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Oxidative Metabolism of 4-Aminobutyrate by Rat Brain Mitochondria: Inhibition by Branched-chain Fatty Acid

J. Cunningham, D. D. Clarke, and W. J. Nicklas*

Abstract: The oxidation of 4-aminobutyric acid (GABA) by nonsynaptosomal mitochondria isolated from rat forebrain and the inhibition of this metabolism by the branched-chain fatty acids 2-methyl-2-ethyl caproate (MEC) and 2,2-dimethyl valerate (DMV) were studied. The rate of GABA oxidation, as measured by $O_2$ uptake, was determined in medium containing either 5 or 100 mM-[K+]. The apparent $K_m$ for GABA was 1.16 ± 0.19 mM and the $V_{max}$ in state 3 was 23.8 ± 5.5 ng-atoms $O_2$·min$^{-1}$·mg protein$^{-1}$ in 5 mM-[K+]. In a medium with 100 mM-[K+] the apparent $K_m$ was 1.11 ± 0.17 mM and $V_{max}$ was 47.4 ± 5.7 ng-atoms $O_2$·min$^{-1}$·mg protein$^{-1}$. The $K_i$ for MEC was determined to be 0.58 ± 0.24 or 0.32 ± 0.08 mM, in 5 or 100 mM-[K+] medium, respectively. For DMV, the $K_i$ was 0.28 ± 0.05 or 0.34 ± 0.06 mM, in 5 or 100 mM-[K+] medium, respectively. The $O_2$ uptake of the mitochondria in the presence of GABA was coupled to the formation of glutamate and aspartate; the ratio of oxygen uptake to the rate of amino acid formation was close to the theoretical value of 3. Neither the [K+] nor any of the above inhibitors had any effect on this ratio. The metabolism of exogenous succinic semialdehyde (SSA) by these same mitochondria was also examined. The $V_{max}$ for utilization of oxygen in the presence of SSA was much greater than that found with exogenously added GABA, indicating that the capacity for GABA oxidation by these mitochondria is not limited by SSA dehydrogenase. In addition, the branched-chain fatty acids did not inhibit the metabolism of exogenously added SSA. Thus, the inhibitors examined apparently act by competitively inhibiting the GABA transaminase system of the mitochondria. Key words: 4-Aminobutyrate (GABA)—Brain mitochondria—2-Methyl-2-ethylcaproic acid—2,2-Dimethylvaleric acid—Succinic semialdehyde.

4-Aminobutyric acid (GABA) is a putative inhibitory neurotransmitter in the mammalian CNS. Its inhibitory activity is believed to be terminated by diffusion away from the synaptic cleft and subsequent active transport into surrounding structures where it is metabolized via GABA transaminase (GABA-T) [4-aminobutyrate:2-oxoglutarate aminotransferase, EC 2.6.1.19] (Roberts and Kuriyama, 1968). It has been proposed (Balazs et al., 1973) that the metabolism of GABA is compartmentalized in the brain; i.e., its synthesis occurs in the presynaptic terminal and its degradation occurs in the cells that take up GABA from the synapse.

Pharmacological manipulations that decrease GABA levels in the brain are often associated with seizure activity (Wood and Peesker, 1974). Conversely, increasing GABA levels by inhibiting its catabolism can protect against the onset of convulsions. Recently, a class of small, branched-chain fatty acids have been shown to inhibit GABA metabolism in vivo and in vitro and to have an

Abbreviations used: AOAA, Aminooxyacetic acid; DMV, 2,2-Dimethylvaleric acid; DPA, Dipropyl acetate (2-Propylpentanoic acid); GABA-T, GABA transaminase; MEC, 2-Methyl-2-ethylcaproic acid; PCA, Perchloric acid; SSA, Succinic semialdehyde; SSADH, Succinic-semialdehyde dehydrogenase.
tiepileptic properties (Simler et al., 1968, 1973; Lespagnol et al., 1972). Some controversy exists as to the mechanism by which these substances act (Harvey et al., 1975). In particular, it is debated whether GABA-T or succinic-semialdehyde dehydrogenase (SSADH) [succinic-semialdehyde:NAD(P)^+ oxido-reductase (EC 1.2.1.16)] is the enzyme inhibited.

