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13. Only neurons that had membrane potentials that were more hyperpolarized than −50 mV and were stable for more than 30 minutes (up to 6 hours) were used. We employed two slices including the middle portion of the Med-AMG from each rat. The responsiveness to the drugs was approximately the same in each slice. To ensure that the results from each neuron were independent, duplicate data obtained from the same rat were eliminated.
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Technical Comments

GABA Receptor–Mediated Chloride Transport in a "Cell-Free" Membrane Preparation from Brain

Harris and Allan (*1*) present evidence that they say demonstrates functional coupling of γ-aminobutyric acid (GABA) receptors to chloride (Cl^-) channels in isolated "cell-free" membranes prepared from brain. However, characterization (*2*) of the "cell-free" membrane preparation used by Harris and Allan (*1*) [initially described by Chasin *et al.* (*3*) and modified by Daly *et al.* (*4*)] has revealed the presence of many intact cells, including neurons. Thus, it is incorrect to conclude that the technique they describe (*1*) measures GABA-barbiturate receptor-effector coupling in "cell-free" brain membranes.

Table 1. Distribution of visible elements in filtered and unfiltered preparations from guinea pig cerebral cortex. The unfiltered particulate material was prepared as described by Harris and Allan (*1*) according to the methods of Chasin *et al.* (*3*) and Daly *et al.* (*4*) from guinea pig cerebral cortex. The filtered particulate material was prepared according to Hollingsworth *et al.* (*2*) by filtering a suspension of unfiltered elements through 100-mesh nylon and then through a 10-μm filter (Millipore). A suspension of both preparations [protein (~1.0 mg/ml) in Krebs-Ringer bicarbonate-glucose buffer] was examined by light microscopy (magnification ×6900) with a reflected-light differential-contrast photomicroscope (Zeiss II) and a video image recorder (*5*). Visible elements were identified as described (*2*) and quantified by counting elements in 100 frames.

Element	Diameter (μm)	Unfiltered	Filtered
Vesicles			
Small	0.5–2.5	671	460
Large	5–30	131	10
Erythrocytes	6–7	18	7
Nonneuronal cells	11–13	44	8
Neuronal cells	(15 × 22)	22	0
Unidentified debris		24	6

Using a modification of the reflected-light differential-interference contrast system (*5*) with a video image recorder (*2*), we have examined and quantified the visible elements present in the crude membranes prepared according to the method used by Harris and Allan (*1, 4*). While both conventional light and electron microscopy are insufficient for visualizing whole cells, reflected-light differential-interference microscopy reveals that this "membrane" preparation contains, in addition to vesicles of various diameters, intact neurons, glia, erythrocytes, unidentified whole cells, and large clumps of unidentifiable "debris" (Table 1). Recently, Hollingsworth *et al.* (*2*) have shown that this crude membrane preparation can be purified by filtration through nylon mesh and a 10-μm filter to yield a relatively "cell-free" preparation of pre- and postsynaptic vesicles. The resulting preparation has been shown to be enriched in "snowman-shaped" entities called "synaptoneurosomes" and to be devoid of intact neurons (Table 1) (*2*). We have reported barbiturate-, muscimol-, and picrotoxin-sensitive $^{36}Cl^-$ flux in this filtered synaptoneurosome preparation (*6*). Figure 1 demonstrates the stimulation of $^{36}Cl^-$ uptake and efflux induced by the GABA receptor agonist muscimol in filtered synaptoneurosomes.

