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Galanin and Glibenclamide Modulate the Anoxic Release of Glutamate in Rat CA3 Hippocampal Neurons

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Abstract

The effects of brief anoxic episodes on intracellularly recorded CA3 pyramidal neurons have been studied in the hippocampal slice preparation. Anoxia induced a depolarization occasionally preceded by a transient hyperpolarization associated with a fall in input resistance. The anoxic depolarization was due to the release of glutamate from presynaptic terminals since it was blocked by tetrodotoxin (TTX) (1 μM) or by the broad spectrum excitatory amino acid antagonist kynurenate (1 mM). In the presence of TTX (1 μM) or kynurenate (1 mM), anoxia only induced a *hyperpolarization* which was due to activation of a K+ conductance. The anoxic *depolarization* was blocked by galanin, a hormone which activates ATP sensitive K+ (K+ATP) channels. Anoxic depolarization was increased by the potent sulfonylurea agent glibenclamide (GLIB) which blocks K+ATP channels. Bath applications of these agents had little effect when applied in oxygenated Krebs solution suggesting that their action may be mediated by K+ATP channels. Since excessive release of glutamate during anoxia is neurotoxic, agents such as galanin which activate K+ATP channels may provide tissue specific protection against anoxic damage.

Introduction

ATP sensitive K+ channels (K+ATP) are recently identified channels that are inhibited by intracellular ATP (Noma, 1983; Sanfield, 1987). K+ATP channels have been shown to exist in pancreatic B cells (Ashcroft et al., 1984; Cook and Hales, 1984), cardiac cells (Noma, 1983; Trube and Hescheler, 1984), skeletal muscle cells (Spruce et al., 1987), and more recently central neurons (Ashford et al., 1988). The presence of K+ATP channels in a variety of excitable cells provides the intriguing possibility of a close link between the metabolism of a cell and its excitability.

Considerable progress has been made in the pharmacology of this type of K+ channel. Thus, hypoglycemic sulfonylureas which are used in the treatment of diabetes release insulin from pancreatic B cells by blocking K+ATP channels (Sturgess et al., 1985; Schmid-Antomarchi et al., 1987). In contrast the hyperglycemic hormone galanin (GAL) (Rökaeus, 1987) blocks the release of insulin by activating K+ATP channels (De Weille et al., 1988). Both GAL immunoreactivity (Chng et al., 1985; Skofitsh et al., 1985; Melander et al., 1986) and high affinity binding sites to glibenclamide (GLIB) (Mourre et al., 1989), the most potent sulfonylurea available, have now been found in the CNS, notably in the CA3 region of hippocampus, but their functional significance is not known. I have now examined the effects of GAL and GLIB on the responses induced by brief anoxic episodes in CA3 pyramidal neurons known to produce a fall in intracellular ATP (Kass and Lipton, 1982); the results suggest that GAL reduces and GLIB augments the anoxic depolarization which is due to presynaptic release of glutamate. Agents which activate K+ATP channels, such as GAL, may be important to prevent the deleterious effects of anoxia. Part of these observations have been reported in brief elsewhere (Ben-Ari, 1989; Ben-Ari and Lazdunski, 1989).

Materials and Methods

Experiments were performed on CA3 hippocampal neurons recorded in slices obtained from adult male Wistar rats. Rats were anaesthetized with ether and decapitated. The brain was removed and submerged in artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl 126, KCl 3.5, CaCl 2.0, $MgCl_2$ 1.3, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11 (pH 7.3), and gassed with 95% O_2 and 5% CO_2. Slices, approximately 500 μm thick, were cut using a McIlwain tissue chopper and incubated at room temperature in ACSF for at least 1 h before use. Individual slices were transferred to a submerged type chamber and superfused with ACSF at 2.5–3 ml/min at 34°C (see Ben-Ari and Gho, 1988 for further details).

Intracellular recordings were made with 3 M KCl-containing microelectrodes (resistance of 60–150 MΩ). Current was passed through the recording electrode by means of an Axoclamp 2 amplifier.

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Bridge balance was checked repeatedly during the experiment and capacitative transients with the electrode tip outside the neuron were reduced to a minimum by negative capacity compensation. Membrane potential was estimated from the potential observed upon withdrawal of the electrode from the cell. Stimulating electrodes (twisted bipolar NiCr insulated wires, 50 m o.d. or bipolar etched tungsten electrodes) were positioned in the hilar zone. Stimulation parameters were 50−100 μs duration, 5−50 V intensity, and 0.05 Hz frequency. Signals were digitized and displayed on a digital oscilloscope and on a computer driven chart recorder. Anoxia was produced by perfusion with an ACSF saturated with N_2 95%, CO_2 5%; this produces a full block of synaptic transmission in 2 min in CA1 and 3−6 min in CA3 (Cherubini et al., 1989). Anoxic tests were repeated at 10−15 min intervals. Drugs were dissolved in ACSF and superfused via a three-way tap system. Drugs used were tetrodotoxin (TTX, 1 μM, Sigma), kynurenic acid (1 mM, Sigma), glibenclamide (GLIB, 0.5−5 μM, gift of Dr Lazdunski), and galanin (GAL, 1 μM, gift of Dr Lazdunski).

