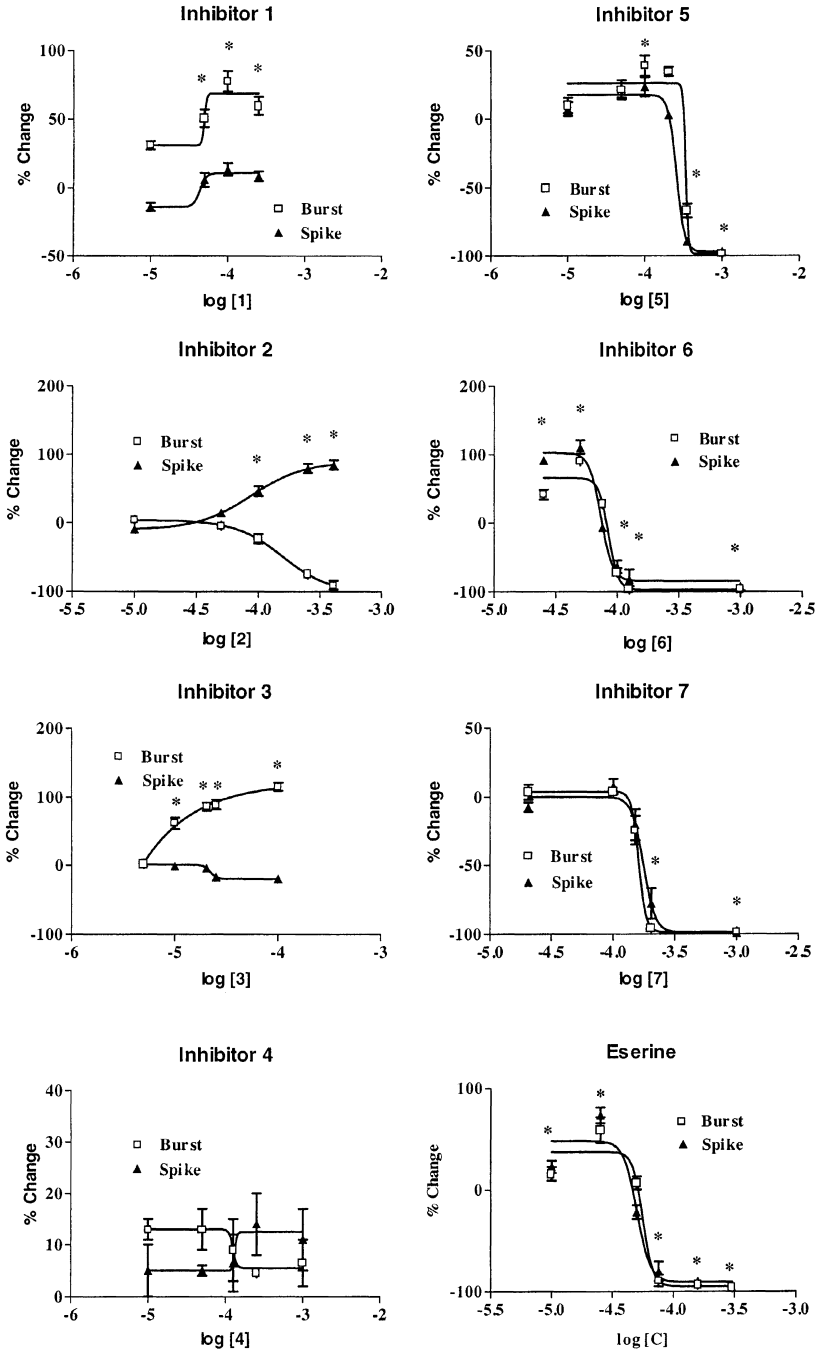

Fig. 3. Dose-response summary for seven novel anticholinesterases and eserine using the network activity variables burst rate and spike rate. Percent change was calculated for a 20–30 min window of activity at the concentration listed (see Section 2), relative to a 30 min reference period of spontaneous activity. Data shown represents mean of two, or in the case of Inhibitors 2 and 6, three experiments. Eserine data originates from four experiments. Vertical bars represent the range of responses for Inhibitors 1, 3, 4, 5, 7 ($n = 2$) and standard error for Inhibitors 2, 6, and eserine. I1 increased burst rate at 10 μM eliciting a maximal increase of 74% at 100 μM. The compound did not change spike rates significantly at any concentration tested. I2 increased spike rates at 100 μM and above with a concomitant inhibition of bursting. I3 was the most efficacious of the compounds, increasing burst rates an average of 98% above baseline at 25 μM. I3 was ineffective at altering spike rates in the range shown, and at concentrations up to 0.5 mM (data not shown). I4 was the least effective agent tested, producing a 27% inhibition of burst rate only at concentrations of 0.5 mM. I5 produced increases in bursting and spiking at 10–200 μM (increases in bursting were significant at 100 and 200 μM, $P < 0.05$) which were transformed into a 79 and 84% inhibition of burst and spike rates, respectively, at 350 μM. This inhibition was irreversible. I6 produced a biphasic effect, increasing both bursting and spiking significantly at 25–50 μM, and reversibly inhibiting both parameters at concentrations above 75 μM. I7 produced effects very similar to I5, although it was more potent, with an irreversible inhibition of both bursting and spiking at concentrations above 150 μM. Response to eserine was biphasic, with excitation of burst and spike rates occurring at the 10 and 25 μM concentrations, and inhibition of both activity measures occurring at 75 μM and above. The suppression of activity by eserine was reversible. Here, '∗' indicates significant difference from control period, $P < 0.05$, one way ANOVA, post hoc Dunnett's test.

Table 1
List of test compounds[a]

Inhibitor	Structure	AChE inhibition constants, K_i (M)	Inhibition type
I1	Ch^+O–CO–S$(CH_2)_2$S–CO–OCh^+	1.0×10^{-6}	Mixed
I2	Ch^+O–CO–S$(CH_2)_3$S–CO–OCh^+	1.4×10^{-6}	Mixed
I3	Ch^+O–CO–S$(CH_2)_4$S–CO–OCh^+	1.0×10^{-6}	Mixed
I4	Ch^+O–CO–S$(CH_2)_5$S–CO–OCh^+	1.4×10^{-6}	Mixed
I5	Ch^+O–CO–S$(CH_2)_6$S–CO–OCh^+	1.4×10^{-6}	Mixed
I6	$DMEA^+O$–CO–S$(CH_2)_4$S–CO–$DMEA^+$	3.6×10^{-7}	Mixed
I7	$DMEA^+O$–CO–S$(CH_2)_6$S–CO–$DMEA^+$	5.0×10^{-7}	Mixed

[a] Ch^+O– represents choline residues; $DMEA^+$ represents *N*,*N*-dimethylethanolamine residues.

