TUBOCURARINE BINDING IN UNDERCUT CEREBRAL CORTEX AND THE EFFECT OF LONG-TERM ELECTRICAL STIMULATION

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INTRODUCTION

Several mechanisms have been proposed to explain prolonged epileptiform afterdischarge, elicited by various forms of stimulation in chronically isolated cerebral cortex: (1) loss of a muscarinic inhibitory mechanism[26], (2) decrease in the enzyme activity responsible for inactivating transmitter substance[21], (3) proliferation of axon collaterals[20], (4) deafferentation of inhibitory stellate cells[22], and (5) denervation (or 'disuse') supersensitivity to putative transmitters[13,24]. Which one or more of these is most implicated is still an open question.

The last mechanism was investigated in the present study because it is related to epilepsy and to the theory of plasticity and learning in the central nervous system. Although undercut cortex is a deafferentated and deefferentated preparation, the characteristic loss of dendritic spines in the surviving neurons is a consequence of deafferentation[19] and of 'disuse' of synapses[22]. This interpretation is supported by Valverde's light deprivation studies[25]. There are also instances of enhanced synaptic responses following deafferentation or disuse in the spinal cord[1,3].

To test the hypothesis of disuse supersensitivity, we have taken advantage of two facts. First, it is possible to isolate fractions rich in 'cholinergic' synaptic membranes by the subcellular fractionation technique of De Robertis *et al.*[7,12], in which the isolated nerve ending complexes often have postsynaptic membranes attached. Second, changes in acetylcholine (ACh) receptor density in 'cholinergic' postsynaptic membranes can be indirectly detected by binding with cholinergic blocking agents such as tubocurarine[8]. Using this approach we observed that the 'cholinergic' synaptic membranes of the undercut cortex bound more dimethyl-[^{14}C]-D-tubocurarine than the corresponding membranes of the contralateral cortex. Furthermore, long-term electrical stimulation tended to prevent the elevation in binding noted in the undercut cortex.

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METHODS

The methods for preparing chronic animals with undercut cortices, the deter-
mination of supersensitivity and the isolation of subcellular fractions are described in
the accompanying paper[6]. Tissue from the same animals (3 with undercut cortices, 5
with stimulated undercut cortices) was used for the binding experiments. Except for a
control experiment, only the membrane fraction 1.0 *M* which is rich in acetylcholin-
esterase (AChE) activity[7,23] was used. The cholinergic blocking agent was dimethyl-
[14C]-D-tubocurarine with a specific activity of 108 mCi/mmole (Amersham/Searle,
Des Plaines, Ill.). The radiochemical purity was determined by obtaining a single
radioactive spot upon thin-layer chromatography on silica gel in ethanol–10%
aqueous HCl (9 : 1, v/v).

The *in vitro* technique of Azcurra and De Robertis[2] was adopted for studying the
uptake of the radioactive cholinergic blocking agent by the 1.0 *M* membranes. Since
the measurements were routinely made in triplicate only 2 tissue level samples each
from the undercut and the contralateral cortex were simultaneously studied within a
single experiment. Twenty-five and 50 μl of the 1.0 *M* membrane suspension at a
protein concentration of about 1 mg/ml were incubated with a known amount of the
radioactive drug in 1 ml of Tris-buffer (0.05 *M*, pH 7.2) in 0.25 *M* sucrose in 11 ml
polyallomer (Spinco Co., Palo Alto, Calif.) centrifuge tubes. The final concentration
of dimethyl-[14C]-D-tubocurarine was $5 \cdot 10^{-7}$ *M*. After incubation at 37° C for 15 min,
the binding reaction was terminated by adding 10 ml of ice-cold 0.25 *M* sucrose. The
tubes were centrifuged at 125,000 $\times$ *g* for 50 min. Two additional sucrose washes and
centrifugations yielded a pellet which was suspended in 1 ml of distilled H_2O. The
radioactivity in the third sucrose wash was close to background. The suspension of the
final pellet was transferred to a scintillation vial to which was added 2 ml of Bio-Solv-
3 (Beckman Co., Fullerton, Calif.) and 10 ml of scintillation fluid (toluene, 1000 ml;
2,5-diphenyloxazole, 4 g; dimethyl-1,4-bis-(5-phenyloxazolyl-2)-benzene, 100 mg).
The radioactivity was determined with a Nuclear Chicago (Des Plaines, Ill.) Unilux II
liquid scintillation spectrometer. The efficiency of counting was 70–80%. Routinely
triplicate samples were counted. It was found that the 'cholinergic' receptors[10,11] lost
their binding capacity for dimethyl-[14C]-D-tubocurarine upon overnight storage at
4°C. All binding experiments were therefore carried out immediately after isolation of
the synaptic membrane preparations.

