Energy Requirements of Glutamatergic Pathways in Rabbit Retina

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***In vitro* rabbit retina was used as an example of CNS tissue in experiments designed to measure the energy requirements associated with the activation of different types of glutamate receptors. Retinas were exposed to glutamate and to four analogs: kainate, 2-amino-4-phosphonobutyric acid (APB), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 2-amino-5-phosphonovaleric acid (APV). The changes in O₂ consumption and lactate production were determined using a recently developed experimental system that permitted simultaneous measurements of the rates at which O₂ was removed from the medium and acid was added.**

The glutamatergic agents had relatively little effect on oxidative metabolism, but they caused large changes in glycolysis. Kainate increased retinal lactate production by 50%, whereas APB, CNQX, and APV reduced it by 23%, 19%, and 35%, respectively. Glutamate increased lactate production by 16% when administered after APB, but decreased it by 12% when administered after CNQX. The changes in energy metabolism coincided with changes in electrophysiological function.

Since the energy metabolism of many retinal cells was presumably not much affected by the glutamatergic agents, the changes measured as a percent of total retinal glycolysis must have reflected considerably larger fractional changes in the cells most affected. From the response to inhibitors, it seems probable that even under resting conditions in darkness, activity in glutamatergic pathways is responsible for more than 50% of the glycolytically derived energy used by the cells involved. It also seems probable (particularly from the response to kainate) that under some circumstances the cells' energy metabolism and/or transport capability cannot meet the requirements imposed by glutamate-induced increases in function. This may account for some aspects of glutamate toxicity.

Decreases in ion gradients, and the active transport needed to restore them, are essential accompaniments of neurotransmission. Under some circumstances, the increases in conductance that occur in response to excitatory transmitters may challenge the cells' capacity to maintain homeostasis—either because the transport requirements exceed the $V_{\max}$ of the NaK ATPase (for instance) or because they exceed the available energy. The pres-

ent study was undertaken to measure the changes in energy requirements associated with the activation of different types of glutamate receptors.

Though it is generally assumed that activation of glutamate receptors increases energy requirements, there is little direct evidence to support this. Several studies have indicated that glutamate can alter the balance between energy supply and demand. Novelli et al. (1988) reported that glutamatergic agonists increased the susceptibility of neurons to ischemic damage. Jacquin et al. (1989) and Ben-Yoseph et al. (1990) found that NMDA caused a reduction in intracellular energy supplies (e.g., phosphocreatine) as measured by nuclear magnetic resonance spectroscopy. However, we are aware of only two studies that provide direct evidence for a change in energy metabolism in response to glutamatergic agents. Nishizaki et al. (1988) reported an increase in the O₂ consumption of hippocampal slices in response to glutamate, kainate, and NMDA. Raley-Susman et al. (1992) found that kainate increased the energy metabolism of cultured hippocampal cells, as assessed by an increase in acid production.

Measuring the rate at which cells use energy is experimentally difficult. The techniques presently available do not provide measurements at the cellular level, and data on the effect of glutamate receptor activation obtained at the tissue level must reflect not only the change in cells with glutamate receptors but also the change in cells downstream from them (and the lack of change in the unresponsive cell types). In spite of these difficulties, the problem seemed sufficiently important to warrant further study with currently available methodology.

The experiments were performed on isolated rabbit retina. Retina incorporates most of the glutamate receptors so far identified (Miller and Slaughter, 1986) in relatively simple circuits; it is thin enough to permit the introduction of test agents at predetermined concentrations, and it can be maintained *in vitro* under conditions that have been shown (Ames and Nesbett, 1981) to preserve quite normal metabolic and electrophysiological function. Measurements of energy metabolism and electrophysiological function were obtained under control conditions; glutamate or a glutamate analog was introduced into the medium, and the measurements were repeated. The test agents were usually introduced alone in darkness, but in some experiments they were introduced in steady or flashing light, or after the retina had already been exposed to another glutamatergic agent.

Energy metabolism was assessed from the rate of O₂ consumption and acid production (from which glycolysis was calculated). These were measured simultaneously using an apparatus with which it was possible to restrict, temporarily, the superfusing medium to a small volume having no contact with the gas phase and to measure the ensuing fall in O₂ and pH.

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Electrical measurements, used to demonstrate electrophysiological responses to the agents being tested, were recorded with extracellular electrodes so that they could be obtained under the same conditions as the metabolic measurements.

We examined the effects of three glutamatergic agonists [2-amino-4-phosphonobutyric acid (APB), kainate, and glutamate] and two antagonists [6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 2-amino-5-phosphonovaleric acid (APV)]. Previously published studies of the electrophysiological effects of these agents in retina provided a basis for predicting their effects on energy metabolism. Thus, as indicated in Figure 1, (1) APB was expected to reduce energy requirements because of its inhibitory effects on ON-bipolar cells (and consequently on the neurons downstream from them), and its effects were expected to be greater in light when less glutamate was being released from the photoreceptors. (2) Kainate was expected to increase energy metabolism, particularly because of its excitatory effects on amacrine and ganglion cells. (3) CNQX was expected to reduce energy requirements by acting as an antagonist, particularly at amacrine and ganglion cell receptors; its effects would be less if it were administered after APB had already reduced the release of endogenous transmitter from ON-bipolar cells, and greater if prior administration of kainate had increased conductance at kainate/CNQX receptors. (4) APV was expected to reduce energy metabolism as an antagonist of NMDA receptors on amacrine and ganglion cells. (It also acts as an inhibitory agonist, like APB, on ON-bipolar cells.) (5) Glutamate would be expected to increase metabolism at some sites and to decrease it at others; its inhibitory effects would be more evident if it were administered after CNQX, and its excitatory effects would be evident if it were administered after APB. All agents were expected to reduce components of the light-evoked response. In general, the results were consistent with these predictions.