Results

This study is based on long lasting (1−4 h) intracellular recordings of 42 CA3 pyramidal neurons with membrane potentials more negative than −58 mV. The mean resting membrane potential (−66±8 mV; mean±SD) and input resistance (63.2±12.9 MΩ) were similar to previous studies (Ben-Ari and Gho, 1988). A brief anoxic episode (90 s to 4 min) induced in over 90% of the cases (n=101 tests in 42 neurons) a depolarization (8.0±5.2 mV; mean±SD) and an increase in synaptic activity (Fig. 1A). In approximately one-third (36%) of the cases anoxic episodes were preceded by a small hyperpolarization (3.7±0.5 mV). The depolarization and hyperpolarization were associated with a fall in input resistance (51±11.1%). Return to oxygenated ACSF elicited a post-anoxic hyperpolarization (14.4±8.4 mV).

Mechanisms of anoxic depolarization

In the presence of TTX (1 μM), the depolarizations were blocked and only hyperpolarizations were observed (Figs 1B and 3B) (15 anoxic tests in five neurons). Furthermore, since excitatory synaptic transmission is mainly mediated by glutamate in the hippocampus (Collingridge et al., 1983; Neuman et al., 1988), anoxic tests were also performed in the presence of the broad spectrum excitatory amino acid antagonist kynurenate at concentrations (1 mM) which fully blocked the mossy fibre epsp (see Fig. 4). In the presence of kynurenate, anoxia induced only a hyperpolarization (Fig. 2B). These observations suggest that in CA3 neurons the depolarization induced by anoxia is due to the release of glutamate from presynaptic terminals. It bears