concentration that caused a change in either bursting or spiking from control of greater than 20%. These categories were further subdivided into reversible (R), irreversible (I), and partially reversible (P). Reversibility was defined as a return to near reference activity after one to two complete medium changes. Partial reversibility represented recovery of activity after washing that deviated more than 20% from control period. Partial reversibility was not encountered in this data set. An effect was considered irreversible if there was no return of any activity after two or more complete medium changes and a subsequent wait of at least 2 h. Table 2 summarizes the structure, minimum effective concentration, and type of effect for each of the compounds.

The results from applying the novel AChE inhibitors to frontal cortex networks are summarized with dose-response graphs in Fig. 3. It is apparent that minor molecular differences in the structures of the inhibitors can have profound effects on the changes in network activity. Two compounds, I1 and I3, caused increases in bursting, with little effect on spike rates, but I3 was significantly more potent in its effects. One compound, I5, had a biphasic effect, increasing bursting activity by significant amounts at 100–200 μM, but causing an irreversible inhibition of both bursting and spiking at concentrations of 350 μM and above. Another compound, I6, increased both bursting and spiking activities at low (<75 μM) concentrations, but reversibly inhibited bursting and spiking at 100 μM and above. I7 was solely inhibitory, producing an irreversible cessation of both bursting and spiking at concentrations of 200 μM and above. Compound I4 had little effect on network activity at concentrations up to 0.5 mM. Inhibitor 2 increased spiking activity, while simultaneously lowering the burst rate, reflecting changes in the organization of network spike activity. It is of interest that the three cultures used in assaying the effects of I2 were 23, 28, and 37 d.i.v. The low standard error in the graph for I2 in Fig. 3 shows that the age of the cultures had no major effect on the responses of the networks.

Fig. 4 illustrates the effect of I5, a choline-based compound with a 6-carbon, hexylene spacer. I5 produced small increases in burst and spike rates at 10–200 μM (the increase in burst rate was significant at 100 and 200 μM, $P < 0.05$) followed by an irreversible inhibition of activity at 350 μM. One of the two networks used to study I5 was monitored for a 12 h period with an inverted microscope and video display. Although no activity was recorded on any channel during the 12 h, the microscope observations showed no discernible cytotoxicity.