Materials and Methods

Tissue preparation. The experiments were performed in a darkroom maintained at 37°C. New Zealand White rabbits weighing about 3 kg were dark adapted for 1 hr, sedated with sodium pentobarbital (15 mg/kg), and brought to a surgical anesthetic level with ether by mask. The eyes were removed and the retinas were isolated under dim red light. The animals were killed while still anesthetized. The retinas were maintained in darkness in a medium that simulated cerebrospinal fluid with respect to electrolytes and 38 organic constituents (Ames Medium; Sigma, St. Louis, MO). A detailed account of the procedures used to isolate the retinas and of the composition of the medium has been presented previously (Ames and Nesbett, 1981), together with evidence that the retinas can be maintained for many hours *in vitro* with their metabolism and electrophysiologic function at near *in vivo* levels.

Metabolic measurements. The retina was mounted with its vitreous surface down on a hemispherical mandrel that was enclosed in a hemispherical chamber so that the tissue was uniformly superfused by a 1-mm-deep layer of medium with no gas phase present. The medium was moved over the retina at 4.6 ml min⁻¹ by a peristaltic pump that moved the medium around a circular system as follows: over the retina; through the pump; through a chamber in which it was equilibrated with the gas phase of 5% CO₂, 40% O₂ and 55% N₂; past an O₂ electrode (Clark) and a pH electrode; and then over the retina again; and so on. The outputs of the O₂ and pH electrodes were conventionally amplified and displayed with a high-speed ink recorder (Gould). To make a measurement of O₂ consumption and acid production, the gas-equilibrating chamber was temporarily bypassed so that the O₂ consumed by the retina could not be replaced and the CO₂ and H₂CO₃ generated could not be removed. In this mode, the volume of recirculating fluid was only 2.3 ml, so that the O₂ consumption of an average retina reduced the O₂ content of the medium by about 12% in the 1 min period over which the measurement was made—an accurately measurable change;

Figure 1. A generic view of the main cell types in retina to show the principal sites of action of the five glutamatergic agents tested, as reported in previously published studies (cf. review by Massey, 1990). The diagram is purposely ambiguous with respect to the type of photoreceptor depicted and with respect to the cell types that are postsynaptic to the horizontal and bipolar cells. Rabbit retina contains predominantly rod photoreceptors and has a large proportion of rod bipolar cells that presumably are ON-bipolars and synapse on amacrine cells. The diagram obscures the actual complexity of retinal circuitry, since each of the five major cell types shown is made up of at least two subtypes (and in some instances many more), and there are many more types of synapses than shown including gap junctions and chemically mediated synapses using transmitters other than glutamate (*Glut*). *KA*, kainate.

the acid generated reduced the pH by about 0.040 units, which also was quite accurately measured (examples of the recordings obtained are shown in Fig. 3). Rates of lactic acid production were calculated assuming that the measured fall in pH is attributable to the CO₂ produced by oxidative metabolism and to the lactic acid from glycolysis, and using the measurements of O₂ consumption to determine the contribution of the former. A more detailed account of the apparatus and its application has been published, including the equation used to calculate lactate production (Ames et al., 1992).

The calculation of lactate production assumes a respiratory quotient of 1 and further assumes that exchanges between retina and medium having a significant effect on pH are limited to the release of CO₂ and lactate. The values calculated for lactate production under control conditions corresponded quite closely with those previously found spectrophotometrically with lactate dehydrogenase (see Results). They fell to less than 2% of the control value within 11 min after omission of glucose from the medium and rose markedly (Pasteur effect) when O₂ was reduced (Ames et al., 1992). The calculated changes in lactate production, in response to the agents tested, were quite large (i.e., increases in acid release of as much as 2.3 mM min⁻¹ with respect to intracellular water and decreases of as much as 2.2 mM min⁻¹), and they were well maintained for as long as measurements were made (often more than 30 min). Thus, the total agent-evoked change in acid release was often more than 60 mM with respect to intracellular water. It therefore seems unlikely that a shift in the ATP/ADP ratio, or proton movements due for example to Na⁺/H⁺ exchange, had a significant effect on the results.

Electrical measurements. Two types of electrical responses were recorded for correlation with the metabolic response to a test agent. (1) The shift in the standing potential across the retina when the test agent was introduced into the medium was measured with transretinal electrodes, and (2) the change in the retina's response to light was measured with transretinal electrodes (electroretinogram, ERG) and with electrodes on the optic nerve stump to record the light-evoked compound action potential.

Some of the ERG recordings were obtained with platinum electrodes incorporated into the apparatus used to measure energy metabolism

Figure 2. Change in transretinal potential following addition of test agent to the medium. The time of addition is indicated by the arrows. All agents were added in darkness except for *B*, in which APB was administered in steady light of $930 \text{ Rh}^* \text{sec}^{-1}$. In records *A*, *C*, *E*, and *F*, a 10 msec flash of about $300 \text{ Rh}^* \text{sec}^{-1}$ was delivered every 5 sec (every 10 sec for *E*) to demonstrate the change, if any, in the light-evoked response. Calibration bars apply to all records, except the vertical bar is $200 \mu\text{V}$ for *E*. In this and the succeeding figures, upward deflection indicates vitreous-positive response. KA, kainate.