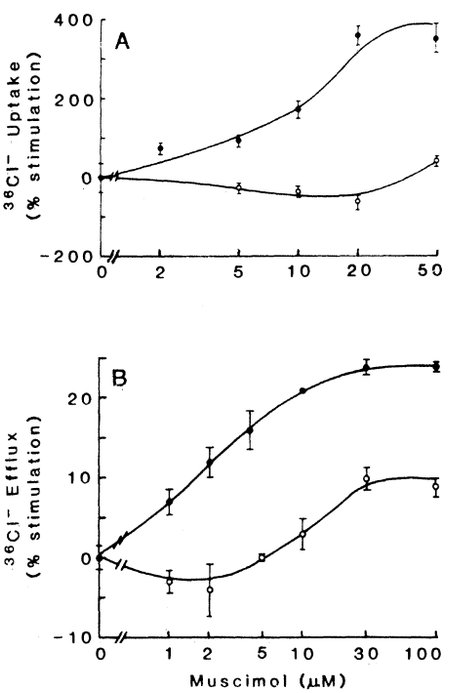

Fig. 1. Muscimol-induced stimulation of $^{36}Cl^-$ uptake (A) and efflux (B) in cerebral cortical synaptoneurosomes. For uptake, synaptoneurosomes prepared from rat cerebral cortex (*2*) were incubated for 5 seconds (30°C) with $^{36}Cl^-$ and various concentrations of muscimol (*6*). The GABA antagonist bicuculline methiodide (100 μ*M*) was added 3 minutes prior to the $^{36}Cl^-$. The reaction was terminated by dilution with ice-cold buffer containing 20 m*M* Hepes/9 m*M* Tris, 118 m*M* NaCl, 4.7 m*M* KCl, 1.18 m*M* $MgSO_4$, and 2.5 m*M* $CaCl_2$ (*p*H 7.4) and by rapid filtration through GF/C Whatman filters. $^{36}Cl^-$ efflux was determined 2 minutes after dilution of preloaded synaptoneurosomes as previously described (*6*). The EC_{50} for muscimol-stimulated $^{36}Cl^-$ uptake and efflux is approximately 3 to 8 μ*M*. Data are the means ± SEM of quadruplicate determination and representative of at least three such experiments.

somes, and the antagonism of both by bicuculline. Thus, even in the absence of intact neurons, $^{36}Cl^-$ flux that is mediated by receptors for GABA and barbiturates can be measured in vitro (*6*). Our results (*6*) are also in good agreement with those obtained from primary cultures of intact embryonic chick neurons (*7*). We would caution, however, that although GABA receptor–mediated Cl^- conductance is believed to be confined to neurons (*8*), even the "filtered" synaptoneurosome preparation used in our experiments contains a few oligodendrocytes and erythrocytes (Table 1), both of which possess specific disulfonylstilbene-sensitive mechanisms for anion transport (*9*). Contrary to the statement made by Harris and Allan (*1*, reference 3), the synaptoneurosome preparation should prove suitable for biochemical and pharmacological studies of GABA receptor–effector function.

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Response: Paul *et al.* raise two points. First, they suggest that the γ-aminobutyric acid (GABA)–stimulated chloride flux reported by us (*1*) may be due to intact cells, rather than isolated membrane vesicles. Second, they assert that a slightly different preparation (synaptoneurosomes) is suitable, and in fact superior to our preparation, for the study of GABA-operated chloride channels.

In support of the first point, Paul *et al.* counted the types of particles in preparations made by their method (*2*) and by the method of Daly *et al.* (*3*). The latter preparation was similar to that used in our study. When one calculates the data of Paul *et al.* as percentages, their preparation contained 1.4% erythrocytes, and the Daly preparation contained 2%. For nonneuronal cells the figures are 1.6% and 4.8%, respectively, and for neuronal cells, 0 and 2.4%, respectively. Thus, the measurements of Paul *et al.* indicate that both preparations contained a small percentage of intact cells, but the Daly preparation contained more than the synaptoneurosome preparation. However, the Daly preparation used is not identical to that used in our study (*1*). Paul *et al.* do not present their methodology, but they do mention that they used guinea pig cortex. We used mouse brain in our studies. Daly *et al.* (*3*) used a different type of homogenization and fewer washes than we did (*1*). To determine the importance of these differences, we examined our preparation by light and electron microscopy. We had great difficulty finding any intact cells in our preparation, and all intact cells together constituted less than 1% of the vesicles (*4*). In addition, we fractionated brain membranes by sucrose and Ficoll density gradient centrifugation (*5*) and found the highest activity of GABA-stimulated chloride flux in the synaptosomal fraction (*4*), a membrane population that clearly does not contain intact cells. Thus, we conclude that the GABA-activated chloride flux reported by us is not due to the presence of intact cells in our preparation.

The second issue raised by Paul *et al.* is whether or not the synaptoneurosome preparation is suitable for measurement of GABA-stimulated chloride flux. We stated (*1*, reference 3) that no one had shown GABA-stimulated chloride flux with a cell-free system and pointed out that Paul's group had shown barbiturates "to stimulate the uptake of $^{36}Cl^-$ by isolated membrane vesicles." Paul *et al.* provide evidence in their comment that a GABA agonist, muscimol, can also enhance chloride flux in their preparation. However, these data had not been published when our report appeared. In addition, the published report of Schwartz *et al.* (*2*) indicates that the GABA receptor antagonist bicuculline reduces flux of chloride across synaptoneurosomes in the absence of added GABA. This suggests that the preparation contains sufficient endogenous GABA to activate the receptor and channel without addition of exogenous agonists. If this is true, it will limit the usefulness of their preparation because it will not be possible to determine accurate concentration-response curves for GABA agonists or kinetics of activation and desensitization if there are significant but unknown amounts of GABA present in the assay mixture. We conclude that additional studies are required to establish the suitability of the synaptoneurosome preparation for the study of GABA-stimulated chloride flux.

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