This study focuses on the oxidative metabolism of GABA by non-synaptic-ending brain mitochondria (Clark and Nicklas, 1970; Walsh and Clark, 1976) and the effect thereon of two such inhibitors, 2-methyl-2-ethyl caproic acid (MEC) and 2,2-dimethyl valeric acid (DMV). These substances were found to be potent inhibitors of GABA oxidation and appeared to act solely on the transamination reaction.

METHODS

Preparation of Rat Brain Mitochondria

Rat brain mitochondria were prepared essentially according to the method of Clark and Nicklas (1970). The final mitochondrial pellet was suspended in ice-cold buffer consisting of 0.32 M sucrose, 0.1 mM EDTA, and 10 mM-Tris·HCl, pH 7.4, to give a final protein concentration of 15 mg/ml. Generally, the yield from eight rats was 18–22 mg mitochondrial protein as determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard. Mitochondria prepared in this manner are primarily of non-synaptic-ending origin (Clark and Nicklas, 1970; Walsh and Clark, 1976).

Incubation Conditions

To study the respiration and respiratory control of a given mitochondrial preparation, an aliquot of the suspension described above (0.5–0.75 mg protein) was pipetted into a final volume of 1 ml incubation medium at either 28°C or 33°C in a chamber with a 2-ml volume. The basic incubation medium consisted of the following: 5 mM-K^+ medium—225 mM-mannitol, 75 mM-sucrose, 5 mM-phosphate, 0.05 mM-EDTA, 5 mM-KCl, adjusted to pH 7.4; 100 mM-K^+ medium—75 mM-mannitol, 27 mM-sucrose, 5 mM-phosphate, 0.05 mM-EDTA, 100 mM-KCl, adjusted to pH 7.4 with 1 M-Tris. Then, 0.05 mM-pyruvate and 2.5 mM-malate were added and state 3 oxidation was initiated by addition of 0.5 mM-ADP. Oxygen uptake was followed utilizing a Clark-type electrode (Yellow Springs Instrument Co.). The oxygen uptake in ng-atoms of oxygen·min^{-1}·mg protein^{-1} under the above conditions was similar to that reported in earlier studies (Clark and Nicklas, 1970) and the respiratory control (Chance and Williams, 1956) for 5 mM or 100 mM-K^+ media was 9–12 or 8–11, respectively, at 28°C, and 4–5.5 and 3.5–5, respectively, at 33°C. Greater respiratory control ratios were obtained at the elevated temperature if 1 mg/ml bovine serum albumin was added but, since this interfered with the later, inhibitory studies, no albumin was used in these experiments.

To study oxygen consumption with GABA as substrate, the protein concentration in the incubation solution was doubled. All studies were carried out at 33°C. As described in Results, a small amount of 2-oxoglutarate was necessary to optimize initial rates of oxygen utilization. Arsenite was added to inhibit direct oxidation of the added 2-oxoglutarate. For determination of the K_m of GABA oxidation, the media used consisted of the basic 5 mM- or 100 mM-K^+ media described above plus 0.25 mM-malate, 0.05 mM-2-oxoglutarate, 1 mM-ADP, and 0.5 mM-arsenite. The concentration of GABA was varied from 0.025 mM to 5 mM, the GABA being added last.

Inhibitory studies were carried out in the same mitochondrial incubation media as those described above. The concentration of GABA was kept constant at either 2.5 mM or 10.0 mM and the inhibitor, either MEC or DMV, was varied in concentration from 0 to 2.5 mM. The inhibitor was added to the mitochondrial preparation just prior to the addition of GABA.