(see Ames et al., 1992), which permitted comparison in the same retina between changes in function and changes in energy consumption. ERGs were also obtained by DC recording in the chamber used to measure the shifts in transretinal standing potential. This chamber consisted of the lower half of a 50 ml beaker onto the bottom of which was sealed a hemispheric, hollow plastic mandrel with perforations at the top. The retina was deployed (vitreous surface down) over the mandrel under 20 ml of gassed medium and held gently in place by a silicone rubber O-ring, so that it covered the perforations and separated the fluid within the mandrel from the fluid outside. One Ag-AgCl electrode with an agar bridge containing half-saturated KCl was immersed in the fluid outside, and another was sealed within a short silicone tube that penetrated the base of the mandrel so that it made contact only with the fluid within. The electrodes, with conventional DC amplification and recording (Gould), were very stable, usually showing only a few microvolts of drift over many minutes. Examples of drug-induced shifts in the transretinal standing potential are shown in Figure 2.

For the optic nerve recordings, the 1 cm of optic nerve that could be removed with the retina was desheathed and drawn through small ($800 \mu\text{m}$) holes in two thin ($250 \mu\text{m}$) silicone membranes separated by 2 mm. The segment of nerve between the membranes was perfused with isotonic mannitol, which played the role of a "sucrose gap" between an Ag-AgCl electrode in electrical contact with the cut end of the nerve and an Ag-AgCl electrode in contact (via an agar bridge and the medium) with the intact nerve. Under control conditions, sharply peaked responses of $200\text{--}400 \mu\text{V}$ were recorded to both the ON and OFF of 1 sec light pulses.

Photostimulation was provided by fluorescent lamps with a phosphor exhibiting rapid ($20 \mu\text{sec}$) rise and fall times. Stimulus intensity was varied by rheostat control of the current to the lamps and by neutral density filters. The intensity of the light was characterized in terms of $\text{Rh}^* \text{sec}^{-1}$, or the number of photoisomerizations per rod outersegment per second (see Ames et al., 1986).

Test agents. Kainate was prepared as a 3 mM sodium salt in medium. It was administered 48 times to 24 retinas at final concentrations from 0.5 to $50 \mu\text{M}$. APB (DL-2-amino-4-phosphonobutyric acid) was prepared as a 15 mM stock solution in medium. It was administered 68 times to 37 retinas at final concentrations of from 0.5 to $50 \mu\text{M}$. CNQX (6-cyano-

7-nitroquinoxaline-2,3-dione) was prepared as a $600 \mu\text{M}$ solution in medium and administered 75 times to 33 retinas at final concentrations of from 0.1 to $40 \mu\text{M}$. L-Glutamate was prepared as a 150 mM sodium salt in medium and administered 55 times to 21 retinas at final concentrations of from 50 to $4000 \mu\text{M}$. APV (DL-2-amino-5-phosphonovaleic acid) was prepared as a 25 mM solution in medium and administered 22 times to 15 retinas at final concentrations of from 20 to $1500 \mu\text{M}$. Stock solutions were neutralized with NaOH, as required. Kainate, APB, and APV were obtained from Sigma (St. Louis, MO). CNQX was obtained from Cambridge Research Biochemicals (Wilmington, DE). Glutamate was obtained from Schwarz/Mann (Orangeburg, NY). The test agents were injected into the gas-equilibrating chamber (28 ml) of the apparatus used for metabolic measurements or into the bubbled medium in direct contact with the retina in the ERG chamber (20 ml) and in the chamber (30 ml) used to record optic nerve responses.

Experimental protocol. The retinas were maintained under control conditions in darkness until stable. For both metabolic and electrical studies, replicate control measurements were obtained, a test agent was introduced, and the measurements were repeated. Then the effect of increasing the concentration of the agent, of introducing another agent, or of returning to control conditions was examined. If (as for most of the agents) the effects were reversible upon returning to control conditions, additional experiments were performed on the same retina. The response of the retina to the agents tested was always prompt, and a new steady state was usually achieved within 1 min with respect both to energy metabolism and electrical activity. Under control conditions, the metabolic measurements and the light-evoked electrical responses were generally stable over periods of many hours.

Results

Energy metabolism under control conditions

Rates of O_2 consumption measured under control conditions in darkness averaged 128 ± 4 ($\pm \text{SE}$) $\text{nmol min}^{-1} \text{retina}^{-1}$ ($n = 12$), and lactate production averaged $146 \pm 9 \text{ nmol min}^{-1} \text{retina}^{-1}$ ($n = 12$). Expressed in terms of dry weight, the values for O_2 consumption and lactate production were 11.6 and $13.3 \mu\text{mol min}^{-1} \text{gm}^{-1}$, respectively. In an earlier study on *in vitro* rabbit retina, using techniques quite different from ours, Cohen and Noell (1960) reported an O_2 consumption per gram dry weight of $7.5 \mu\text{mol min}^{-1}$ and lactate production of $14.3 \mu\text{mol min}^{-1}$.

Repeated measurements, on the same retina, were quite reproducible, the SD for measurements of O_2 consumption averaging 1.5% of the mean value ($n = 13$) and the SD for measurements of lactate production averaging 3.7% of the mean. The variation between measurements obtained on different retinas was appreciably larger, attributable in large part to their differences in size. Therefore, the retinas have been used as their own controls, and in the following presentation of data, the changes observed in response to an experimental variable have been expressed and averaged as percentage changes from the control state of the retina being tested. Each comparison between test and control is based on the averaged values for two or more measurements under each condition.