Table 2
Network responses to test compounds

Inhibitor	Spacer	Base	Excitatory (μM)	Inhibitory (μM)	Reversibility	n[a]
I1	2-C	Choline	10		R[b]	2
I2	3-C	Choline	50		R	3
I3	4-C	Choline	10		R	2
I4	5-C	Choline	NE[c]	NE	–	2
I5	6-C	Choline	100	350	I[d]	2
I6	4-C	Ethanolamine	25	125	R	3
I7	6-C	Ethanolamine		200	I	2

[a] Number of cultures tested.
[b] R: reversible after one or two medium exchanges.
[c] NE: no effect.
[d] I: irreversible after two or more medium exchanges and at least a 2 h wait.

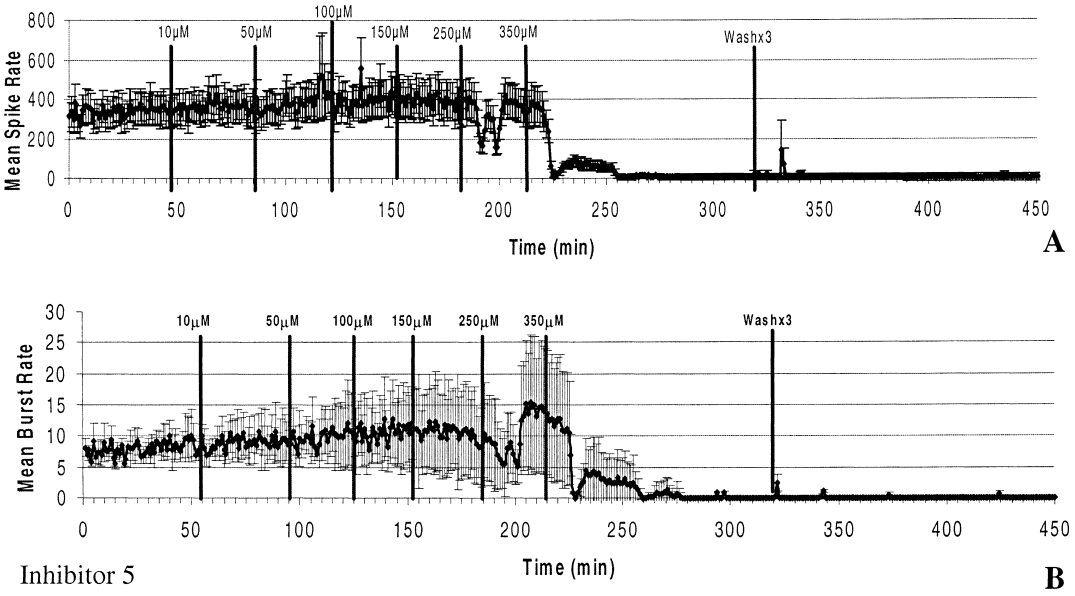

Fig. 4. Irreversible inhibition of network activity in response to I5. (A) Mean ± S.D. of 28 discriminated units. Titration to 200 μM produces very little effect on spike rates, raising the concentration to 350 μM causes a rapid reduction in spiking. All spike activity is abolished within approximately 30 min. (B) The effect on spike rates is paralleled by burst rate inhibition (mean ± S.D. of 12 channels). However, the standard deviation of the burst rates begins to increase at 50 μM, indicating a lessened cross-channel coordination. The inhibition is not reversible by three complete medium changes at 320 min.

Inhibitor 6 (a DMEA-based compound), produced a biphasic effect on network activity (Fig. 5). At 25 μM, I6 caused an increased spike production of greater than 90%. Burst rate was increased by 80% at the same concentration. Increasing the concentration to 50 μM resulted in a spike rate increase averaging 101%, with a concomitant increase in burst rates of 73%. The excitatory window for I6 was rather narrow; at 75 μM the spike rate was inhibited by 28% while burst rates were

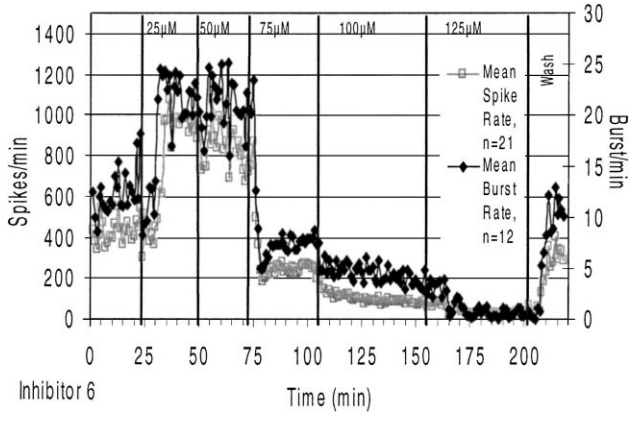

Fig. 5. With a 4-C spacer and a DMEA residue, I6 had a biphasic effect on spiking and bursting, increasing both activity measures at concentrations of 25–50 μM. This activity increase was reversed at concentrations of 75 μM and above. Only minor spiking remained at 125 μM. The inhibitory effect was reversible by washing.