In some cases, samples of the incubation mixtures were assayed for glutamate and aspartate content. After monitoring the O_2 uptake for 3 min after addition of GABA, both in the presence and absence of inhibitors, 1 ml of the total mixture was mixed with 0.04 ml 70% perchloric acid (PCA). Glutamate and aspartate in the PCA extract were determined either by enzymatic fluorometric procedures (Williamson and Corkey, 1969) or with a Technicon Autoanalyzer (Nicklas and Browning, 1978). Comparable results were obtained with either method.

Kinetic Parameters

The kinetic parameters of GABA catabolism were determined by regression analysis of an S/V versus S plot using the internal program of the Hewlett Packard 9805A Statistic Calculator. The S/V versus S plot was chosen because of its more even weighing of the points throughout the substrate concentration range (Wilkinson, 1961). The kinetics in the presence of the inhibitors were evaluated using regression analyses on Dixon plots (Dixon, 1953).

Chemicals

All reagent chemicals were of the highest purity commercially available. Ficoll, malate, aminooxyacetic acid, and GABA were obtained from Sigma Chemical Co., St. Louis, Missouri. Pyridine, 2-oxoglutarate, and adenine nucleotides, as well as the enzymes used in the fluorometric assays, were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Indiana. Pyruvic acid (Calbiochem) was redistilled under vacuum and stored at 2–5°C. Sodium arsenite was a product of Fisher Scientific Company, Fair Lawn, New Jersey. 2-Methyl-2-ethyl caproic and 2,2-dimethyl valeric acids were from ICN Pharmaceuticals Inc., Plainview, New York. Their purity was checked by nuclear magnetic resonance spectrometry. Working solutions of these acids were neutralized with Tris (pH 7.0) prior to addition to incubation mixtures.

RESULTS

Oxidation of GABA by Brain Mitochondria

Addition of GABA to mitochondrial preparations caused an increase in state-3 oxygen uptake which
was measurable, but small, in comparison to pyruvate or succinate oxidation. The procedure finally adopted is illustrated in Fig. 1 and gives reproducible GABA oxidation rates for kinetic studies. Similar to findings of Walsh and Clark (1976) in their studies of GABA transamination in intact mitochondria, it was necessary to add 2-oxoglutarate to the medium for optimal GABA utilization. The apparent \( K_m \) for 2-oxoglutarate for this effect was approximately 10 \( \mu M \); 50 \( \mu M \)-2-oxoglutarate was added in the kinetic studies to give maximal activation. This concentration of 2-oxoglutarate is one-fiftieth that used by Walsh and Clark (1976) and is much less than the concentration that has been shown to cause substrate inhibition of GABA-T (Maitre et al., 1975).

![Diagram](image)

**FIG. 1.** Oxygen uptake by rat brain mitochondria with GABA as substrate. Mitochondria (1.8 mg protein) were placed in 1 ml of incubation medium at 33°C. (A) GABA oxidation in the absence of inhibitor and (B) GABA oxidation in the presence of methyl ethyl caproate (MEC). Initially, the medium contained 5 mM-[K+] incubation medium (see Methods), 0.5 mM-sodium arsenite, 50 \( \mu M \) 2-oxoglutarate, and 0.25 mM-malate. Adenosine diphosphate (ADP), GABA, aminooxyacetate (AOAA), and succinate were added where indicated by the arrows. Oxygen consumption is in the downward direction. The numbers above each line segment represent the oxygen utilization in ng-atoms \( \cdot \) min \(^{-1} \cdot \) mg protein \(^{-1} \).