It should be noted that, when both are expressed as percentages, a change in O_2 consumption represents a considerably larger change in energy generated than the same change in lactate production. Assuming a P:O ratio of 3, a molecule of O_2 consumed represents six times as much high-energy phosphate ($\sim \text{P}$) generated as a molecule of lactic acid produced. Since, as indicated above, control levels of O_2 consumption and of lactic acid production were similar when expressed on a molar basis, percentage changes are roughly comparable in terms of energy generated if the change in O_2 consumption is multiplied by 6.

Kainate

Kainate caused an increase in lactate production but no change in O_2 consumption (Fig. 3*A*, Table 1). It caused a vitreous-

Figure 3. Simultaneous recordings of O_2 and pH electrodes showing fall in medium $[O_2]$ and rise in medium $[H^+]$ when the gas equilibrating chamber was bypassed at times indicated by arrows. The initial step in the fall in O_2 and the rise in H^+ reflects the shift from "arterial" to "venous" medium following the lung bypass. Subsequent steps are markedly damped by admixing within the recirculating medium. *A*, Traces recorded in darkness, under control conditions and after $5 \mu M$ kainate (KA). From the fall in $[O_2]$ during 1 min, O_2 consumption was calculated to be $0.141 \mu mol \min^{-1}$ (control) and $0.140 \mu mol \min^{-1}$ (test). From the rise in $[H^+]$, lactate production was calculated to be $0.116 \mu mol \min^{-1}$ and $0.158 \mu mol \min^{-1}$ —a 36% increase in response to the kainate. *B*, Traces recorded in the presence of 10 msec light flashes of about $300 \text{ Rh} \cdot \text{sec}^{-1}$ delivered at 4 Hz, in control medium and after APB ($20 \mu M$), CNQX ($20 \mu M$), and APV ($1000 \mu M$). O_2 consumption was calculated to be 0.116 and $0.115 \mu mol \min^{-1}$. Lactate production was calculated to be 0.276 and $0.158 \mu mol \min^{-1}$ —a 43% decrease in response to the inhibitory analogs. The effects of kainate and of the inhibitory analogs were fully reversible upon return to control medium.

positive shift in transretinal potential (Fig. 2*C,D*) that was dose related as the concentration of kainate was increased to $20 \mu M$, and probably to $40 \mu M$ (Fig. 4). The glycolytic response was also dose related, but only at concentrations up to 5 or $10 \mu M$. It then plateaued, about 50% above the control level, and the rate of lactate production with $25 \mu M$ kainate was not significantly greater than with $5 \mu M$ kainate (Table 1, Fig. 4).

Kainate ($20 \mu M$) abolished the light-evoked response of the ganglion cells as recorded from the optic nerve (not shown), but it had less effect on the response of ON-bipolar cells, as revealed by the ERG. It sometimes increased the vitreous-positive light-

Figure 4. Effect of kainate on transretinal potential and on lactate production. Averaged values for the amplitude of the vitreous-positive potential shift when lactate was added to the medium (e.g., Fig. 1*C,D*) are shown by open symbols. Averaged values for the kainate-induced increase in lactate production, expressed as percentage of control rate, are shown by solid symbols. Lines were drawn by eye. Error bars show SE. Numbers of experiments are in parentheses.

evoked response at low concentrations (Fig. 2*C*) but always reduced it at higher concentrations (not shown). The latter effect was largely reversed by CNQX. Kainate receptors have been found on amacrine and ganglion cells (Bloomfield and Dowling, 1985; Aizenman et al., 1988; Massey and Miller, 1988, 1990), and 6,7-dinitroquinoxaline-2,3-dione (DNQX) has been reported to block both light-evoked and kainate-evoked ACh release from rabbit amacrine cells (Massey, 1990).

The EC_{50} for kainate was about $1 \mu M$ for the metabolic response. It appeared to be much higher for the electrical response (see Fig. 4).

APB

APB has been shown to act, selectively, as an inhibitory agonist on ON-bipolar cells (see Discussion). When APB was added to the medium in our experiments, there was a prompt, vitreous-

Table 1. Effect on energy metabolism and transretinal potential of glutamatergic agonists and antagonist added in darkness

Agent		% Change in energy metabolism		Change in transretinal potential (μV)
		Lactate production	O_2 consumption	
APB	$20 \mu M$	-23.4 ± 2.7 (13)***	-1.2 ± 0.5 (13)*	-107 ± 8 (11)***
Kainate	$5 \mu M$	$+43.3 \pm 2.8$ (7)***	$+0.6 \pm 0.7$ (7)	$+117 \pm 12$ (3)*
Kainate	$25 \mu M$	$+45.5 \pm 8.5$ (2)	$+0.0 \pm 1.8$ (2)	$+336 \pm 103$ (5)*
CNQX	$20 \mu M$	-19.3 ± 3.7 (6)**	$+2.1 \pm 0.3$ (6)***	Triphasic
Glutamate	$500 \mu M$	$+2.3 \pm 5.3$ (3)	$+4.4 \pm 1.0$ (3)*	$+255 \pm 25$ (2)

Results are expressed as mean $\pm$ SE. Number of experiments is in parentheses. *p* values are for comparison with controls. Results without an asterisk did not differ significantly from control. For change in transretinal potential, minus sign indicates vitreous-negative shift. The response of transretinal potential to the higher concentration of kainate was determined using $20 \mu M$ instead of $25 \mu M$. In two experiments, kainate was added in light, and in three experiments it was added after APB. Neither of these changes appeared to affect the metabolic or the potential response significantly, so the data have been combined.

* $p < 0.05$.