29% below baseline. Maximal inhibition of mean spike rate by 92% was produced by concentrations of 125 μM and above. The burst rate was reduced to zero by concentrations exceeding 100 μM. The inhibition of network activity was reversible by washing, in contrast to that produced by Inhibitors 5 and 7.

The other DMEA-based compound, Inhibitor 7, produced only inhibitory effects on network activity, suppressing spike and burst rates at 150 μM by an average of 50 and 43%, respectively. I7 totally stopped all activity at 200 μM (Fig. 6). This effect was irreversible with only transient activity recovery after two complete medium changes, followed by a complete cessation of activity for a 3 h observation period. There was no overt cytotoxicity associated with this functional (electrophysiological) toxicity. Neuronal somata remained phase-bright, without cytoplasmic granularity throughout the observation period.

DISCUSSION

We tested the responses of murine frontal cortex networks cultured on multielectrode arrays to seven newly synthesized compounds designed to block AChE, the primary effector of ACh removal from the synaptic cleft. The presence of this enzyme in the cortical cultures was deduced from the change in

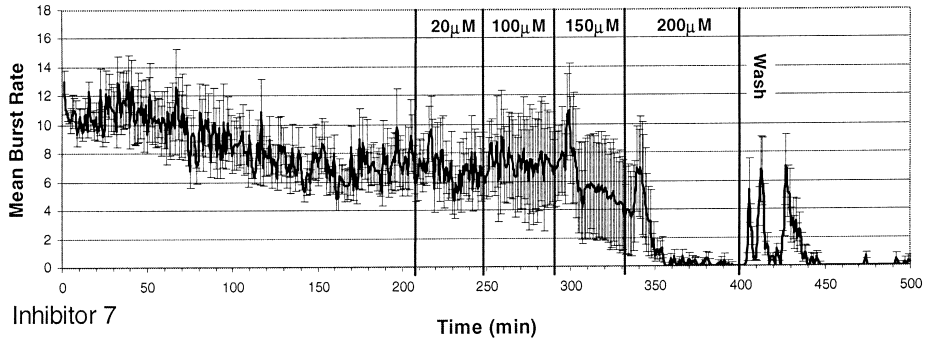

Fig. 6. Inhibitor 7 (DMEA-based compound with a hexylene spacer) caused only moderate increases in the standard deviation of the burst rate, until the concentration was increased to 150 μM, at which point the burst rate was depressed. At 200 μM, Inhibitor 7 totally suppressed all network activity. The inhibition was not reversed by two complete medium changes.

activity elicited by eserine, a specific blocker of AChE. Eserine was effective in increasing network activity at a concentration range of 10–25 μM, while it suppressed network activity at higher concentrations. This biphasic activity of eserine has been seen at the neuromuscular junction where eserine, at low concentrations (1–20 μM), increased the amplitude and prolonged the decay time of endplate-currents, but at 20 μM to 2 mM decreased the decay times (Dudel et al., 1999). The 1–20 μM effects were attributed to inhibition of AChE, while the high dose effects were thought to be a result of a direct blocking action of eserine on the open channel configuration of the nicotinic receptor. Blocking of nicotinic ACh receptors by eserine and four other AChE inhibitors was also seen by van den Beukel et al. (1998) in a variety of cell types. In the feline medulla, chemosensitive respiratory neurons also responded biphasically to eserine, with an initial augmentation of neuronal activity at low concentrations which converted to a depression in firing rates at higher concentrations in an apparently opioid-receptor dependent fashion (Trouth et al., 1993).

Although eserine is approved for clinical applications in the treatment of Alzheimer's, the therapeutic window for this drug is relatively narrow. This is also the case for I6, the novel compound that most closely resembled eserine in its effects. Possible therapeutic windows are wider for compounds I2 and I3, which demonstrate increases in spike production and burst production, respectively. Although they show no general inhibition, the compounds change spontaneous activity differently. Whereas I2 transforms bursting into spiking, I3 is very effective at organizing existing spikes into bursts. As increased bursting is generally associated with epileptiform activity, I3 may indeed trigger epilepsy, whereas I2 may stabilize epileptic tissue. Animal experiments are clearly required to determine the final behavioral response, however, the dose-response curves of Fig. 3 provide valuable data that can be used to increase the efficiency of drug screening procedures. The irreversible inhibitory effects of I5 and I7 would seem to make them unsuitable for possible clinical applications. Such inhibition of network activity should be considered acute functional toxicity despite a lack of cytotoxicity. Tetrodotoxin would be a compound in this category.