It was also necessary to add malate (0.25 mM) to the system, probably to maximize 2-oxoglutarate transport into the mitochondria. Aminoxyacetic acid at 1 mM added to the system resulted in a 95% inhibition of the respiration rate under these conditions. When 10 mM-succinate was added, an immediate increase in oxidation to 160–180 ng-atoms oxygen \( \cdot \) min \(^{-1} \cdot \) mg protein \(^{-1} \) resulted, comparable to succinate oxidation found in intact, uninhibited mitochondria. Increasing the [K+] (Table 1) in the medium from 5 to 100 mM altered the kinetics of GABA oxidation. The \( K_m \) for GABA metabolism as measured by \( O_2 \) uptake was similar in both incubation media, but there was a doubling in \( V_{max} \) for GABA oxidation in going from 5 to 100 mM K+ medium (Table 1).

**TABLE 1. Kinetic parameters \( (K_m, V_{max}, K_i) \) for GABA oxidation and its inhibition by DMV and MEC in rat brain mitochondria**

<table>
<thead>
<tr>
<th>Parameter, species</th>
<th>Incubation Medium</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mM-[K+]</td>
<td>100 mM-[K+]</td>
<td></td>
</tr>
<tr>
<td>( K_m ), GABA (mM)</td>
<td>1.16 ± 0.19</td>
<td>1.11 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>( V_{max} ), GABA (ng-atoms ( O_2 )/min/mg protein)</td>
<td>23.8 ± 5.5</td>
<td>47.4 ± 5.7</td>
<td></td>
</tr>
<tr>
<td>( K_m ), 2-oxoglutarate (( \mu M ))</td>
<td>8.6 ± 0.5</td>
<td>0.13 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>( K_m ), succinic semialdehyde (mM)</td>
<td>0.13 ± 0.02</td>
<td>0.10 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>( V_{max} ), succinic semialdehyde (ng-atoms ( O_2 )/min/mg protein)</td>
<td>52.9 ± 6.6</td>
<td>66.7 ± 5.5</td>
<td></td>
</tr>
<tr>
<td>( K_i ), MEC (mM)</td>
<td>0.58 ± 0.24</td>
<td>0.32 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>( K_i ), DMV (mM)</td>
<td>0.28 ± 0.05</td>
<td>0.34 ± 0.06</td>
<td></td>
</tr>
</tbody>
</table>

\( K_m \) and \( V_{max} \) were obtained by extrapolation of \( S/V \) vs. \( S \) plots. \( K_i \)'s for inhibitors of GABA oxidation were determined by extrapolation of Dixon plots for two concentrations of GABA, 2.5 mM and 100 mM.

All parameters are mean values from three experiments ± S.D.
The oxidation of GABA requires the enzymes GABA-T and SSADH. To determine whether the activity of the mitochondrial SSADH was sufficient for oxidation of SSA produced by the GABA-T under these conditions, the rate of oxidation for exogenous succinic semialdehyde (SSA) was determined. The $K_m$ for exogenous SSA oxidation was found to be an order of magnitude less than that for GABA (Table 1). The $V_{\text{max}}$ for exogenous SSA oxidation was 100% greater than that of GABA in 5 mM K+ incubation medium and 50% greater in 100 mM K+ medium. The oxidation of SSA was completely inhibited by rotenone (0.2 mM).

**Effect of MEC and DMV on GABA and SSA Oxidation**

Both MEC and DMV inhibited GABA-supported respiration by the brain mitochondria (Tables 1, 2, Fig. 2). Dixon plots indicated that the inhibition, in both cases, was competitive with exogenous GABA (illustrated for inhibition by DMV in Fig. 2). There was no substantial difference in the inhibitory kinetics between 5- and 100-mM [K+] medium (Table 1, Fig. 2). Similar kinetic changes were obtained when the inhibitor was added before or after the GABA. In the results reported here, the inhibitors were added before GABA. The $K_m$ of SSA as well as the $V_{\text{max}}$ of its oxidation were unaffected by concentrations of DMV and MEC up to 1.0 mM.