** $p < 0.005$.

*** $p < 0.001$.

Figure 5. Changes in lactate production and in O₂ consumption on shifting from darkness to flashing light, under control conditions and in the presence of glutamatergic agents. Flashes of about 300 Rh⁺sec⁻¹ and 10 msec in duration were delivered at 4 Hz. CNQX and APB were 20 μM; APB was 1000 μM. Error bars indicate SE. Numbers of experiments are in parentheses.

negative shift in transretinal potential (Fig. 2*A,B*), and this was accompanied by a large reduction in glycolysis and a small reduction in O₂ consumption (Table 1). These effects were dose related, reaching a maximum with 10 or 20 μM APB. APB also prevented much of the increase in lactate production normally observed (see Ames et al., 1992; see also, Bill and Sperber, 1990) in response to flashing light. As shown in Figure 5, light flashes (of 10 msec duration at 4 Hz) caused a 26% increase in lactate production under control conditions, but only a 15% increase in the presence of 20 μM APB (a reduction of 42%; $p < 0.005$ with each retina as its own control).

When APB (20 μM) was administered in darkness (Fig. 2*A*, Table 1), the vitreous-negative potential shift averaged 107 μV, and the reduction in glycolysis averaged 23.4%. When APB was administered in light of sufficient intensity (930 Rh⁺sec⁻¹) to

Table 3. Effect on energy metabolism in light of CNQX and APB added alone and in sequence

Agents	% Change in energy metabolism	
	Lactate production	O ₂ consumption
CNQX	-12.5 ± 3.7 (3)	+1.1 ± 1.0 (3)
CNQX after APB	+1.4 ± 1.4 (2)*	+0.4 ± 5.2 (2)
APB	-27.7 ± 5.3 (3)**	-2.6 ± 0.3 (3)***
APB after CNQX	-16.7 ± 0.2 (2)***	+0.7 ± 0.7 (2)

The change in lactate production and O₂ consumption following each addition has been calculated as a percentage of the control value in darkness. The concentration of APB and CNQX was 20 μM. The agents were administered in darkness, and measurements of lactate production performed 4 and 6 min after introduction of steady light of about 700 Rh⁺sec⁻¹ were averaged for each experiment.

* $p < 0.05$ compared with CNQX alone.

** $p < 0.05$ compared with control.

*** $p < 0.025$ compared with control.

have greatly reduced the release of endogenous transmitter from the photoreceptors (see Fig. 2*B*, Table 3), the transretinal potential shift was 50% larger ($160 \pm 20 \mu V$; $n = 2$; $p < 0.05$ for difference) and the reduction in glycolysis was 18% larger ($p < 0.05$ for difference when each retina was used as its own control).

When APB (20 μM) was administered with CNQX (20 μM) already present in the medium, in either darkness (Table 2) or in light (Table 3), the reduction in glycolysis was only about half as great as when APB was administered alone.

APB abolished the ERG b-wave, that is, the vitreous-positive component of light-evoked response of the transretinal potential (Fig. 2*A*), and it abolished the optic nerve response to the ON of the light but not to the OFF of the light (not shown).

The EC₅₀ for APB was about 5 μM for both the metabolic and the electrical response.

CNQX

CNQX was used as an antagonist of kainate and quisqualate receptors (Honore et al., 1988). It caused a dose-related reduction in glycolysis that reached a maximum of 19.3% with 20 μM CNQX, and, surprisingly, it caused a small but significant increase in O₂ consumption (Table 1). CNQX appeared to reduce by about half the increase in lactate production caused by

Table 2. Effect on energy metabolism in darkness of glutamatergic agonists and antagonists added alone and in sequence

Agent	% Change in energy metabolism	
	Lactate production	O ₂ consumption
APB 20 μM	-23.4 ± 2.7 (13)	-1.2 ± 0.5 (13)
APB 20 μM after CNQX 20 μM	-8.9 ± 1.0 (3)****	-2.2 ± 1.3 (3)
CNQX 20 μM	-19.3 ± 3.7 (6)	+2.1 ± 0.3 (6)
CNQX 20 μM after APB 20 μM	-1.7 ± 2.5 (4)***	-0.1 ± 0.7 (4)
CNQX 20 μM after kainate 5 μM	-46.0 ± 4.1 (2)***	+3.8 ± 0.4 (2)
Glutamate 500 μM	+2.3 ± 5.3 (3)	+4.4 ± 1.0 (3)
Glutamate 500 μM after APB 20 μM	+15.8 ± 4.3 (3)	+0.5 ± 0.6 (3)
Glutamate 500 μM after CNQX 40 μM	-11.9 ± 1.0 (3)**	+1.5 ± 0.4 (4)*

The change in lactate production and O₂ consumption following each addition has been calculated as a percentage of the control value. Data in italics are repeated from Table 1 for comparison.

* $p < 0.05$ compared with control.

** $p < 0.01$ compared with control and $p < 0.005$ compared with glutamate after APB.

*** $p < 0.005$ compared with CNQX alone.

**** $p < 0.001$ compared with APB alone.

a flashing light (Fig. 5), though the number of experiments was small and the results do not meet the usual criteria for significance ($p < 0.2$).

If the retina had already been exposed to APB, the administration of CNQX caused little or no further reduction in lactate production in darkness (Table 2), in steady light (Table 3), or in flashing light (Fig. 5). These observations are consistent with the finding (see above) that, when APB was administered after CNQX, the reduction in glycolysis caused by APB was reduced by an amount similar to the reduction caused by CNQX alone. They suggest that most of the CNQX-sensitive receptors in rabbit retina are on cells of the ON system, but they do not exclude the possibility of CNQX receptors at other sites where the metabolic effects are of opposite sign and result in no net change (e.g., on inhibitory interneurons and also on excitatory neurons in the OFF system).