Cholinergic neurons intrinsic to the frontal cortex have been labeled immunohistochemically with antibodies to the vesicular acetylcholine transporter (Arvidsson et al., 1997) and choline acetyltransferase (ChAT) (Johnston et al., 1981). Cossette et al. (1993) demonstrated that ChAT activity recovered to near normal levels in the fronto-parietal cortex within 3 months of ibotenic acid lesion of the nucleus basalis in adult rats. These results, along with the effects of eserine that we observed, imply that cholinergic neurons are intrinsic to the cortex of intact animals and that these neurons survive in our cultures and make functional synapses.

It is interesting to compare the effects on neuronal activity of the seven compounds with their inhibitions of AChE (Tables 1 and 2). All the test compounds were classified as giving mixed inhibition, although double reciprocal plots suggest inhibitions of a non-competitive nature. This phenomenon has been addressed by others, who contend that certain types of AChE inhibitors exhibit mixed inhibition (Krupka and Laidler, 1961). From Table 1, it can be argued that the DMEA derivatives (compounds 6 and 7) are better AChE inhibitors than the choline derivatives (compounds 1–5). The former have K_i values in the 10^{-7} M range, while the latter derivatives have K_i values in the 10^{-6} M range. Further, there is no apparent relationship between alkylene length separating the thiocarbonate moieties

and the inhibitory potencies of the choline or the DMEA derivatives. This also suggests that inhibitory potencies of the compounds are not dependent upon binding with both the peripheral binding site and the active site of AChE, since the greater the separation between the thiocarbonate moieties the greater the anticipated binding with both sites (Pang et al., 1996).

For the choline-containing compounds, Table 2 shows that there is an apparent relationship between alkylene length and whether or not the spontaneous network activity is increased or inhibited. With linker chains having two to five carbon atoms (compounds 1–4), no inhibitory action is found, but excitatory activity does occur with demonstrated reversibility. The one exception is compound I4 (5-C spacer), which shows neither excitatory nor inhibitory activity, and apparently is in a transitory position between excitatory and inhibitory behavior. When the choline-bearing bis-thiocarbonate has a spacer length of six carbon atoms (compound 5), all excitatory activity is lost at 350 μM and above, and irreversible inhibition of spontaneous network activity occurs. The reversible and irreversible cessation of network activity in response to three of the seven compounds could not be anticipated from the biochemical data. The fact that two of these compounds are irreversibly toxic is surprising and emphasizes the importance of tissue-based screening early in the drug development process.

For the better AChE inhibitors, the DMEA derivatives, Table 2 shows that compound I6 (4-C spacer) demonstrates both reversible excitatory and reversible inhibitory effects on neuronal networks. This contrasts with the activity of choline-containing compound I3 (4-C spacer), which has only reversible excitatory activity. The DMEA-containing compound 7 (6-C spacer) has only irreversible inhibitory effects, similar to that of the choline-containing compound 5 (6-C spacer). A logical conclusion from these data is that the effects of the inhibitory bis-thiocarbonates on the neuronal networks reflect varying combinations of their actions on AChE activity and their unexpected interaction with neuronal ionotropic receptor sites.

Acetylcholine in the frontal cortex is primarily a neuromodulator that has been implicated in arousal, attention, learning and memory. The effects of ACh are exerted pre- and post-synaptically and have been shown to regulate release of neurotransmitters and post-synaptic excitability (McGehee and Role, 1996). The therapeutic strategy of increasing cholinergic activity by inhibiting the enzymatic hydrolysis of ACh, thereby increasing the constitutive amount of neurotransmitter available, can have multiple outcomes depending upon the receptor subsequently activated. Increased ACh levels that result in an activation of certain presynaptic nicotinic receptors such as the $\alpha 7$ and $\alpha 4\beta 2$ subtypes could lead to increased release of ACh from the cell stimulated, especially in conditions of impaired cholinergic function as exists in AD (Marchi and Raiteri, 1996). On the other hand, increasing the activation of presynaptic m2 muscarinic receptors could result in lower levels of ACh release (Vizi et al., 1989; Rouse et al., 1997). Such antagonistic responses make it difficult to predict physiological effects from biochemical or even single cell data. However, a spontaneously active network reflects the interactions of all transmitter systems present. The use of such networks as an intermediate step between biochemical analyses and animal experiments holds the promise of greater research efficiency through a rapid recognition and quantification of physiological tissue responses.

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