**Comparison of Oxygen Utilization with Production of Glutamate and Aspartate**

A comparison was made of oxygen utilization to the amount of glutamate plus aspartate produced during GABA oxidation (Table 2). The ratio of oxygen utilization (ng-atoms O$_2$·min$^{-1}$·mg protein$^{-1}$) to amino nitrogen transfer (nmol [glutamate + aspartate]·min$^{-1}$·mg protein$^{-1}$) from GABA to glutamate and then via further transamination to aspartate was $3.35 \pm 0.34$. This value was slightly greater than the theoretical value of 3 for flow of carbon from GABA to oxaloacetate via GABA-T, SSADH, and the tricarboxylate cycle. Alteration in the [K+] had no effect on this ratio. The rate of glutamate plus aspartate production during GABA oxidation was inhibited by MEC and DMV and the percentage inhibition was similar to the percentage inhibition of O$_2$ uptake. MEC or DMV (0.25 mM) inhibited O$_2$ uptake and amino acid production by about 50% in both 5 mM- and 100 mM-K+ media.

**DISCUSSION**

The metabolism of GABA by intact brain mitochondria is subject to regulation at several levels. The first is at the point at which it is transported across the mitochondrial inner membrane. Previous studies (Brand and Chappel, 1974; Walsh and Clark, 1976) have shown this transport to occur by passive diffusion. Therefore, the rate of GABA oxidation must be regulated at the level of GABA catabolism, i.e., GABA-T, SSADH, and the enzymes of the tricarboxylate cycle. As has been discussed by Walsh and Clark (1976), studies of GABA metabolism in mitochondrial preparations have been confusing, especially with regard to the extent to which GABA can serve as a substrate for mitochondrial respiration. The above investigators showed various kinetic and regulatory differences in GABA metabolism in "synaptic" versus "free" brain mitochondria. Several of the conclusions of that study are corroborated by the data presented here. However, there are several points of divergence worth noting.

Whereas Walsh and Clark (1976) used measurements of glutamate production as a measure of GABA oxidation, we have used both oxygen utilization and the rate of formation of the sum of

**TABLE 2. GABA metabolism by rat brain mitochondria: Comparison of oxygen utilization vs. glutamate—aspartate production**

<table>
<thead>
<tr>
<th>[K+]</th>
<th>O$_2$ uptake</th>
<th>(Glu + Asp)</th>
<th>% GABA oxidation, O$_2$ uptake</th>
<th>% GABA oxidation (Glu + Asp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 mM GABA</td>
<td>23.1 ± 2.8</td>
<td>7.1 ± 0.8</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2.5 mM-DMV</td>
<td>46.4 ± 3.6</td>
<td>13.4 ± 1.6</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2.5 mM-GABA + 0.25 mM-MEC</td>
<td>11.7 ± 2.0</td>
<td>3.2 ± 0.1</td>
<td>50 ± 2</td>
<td>45 ± 4</td>
</tr>
<tr>
<td>2.5 mM-DMV + 0.25 mM-MEC</td>
<td>17.1 ± 1.9</td>
<td>5.6 ± 0.3</td>
<td>48 ± 4</td>
<td>42 ± 3</td>
</tr>
</tbody>
</table>

Mitochondria were incubated in either 5 mM- (A) or 100 mM-[K+] (B) medium with GABA or GABA + inhibitor. The oxygen uptake was measured for 3 min, at which point the reaction was terminated with PCA. The results are averaged from three different preparations ± s.d. O$_2$ uptake is measured in ng-atoms·min$^{-1}$·mg protein$^{-1}$.