CNQX blocked most of the retina's response to kainate, both electrical and metabolic. If CNQX was already present in the medium at 20 μM , the vitreous-positive potential shift caused by the addition of 20 μM kainate was reduced by 75% (from $336 \pm 103 \mu\text{V}$, $n = 5$ to $83 \pm 9 \mu\text{V}$, $n = 3$; $p < 0.05$ for difference). When CNQX was administered after kainate, the reduction in lactate production caused by the CNQX was 2.4-fold greater than when CNQX was administered alone (Table 2). By comparing the increase in lactate production caused by kainate with the reduction in lactate production caused by CNQX, when administered after kainate and when administered alone, we calculated that CNQX reduced the kainate-evoked increase in lactate production by $63 \pm 13\%$ ($n = 4$; $p < 0.025$). It seems evident that the majority of the kainate-sensitive receptors in rabbit retina are also sensitive to CNQX. However, our data provide little evidence about the fraction of CNQX-sensitive receptors that is sensitive to kainate.

The effects of CNQX on our electrical recordings were less consistent and less definitive than the effects of the other agents tested. It caused a relatively small, usually triphasic, change in the transretinal potential that was predominantly vitreous negative (Fig. 2E). It slowed the vitreous-positive light-evoked response of the transretinal potential (the ERG b-wave) and reduced the amplitude of the response to low-intensity stimuli (Fig. 2E) but increased the amplitude of the response to high-intensity stimuli (not shown). At higher concentrations (20 μM), CNQX reduced or abolished the light-evoked responses recorded from the optic nerve (not shown). The effects of CNQX on the light-evoked electrical responses were not significantly altered by 500 μM glycine. This latter observation, and the clear antagonism between CNQX and kainate noted above, make it unlikely that CNQX was acting primarily at the glycine site on the NMDA receptor (e.g., Lester et al., 1989).

Though the effects of CNQX were reversible following short exposures, there appeared to be incomplete recovery of light-evoked electrical responses following exposures of more than 1 hr to 20 μM or 40 μM CNQX.

The EC_{50} for CNQX was about 7 μM .

APV

APV was used as an antagonist at NMDA receptors. It caused a dose-related reduction in glycolysis between 100 and 1000 μM , averaging $35.2 \pm 3.9\%$ ($n = 5$; $p < 0.001$) at 500 μM . APV had little effect on O_2 consumption, but if data obtained with 250 and 500 μM APV administered alone and after APB are combined ($n = 8$), it appears to have caused a small ($+2.9 \pm$

1.0%) but significant ($p < 0.05$) increase. APV blocked the vitreous-positive light-evoked response (ERG b-wave) at concentrations between 500 and 1000 μM . On the basis of its effect on the b-wave and on the basis of published studies on rabbit (Neal et al., 1981; Massey and Miller, 1990) and mudpuppy (Slaughter and Miller, 1985), it seems likely that APV was acting in part as an agonist at the APB receptors on the ON-bipolar cells. When administered alone, the effects of APV are therefore difficult to interpret. [The racemic mixture was used in these experiments. In retrospect, it would have been desirable to have used the D-isomer since it is less effective than the L-isomer as an agonist at APB receptors and more effective as an antagonist of NMDA receptors (cf. Massey and Miller, 1990).] When administered after APB and CNQX, the effects of the APV probably can be attributed to the blockade of NMDA receptors. APV (1000 μM), administered in addition to 20 μM APB and 20 μM CNQX, caused an additional reduction in glycolysis in darkness of 18% ($p < 0.01$ for the difference) and reduced to zero ($p < 0.01$) the increase in glycolysis evoked by a flashing light (Fig. 5). NMDA receptors have been found on third-order neurons (Bloomfield and Dowling, 1985; Aizenman et al., 1988; Massey and Miller, 1988, 1990) and recently on rod bipolar cells (Karschin and Wässle, 1990).

Glutamate

Glutamate itself caused a vitreous-positive shift in transretinal potential. It increased O_2 consumption, but had no significant effect on glycolysis (Fig. 2F, Table 1).

The response to glutamate was quite different when it was administered after the inhibitory agonist APB and when it was administered after the antagonist of excitatory receptors, CNQX. When administered after APB, glutamate (at 500 μM) caused a larger potential shift ($317 \pm 64 \mu\text{V}$; $n = 3$) than glutamate alone, and it increased glycolysis by 15.8% (Table 2). If the data obtained with 500 μM and 1000 μM glutamate are combined, the increase in glycolysis was significant ($p < 0.05$). When glutamate was administered after CNQX, it caused a smaller potential shift ($195 \pm 5 \mu\text{V}$; $n = 2$), and it caused an 11.9% reduction in glycolysis ($p < 0.05$), as shown in Table 2. In the one experiment in which this was tested, progressive increases in the concentration of glutamate administered after 20 μM CNQX caused a 10% reduction in glycolysis at 500 μM glutamate, but a 46% increase in glycolysis when the glutamate had been increased to 2000 μM —consistent with CNQX having acted as an antagonist at excitatory receptors but having been displaced by the higher concentration of glutamate.

At concentrations of 500 μM and above, glutamate abolished the vitreous-positive light-evoked response of the transretinal potential (ERG b-wave) and abolished the optic nerve response to both the ON and OFF of the light (not shown).

Discussion

The metabolic responses to the glutamate analogs were generally as expected. The excitatory agonist (kainate) caused a dose-related increase in energy metabolism whereas the antagonists (CNQX and APV) and the inhibitory agonist (APB) caused dose-related decreases. Glutamate itself caused a small increase in energy metabolism when administered alone, but caused a large increase when it was administered after APB had already occupied receptors at which the endogenous transmitter is inhibitory. It caused a reduction in metabolism when it was ad-

ministered after CNQX had already occupied some of the receptors at which the endogenous transmitter is excitatory.