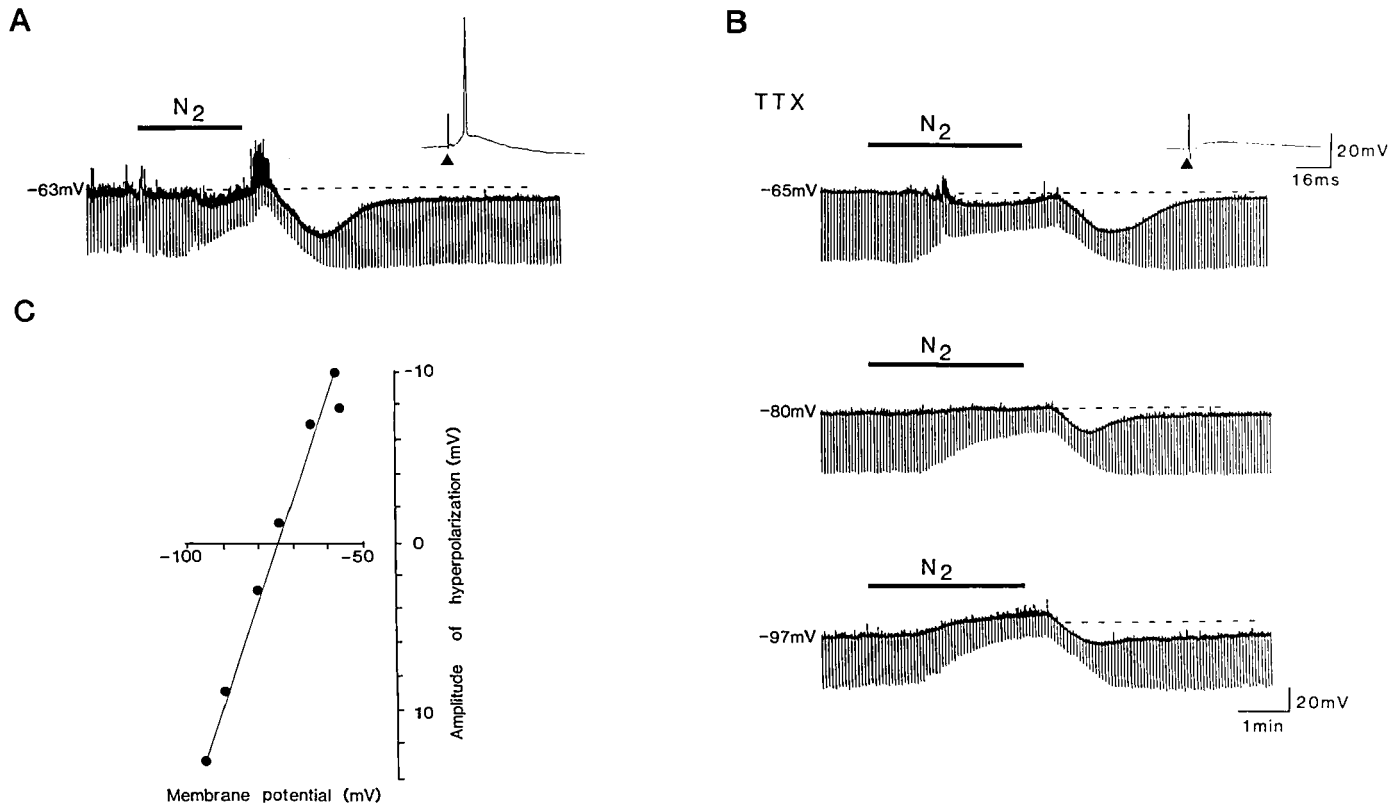

FIG. 1. Effects of blocking synaptic transmission on hypoxia induced hyperpolarization and depolarization. A,B,C data obtained from the same CA3 neuron. In this and the following figures, the slices were exposed to anoxic episodes as indicated by the dark line, and downward deflections are hyperpolarizing potentials elicited by anodal current pulses (0.5 nA−200 ms, 0.2 Hz); resting potential was −63 mV.

(A) In control ACSF hypoxia induced a small hyperpolarization followed by a depolarization and a post-anoxic hyperpolarization.

(B) Bath application of tetrodotoxin (TTX, 1 μM) blocked the excitatory postsynaptic potential (epsp), see insert in A and B. In the second and third recording membrane potentials were held at −80 and −97 mV respectively by passing anodal d.c. currents. Note anomalous rectification at hyperpolarizing potentials.

(C) Amplitude of the anoxia induced hyperpolarization versus membrane potential. Reversal of the anoxic hyperpolarization occurred at −75 mV. In this and the following figures, neurons were impaled with potassium chloride electrodes.

stressing that this 'early' depolarization is not identical to that commonly observed following prolonged anoxic episodes which are associated with neuronal loss (e.g. Benveniste et al., 1984; Choi, 1988).

Mechanisms of anoxic hyperpolarization

The experiments illustrated in Figures 1 and 2 indicate that in contrast to the depolarization, the anoxic hyperpolarization is generated post-synaptically. As in CA1 neurons (Hansen et al., 1982; Fujiwara et al., 1987), the hyperpolarizations were due to the activation by anoxia of a K+ conductance since they reversed polarity at a membrane potential of -77 ± 6.24 mM (Fig. 1B,C; n=3 neurons) in the presence of TTX and -75 ± 5.2 mV in the presence of kynurenate (Fig. 2B,C; n=3 neurons). To examine the possible contribution of a K+ATP conductance in the anoxic hyperpolarization the effects of GLIB (5 μM) or GAL (1 μM) on the anoxic response were tested in three neurons in the presence of TTX. As shown in Fig. 3B,C, the amplitude or reversal potential of the anoxic hyperpolarization were not changed by GLIB or GAL. Similar observations were made in the presence of kynurenate (1 mM, n=2, not shown) suggesting that a K+ATP conductance does not contribute to the anoxic hyperpolarization.

GLIB augments and GAL reduces anoxic depolarization

Figure 4A–D shows that bath application of GLIB (5 μM) in an oxygen-deprived ACSF considerably increased the anoxic depolarization. In 44 tests (in 14 neurons), the amplitude of the anoxic depolarization in the presence of GLIB (13.2 ± 8.6 mV) was significantly different from that recorded during similar anoxic periods in control ACSF ($p < 0.005$, unpaired t-test). Figure 4E and F shows that in the same neuron, GLIB had no effect on the anoxic response in the presence of kynurenate, suggesting (see also Fig. 3) that the drug was acting at a presynaptic site to augment the release of glutamate. Furthermore, as shown in Figure 5, in neurons in which anoxia produced a hyperpolarization (Fig. 5A), GLIB induced an increase in synaptic noise which masked the hyperpolarization (Fig. 5B). Similar applications of GLIB in control oxygenated ACSF produced either no effect (n=5 tests in three neurons) or a small (15%) increase in input resistance (n=3 tests in two neurons). GLIB had no effect on membrane potential.