RESULTS

It was found that the AChE-rich 1.0 *M* membranes had the highest binding
capacity for dimethyl-[14C]-D-tubocurarine. In a control study of rat brain cortex,
binding of dimethyl-[14C]-D-tubocurarine by 1.0 *M*, 1.2 *M* and 1.4 *M* membranes
amounted to 7,458, 2,740 and 2,068 disint./min/mg protein respectively, under stan-
dard incubation conditions. In a comparable control experiment using the cerebral
cortex of a cat samples of 1.0 *M* and 1.2 *M* membranes bound dimethyl-[14C]-D-tubo-
curarine to the extent of 17,042 and 9,518 disint./min/mg protein. In the same experi-

ment, boiling of the membrane preparations for 5 min reduced the binding to 6,292 disint./min/mg protein in the 1.0 M fraction and to 4,682 disint./min/mg protein in the 1.2 M fraction. The relationship between binding and amount of tissue was close to linear within the range of protein values tested. Other experiments, using rat cerebral cortices, showed that chloralose anesthesia did not affect the binding of dimethyl-[14C]-D-tubocurarine by the 1.0 M synaptic membranes.

Since the above experiments showed that the AChE-rich synaptic membranes of the 1.0 M fraction[6,23] also had the highest binding capacity for dimethyl-[14C]-D-tubocurarine, this fraction was used throughout this study to compare the undercut and the contralateral, homotopic cortex. Table I shows the binding of dimethyl-[14C]-D-tubocurarine by the 1.0 M membranes of cat cortex. Cortex contralateral and homotopic to the undercut side had a binding capacity of 18,000 – 19,000 disint./min/mg protein which corresponds to about 10^{-7} mmole/mg protein, a value similar to that reported by De Robertis *et al.*[10]. Partial isolation of the feline cerebral cortex by undercutting increased the binding capacity of the 1.0 M membranes by 63% as compared with that of the contralateral cortex (Table I). Tissues treated by long-term electrical stimulation showed increased binding of only 26%, comparing stimulated cortex with contralateral cortex. Although these differences could not be statistically tested for significance, because the number of experiments was small, the trend seems clear. Expressing binding in terms of the specific activity of AChE[6] diminishes the difference between the undercut cortex and the contralateral cortex from 63% to 41% (Table I). The effects of long-term stimulation are also not as great, only a 10% difference as compared with the binding of the undercut group.

The two 'most supersensitive' undercut cortices[6] had a high binding capacity averaging 38,694 disint./min/mg protein for the undercut cortex and 18,239 disint./min/mg protein for the contralateral cortex. For two of the three 'least supersensitive'

TABLE I

BINDING OF DIMETHYL-[14C]-D-TUBOCURARINE TO THE SYNAPTIC MEMBRANES (FRACTION 1.0 M) OF CAT CEREBRAL CORTEX

UC = undercut cortex; SUC = stimulated undercut cortex; CC = contralateral cortex.

CC (3)*	UC (3)	% change	CC (5)	SUC (5)	% change
Bound dimethyl-[14C]-D-tubocurarine (disint./min/mg protein)					
18,144	29,678	+63	19,172	24,366	+26
±494**	±928		±368	±701	
Bound dimethyl-[14C]-D-tubocurarine (disint./min/AChE)***					
228.2	321.8	+41	234.6	306.2	+31
±38.7	±89.4		±22.1	±24.7	

* Number of experiments in parentheses.
** Standard error of the mean.
*** Δ O.D.$_{412}$/20 min/mg of protein[6].

stimulated undercut cortices the binding for both undercut and contralateral cortices was nearly equal. The third animal was equivocal.

DISCUSSION

The specific binding of dimethyl-[^{14}C]-D-tubocurarine is suggested by the high affinity of the drug for the 1.0 *M* fraction, by the linearity between binding and the protein concentration and by the drastic reduction of binding after boiling. In spite of these consistencies, the limitation of the *in vitro* technique should be considered. The osmotic shock used to rupture the cells and the repeated homogenizations are likely to change the accessibility of the subsynaptic receptors to the cholinergic blocker. Furthermore, the present technique did not allow us to detect changes in the sensitivity of non-synaptic neuronal membranes, such as that observed in skeletal muscle after denervation[18].