All agents caused changes in electrophysiological function over the dose ranges that elicited changes in metabolism. As expected, they reduced or abolished light-evoked responses, whether recorded transretinally (i.e., the b-wave of the ERG) or from the optic nerve stump; and they caused shifts in the transretinal standing potential when they were introduced into the medium. The agents that were expected to decrease Na^+ conductance (APB and CNQX) caused vitreous-negative shifts in the transretinal potential, and agents expected to increase Na^+ conductance (kainate and, at some sites, glutamate) caused vitreous-positive shifts. Because of the close association between the metabolic and the electrical responses, it seems reasonable to interpret the changes in energy metabolism as reflecting changes in energy requirements that resulted from the drug-induced changes in electrophysiological function. In a few experiments (not shown), the test agents were administered to retinas in which neurotransmission had been blocked by Co^{2+} . Both kainate and glutamate caused an increase in the amplitude of PIII reversed by CNQX (suggesting that phototransduction may be modulated by activation of glutamate receptors on photoreceptor cells). However, the effect of these agents on PIII was small relative to their effect on the optic nerve response of unblocked retinas, so it seems likely that most of the metabolic effects observed reflected changes in neurons postsynaptic to the photoreceptor cells and were due to effects of the agents on neurotransmission.

Glutamate appears to have much larger effects on the energy metabolism of retina than other excitatory transmitters. Both histological and electrophysiological studies have indicated that a large fraction of retinal cells—photoreceptor, horizontal, bipolar, amacrine, and ganglion—are presynaptic and/or postsynaptic to glutamatergic synapses (e.g., Massey, 1990). Conversely, though many other potentially excitatory transmitters have been identified in retina, they appear to be largely restricted to subgroups of amacrine cells (e.g., Daw et al., 1989; Wässle and Boycott, 1991). In a few experiments (not shown), we tested the effects of two cholinergic antagonists (curare at 0.5–12.5 μM and atropine at 10–1000 μM) and two adrenergic antagonists (propranolol at 2–50 μM and phentolamine at 2–50 μM). None caused a significant reduction in either O_2 consumption or lactate production.

Glycolytic versus oxidative metabolism

It was characteristic of the response of energy metabolism to the glutamatergic agents that the predominant change was in glycolysis rather than in oxidative metabolism. Thus, as shown in Table 1, the changes in glycolysis in response to three analogs (APB, kainate, CNQX) ranged from –23.4% to +45.5%, whereas the changes in O_2 consumption ranged only from –1.2% to +2.1%. Even if account is taken of the larger amount of high-energy phosphate ($\sim\text{P}$) generated per percentage change in O_2 consumption (a factor of about 6), it is clear that the great majority of the change in energy requirements was met by glycolysis. This is consistent with the results of a previous study on rabbit retina that showed that, though phototransduction was supported primarily by oxidative metabolism, neurotransmission was supported primarily by glycolysis (Ames et al., 1992). It is not clear whether this merely represents an adaptive response to the hypoxia normally present in the inner retina of many species (including rabbit), or whether processes involved

in neurotransmission are preferentially energized by glycolysis. There is some recent evidence to suggest the latter (Lipton and Robacker, 1983; Fox et al., 1988; Ackerman and Lear, 1989). It would be of interest to determine if neurotransmission through more vascular retinas (e.g., primates) is equally dependent on glycolytically derived energy.

Though the changes in oxidative metabolism were always small, they were often sufficiently reproducible to be statistically significant. If all the data on APB administered in darkness or steady light and with or without CNQX ($n = 21$) are combined, there was a small ($-1.4 \pm 0.4\%$) but significant ($p < 0.005$) reduction in O_2 consumption in addition to the reduction in lactate production. CNQX, paradoxically, increased O_2 consumption (Table 1). This might be attributable to disinhibition of photoreceptor cells due to the hyperpolarization of horizontal cells by CNQX. Since, of the three analogs, the agonist (APB) decreased O_2 consumption and the two antagonists (CNQX and APV) increased it, it was expected that glutamate would decrease it. Surprisingly, the combined data obtained with 500 μM glutamate (see Table 2) showed a significant increase in O_2 consumption ($+2.1 \pm 0.6\%$; $n = 10$; $p < 0.025$). Oxidative energy may be used for glutamate reuptake, or there may be neurons using oxidative metabolism that have glutamate receptors that were not affected by the glutamate analogs tested in these experiments.

Metabolic burden of glutamatergic transmission

All analogs tested caused relatively large changes in the rate of glycolysis. The response to APB can be attributed to changes in cells of the ON system. APB has been shown to be selective for receptors on ON-bipolar cells where it acts like the endogenous transmitter (as an inhibitory agonist) leading to hyperpolarization of the cell and loss of its light-evoked response (e.g., Shiells et al., 1981; Slaughter and Miller, 1981; Massey et al., 1983; Attwell et al., 1987). The response to APB would be expected to be maximum in bright light, when transmitter release from photoreceptors is markedly reduced (and perhaps near zero; Nawy and Copenhagen, 1987), and it would be expected to be minimal in darkness, when many receptors are already occupied by endogenous transmitter. APB reduced energy consumption by 52 nmol of $\sim\text{P}$ min^{-1} retina^{-1} when administered in light and by 38.8 nmol min^{-1} retina^{-1} when administered in darkness, a reduction of only 25%. (For these calculations, the reductions in lactate production and O_2 consumption were expressed in terms of nmol min^{-1} retina^{-1} , and three $\sim\text{P}$ were assumed to be generated per atom of O_2 consumed.) We interpret this as evidence that a considerable fraction of the ON-bipolar receptors is unoccupied by glutamate even in darkness, so that they are poised to respond not only to a reduction in transmitter due to light at the center of the receptive field but also to an increase in transmitter due to light on the surround (e.g., Toyoda, 1973). However, maintaining this two-way responsiveness is energy expensive. As shown in Table 1, activity in the ON system in its basal state in darkness was responsible for 23.4% of the total glycolytic energy generated by the retina.