(Glu + Asp) is the increase in glutamate and aspartate over the concentration of these amino acids in the absence of GABA. The units of this quantity are nmole·min$^{-1}$·mg protein$^{-1}$.  

glutamate plus aspartate. Since varying the rate of oxygen utilization (either by using inhibitors or by changing \([K^+]\)) did not alter the ratio of oxygen uptake to formation of dicarboxylic amino acids, measurement of either parameter is apparently a valid indicator of oxidative GABA metabolism. It is interesting to note that there was a doubling in state-3 GABA oxidation in going from 5 to 100 mM-[\(K^+\)]; no such increase was seen by Walsh and Clark (1976) in their studies. Stimulation of brain mitochondrial respiration by \(K^+\) has been reported by several authors (see Nicklas et al., 1971). In the present studies, the mitochondrial transaminases were coupled; i.e., amino groups from GABA were transferred to 2-oxoglutarate via GABA-T and then on to oxaloacetate via aspartate aminotransferase. Perhaps, the intactness of this system allowed the \(K^+\)-enhanced oxidation and transamination to occur.

Walsh and Clark (1976) suggested that mitochondrial aspartate aminotransferase was inhibited by the relatively high concentration of 2-oxoglutarate (2.5 mM) used in their experiments. Others (Maitre et al., 1975; van der Laan et al., 1979) have shown that 2-oxoglutarate can exert a substrate inhibition on GABA-T activity in vitro, with an estimated \(K_i\) of about 3 mM. The coupling of GABA oxidation and concomitant "irreversible" formation of dicarboxylic amino acids is consistent with proposed models of brain amino acid metabolism (van den Berg and Garfinkel, 1971). The maximal flux of the "GABA shunt" in these experiments was 8–11% of the pyruvate utilization by these mitochondria, which is also in agreement with calculations on the in vivo and in vitro GABA flux relative to total tricarboxylate cycle flux in brain (Balazs et al., 1973).

Dysfunctions in GABAergic systems have been postulated to play a role in several disorders, e.g., epilepsy (van Gelder et al., 1972), Huntington's chorea (Perry et al., 1973; Bird and Iversen, 1974; Enna et al., 1976), meningitis (Bukakova et al., 1975), cerebrovascular disease (Achar et al., 1976), and psychiatric illness (Roberts, 1972, 1976; Langer et al., 1975). Experimentally, elevating brain GABA levels has been shown to be especially useful in protecting against convulsions (Simler et al., 1968). No method has yet been devised to increase GABA levels by stimulating synthesis per se. However, inhibiting GABA catabolism has been successful. Specifically, several branched-chain fatty acids have been shown to protect against seizures (Simler et al., 1968, 1973; Lespangnol et al., 1972). One of these, sodium \(n\)-dipropyl acetate (DPA) has been introduced as a clinical anticonvulsant (Jeavons and Clarke, 1974). This substance produces a rise in total brain GABA, which has been suggested to occur by inhibition of either GABA-T (Simler et al., 1973) or, more likely, SSADH (Harvey et al., 1975; van der Laan et al., 1979). In preliminary experiments with brain mitochondria, DPA had no appreciable effect on GABA catabolism at levels similar to those used for MEC and DMV; concentrations of DPA that inhibit GABA-T (\(K_i = 18 \text{ mM}\), Maitre et al., 1974) uncoupled brain mitochondrial respiration (Dziedzic and Nicklas, unpublished observations). However, the two analogues of DPA used in the present studies were found to be
potent competitive inhibitors of GABA oxidation in intact mitochondria. Maitre et al. (1974) found that these substances, which protect against seizures induced audiogenically (Maitre et al., 1974) or by electric shock, pentetrazol, or strychnine administration (Lespagne et al., 1972), were more potent GABA-T inhibitors than was DPA.

The results of these experiments indicate that the study of the oxidation of GABA by an intact mitochondrial preparation is a useful means of assessing various parameters of GABA metabolism. As such it can bridge the gap between studies *in vivo*, in which results are often difficult to interpret, and those with enzyme preparations, whose applicability to the intact state is often questioned.

**ACKNOWLEDGMENT**

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**REFERENCES**


Roberts E. (1972) An hypothesis suggesting that there is a defect in the GABA system in schizophrenia. *Neurosci. Res. Program Bull.* 10, 468–482.