Micro-iontophoretic study has indicated that cholinoceptive cortical neurons possess muscarinic instead of nicotinic receptors[15]. In preliminary experiments, the binding of tritiated atropine to the 1.0 *M* fraction was found to be low and the undercut cortex did not bind in excess of 10 % more drug than control preparations. It is possible, however, that the binding of atropine *in vitro* was too weak to survive the isolation procedures.

It is interesting to note that protein of the synaptic membranes was more closely representative of the changes in tubocurarine binding than was the AChE activity of the membranes. Expressing the binding capacity in terms of the specific activity of AChE thus merely complements the expression of disint./min/mg protein without inferring any direct association of the drug with enzyme protein. Yet, since the proteins of the 1.0 *M* fraction are obviously derived from more than one type of synaptic membrane (even though, admittedly, those constituting the 'cholinergic' AChE-rich type predominate), it is possible that the enhanced binding capacity of the undercut cortex represents a non-specific increase in sensitivity of admixed non-cholinergic synaptic membranes to a cholinergic blocking agent. However, De Robertis *et al.* have recently described the extraction from AChE-rich synaptic membranes, of a special type of proteolipid implicated as the receptor substance for tuboburarine[10,11]. These workers further observed the relative independence of AChE and of the overall capacity of the synaptic membranes to bind tubocurarine[9].

The phenomenon of supersensitivity of the neuronally isolated cortex may be explained by an increased sensitivity of the subsynaptic and, possibly, non-synaptic membranes as a consequence of denervation. This is analogous to the supersensitivity observed in denervated muscle. The suggestion was made more than 30 years ago by Cannon[5], that abnormality of cortical cells in epilepsy might be the result of loss of innervation of cells — another example of increased sensitivity in denervated structures. A reasonable explanation of the hyperexcitability, repetitive burst firing and of the increased spontaneous firing of neurons near an epileptic irritative focus, indeed seems to be through denervation of the affected cells. This and similar causative mechanism were recently reviewed by Ward[27].

It certainly seems unlikely that epileptiform activity of traumatized and denervated mature cortex can be explained by proliferation of axon collaterals[22] or directly as a result of a decrease in transmitter inactivating enzyme. Loss of an inhibitory mechanism involving stellate cells[22] as a single responsible mechanism seems less likely than a denervation-disuse hypothesis, to explain epileptiform signs of supersensitivity. Pertinent to this point are the recent important findings of Krnjević et al.[17] showing that normal inhibitory mechanisms are still present in chronically isolated cortical slabs. Marked changes in levels of several amino acids following undercutting of the cortex have been noted[4] but since these were restored with time, the supersensitivity of chronic isolation cannot be associated with such changes. Although the hyperexcitability of neurons in chronic undercut cortex may be explained by increased sensitivity of postsynaptic receptor or receptors, other changes are most certainly associated. It was recently suggested by Green et al.[14] that since the Na^+–K^+-dependent ATPase activity was low in the isolated cortex, an impaired K^+ uptake could likely result with consequent increase in neuronal excitability. Finally, the recent micro-iontophoretic study by Krnjević et al.[16] in part indicated that there were fewer ACh sensitive neurons in isolated cortex and their responses were atypical.

However, our present data do suggest that in a cortical cholinergic system deafferentation and 'disuse' of synapses increases the sensitivity of subsynaptic membranes to putative transmitters. Conversely, long-term electrical stimulation and synaptic 'use' reduces this 'disuse'-elicited increase in sensitivity. This research thus adduces additional evidence to support the contention that electrical stimulation exerts its effect via synaptic sites.

SUMMARY

(1) An *in vitro* technique was used to study the binding capacity of the acetylcholinesterase (AChE)-rich synaptic membrane fraction of cat cerebral cortex for dimethyl-$[^{14}C]$-D-tubocurarine. Observations were made on the effect of undercutting and long-term electrical stimulation of the undercut cortex upon this binding.

(2) Undercutting increased the binding of dimethyl-$[^{14}C]$-D-tubocurarine by 63% as compared with the contralateral control cortex. In parallel studies long-term electrical stimulation of undercut cortex appreciably reduced this expected increase.

(3) The binding of dimethyl-$[^{14}C]$-D-tubocurarine to the membranes was more directly related to membrane protein levels than to the AChE activity associated with them.

(4) Supersensitivity of undercut cerebral cortex is likely the result of deafferentation resulting in synaptic disuse and in a concomitantly increasing sensitivity of a postsynaptic receptor or receptors.

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