A substantial amount of energy consumption in the OFF system also appears to be dependent on glutamatergic transmission. The 26% increase in glycolysis caused by flashing light under control conditions was (surprisingly) only reduced by 11% by APB (which presumably eliminated the contribution of the ON system to the flash response), but it was eliminated by APV

(Fig. 5). We interpret these findings as evidence of neurons in the OFF system whose activity depends on NMDA receptors and whose glycolytic metabolism is increased by phasic stimulation by an amount equivalent to 15% of the total glycolytic metabolism of the resting retina. NMDA receptors have been reported to be particularly responsive to phasic stimuli (Herron et al., 1986; Salt, 1986).

Since the responses to the agents tested were presumably limited to subgroups of retinal neurons (and cells downstream from them), the percentage change in the rate of glycolysis in the neurons most affected must have been much larger than the percentage change in total glycolysis. Thus, the 50% increase in total glycolysis caused by kainate may have reflected a two- or threefold increase in the cells most affected. If APB affected (directly or indirectly) a third of the cells in the retina, the 23% reduction it caused in total retinal glycolysis in darkness would represent a 69% reduction in the affected cells (assuming some uniformity of glycolysis initially among the different cell types). Similarly, the 47% decrease in glycolysis seen with the combination of inhibitors must have represented a very large decrease in the cells most affected. The response to the inhibitors is of particular interest as it provides an indication of the energy requirements associated with the normal physiological activity of the various glutamate receptors and of the pathways they influence. It seems reasonable to conclude that there are groups of retinal neurons that, under physiological conditions, must devote most of their glycolytic metabolism to meet energy requirements determined directly or indirectly by the activity of glutamate receptors. Experiments in which NaK ATPase was inhibited with ouabain or strophanthidin have indicated that about 60% of the glycolytic energy generated by the retina is devoted to Na⁺ transport (Winkler, 1981; Ames et al., 1992). A major portion of the energy required for glutamatergic transmission is probably used for this purpose. Definitive identification of the cell types, and the metabolic processes, responsible for function-related changes in energy requirements will require improvements in the resolution of our methods for measuring energy usage (see, e.g., Van der Laarse et al., 1989).

It is likely that activation of glutamate receptors has the potential of increasing neuronal activity to levels that exceed the cells' capacity to maintain homeostasis. As shown in Figure 4, the electrophysiological response to kainate paralleled increases in concentration to 40 μ M, but the glycolytic response reached a maximum at 10 μ M. This suggests that there are limitations (perhaps in energy generation or in pump capacity) that prevent the metabolic response from meeting the functional demands of the higher concentrations of kainate.

More direct evidence that retinal cells are unable to maintain ionic homeostasis in the face of glutamate-induced increases in conductance has been obtained by measuring changes in the concentrations of intracellular ions (Ames, 1956; Ames et al., 1967). In these experiments, also on rabbit retina, the addition of glutamate to the medium caused dose-related (from 0.5 to 5 mM) increases of up to 2.3-fold in intracellular Na⁺, which were accompanied by decreases of up to 36% in intracellular K⁺ and increases of up to 65% in intracellular Cl⁻. The electrolyte shifts were evident within 5 min and maximum by 10 min, after which they returned somewhat toward normal. Glutamate (at 5 mM) caused a twofold increase in total intracellular Ca²⁺; intracellular Mg²⁺ was not affected. The electrolyte shifts were presumably even larger in the cells most affected, but it should be recognized that it is unlikely that all the changes were due to increases in

conductance and that some of them were probably the result of the electrogenic carrier-mediated uptake of glutamate.

On the basis of the above considerations, it seems likely that the toxic effects of excitatory glutamatergic agents can be attributed, at least in part, to an imbalance between the metabolic demands imposed by the conductance increases and the cells' ability to meet these demands. If the imbalance is sustained, the loss of the normal intracellular ionic pattern or the continued depletion of ~P may lead, through a variety of mechanisms, to irreversible damage. This may account at least in part for the toxicity of exogenously administered agonists (Lucas and Newhouse, 1957; Olney et al., 1986) and perhaps for the neuronal loss observed in pathological conditions such as Huntington's disease (Beal et al., 1986; Young et al., 1988) and ischemia (e.g., Benveniste et al., 1989). Ischemia can increase the extracellular concentration of glutamate (Benveniste et al., 1984). Experiments on cultured cerebellar neurons have demonstrated that both cardiac glycosides and ischemia-type insults act synergistically with glutamatergic agonists in producing irreversible damage (Novelli et al., 1988).

While it seems quite certain that there is a relationship between the effects of glutamate and the effects of ischemia, the nature of the relationship is less clear. Experimental results have commonly been interpreted as indicating that the damage caused by ischemia is mediated by glutamate (e.g., Choi, 1990). We suggest that this causal sequence may also act in reverse, and that the damage caused by glutamate may be mediated by an ischemic-like condition in which the cells' energy requirements have been increased beyond the cells' capacity to generate energy. It seems likely that both types of response can occur—with the potential for a positive feedback that would have a devastating effect on cell survival.

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