Bath applications of GAL (1 μM) in oxygenated ACSF produced no change in membrane potential or input resistance (n=6 tests in four neurons. Fig. 6B–D) or a small hyperpolarization (2–4 mV; n=3 tests in two neurons, not shown). In contrast, application of GAL via oxygen-deprived ACSF blocked the anoxic depolarization (Fig. 6B–D). Similar experiments (n=8 anoxic tests in four neurons) confirmed a highly significant reduction of the anoxic depolarization by GAL (1.1 ± 1.4 mV; $p < 0.005$, unpaired t-test). As shown in Figure 6C, application of GLIB (5 μM) augmented the depolarization which was almost completely blocked by a second application of GAL (Fig. 6D). As for GLIB (e.g. Fig. 3B), GAL had no effect on the anoxic

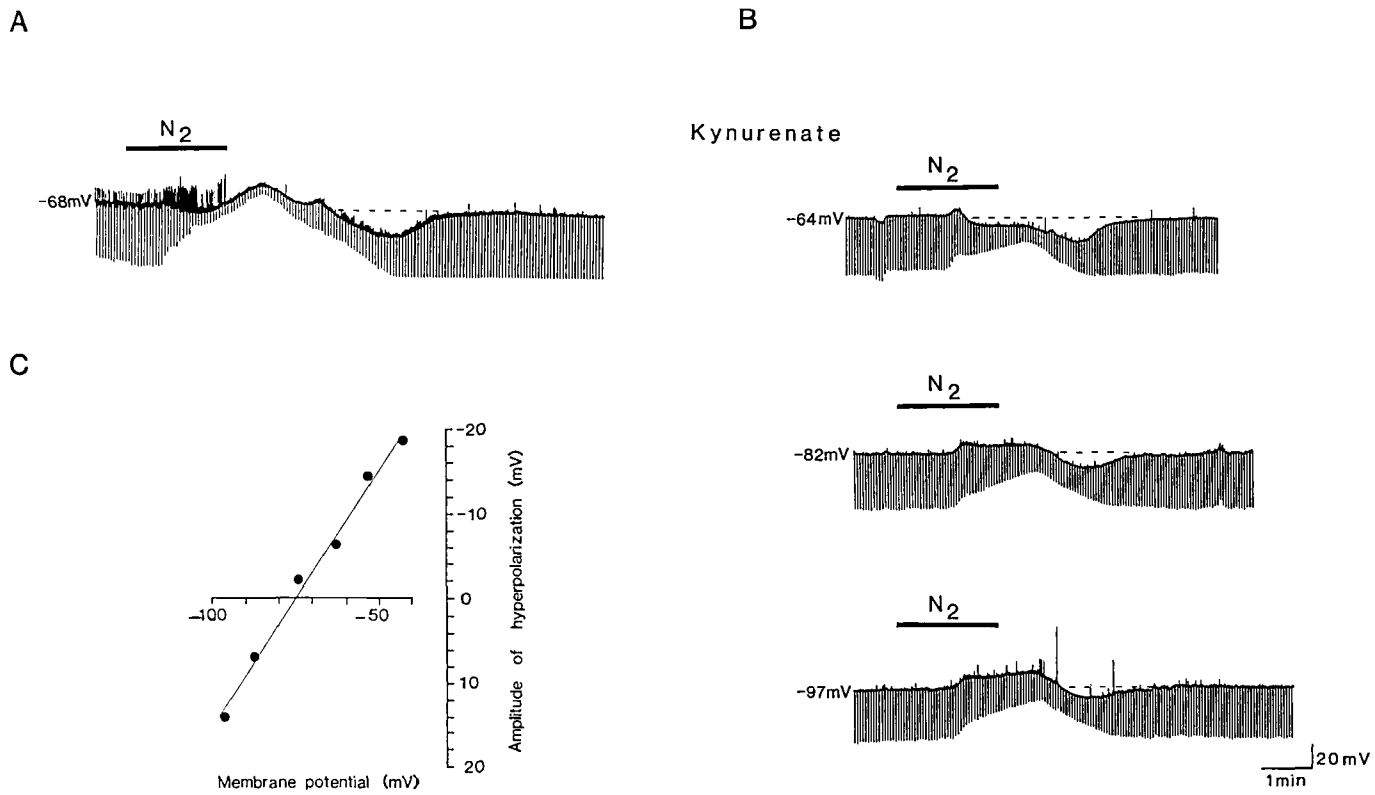

FIG. 2. Effects of bath application of the broad spectrum excitatory amino acid antagonist kynurenate (1 mM) on the hypoxic response. Same organization as Figure 1.

(A) In control ACSF, hypoxia induced a small hyperpolarization followed by a depolarization and a post-anoxic hyperpolarization.

(B) Kynurenate blocked the mossy fibre epsp (not shown) and the anoxic depolarization. The anoxic hyperpolarization shifted to the depolarizing direction at −82 and −92 mV.

(C) The amplitude of the hyperpolarizing response is plotted against membrane potential. Reversal occurred at −75 mV.

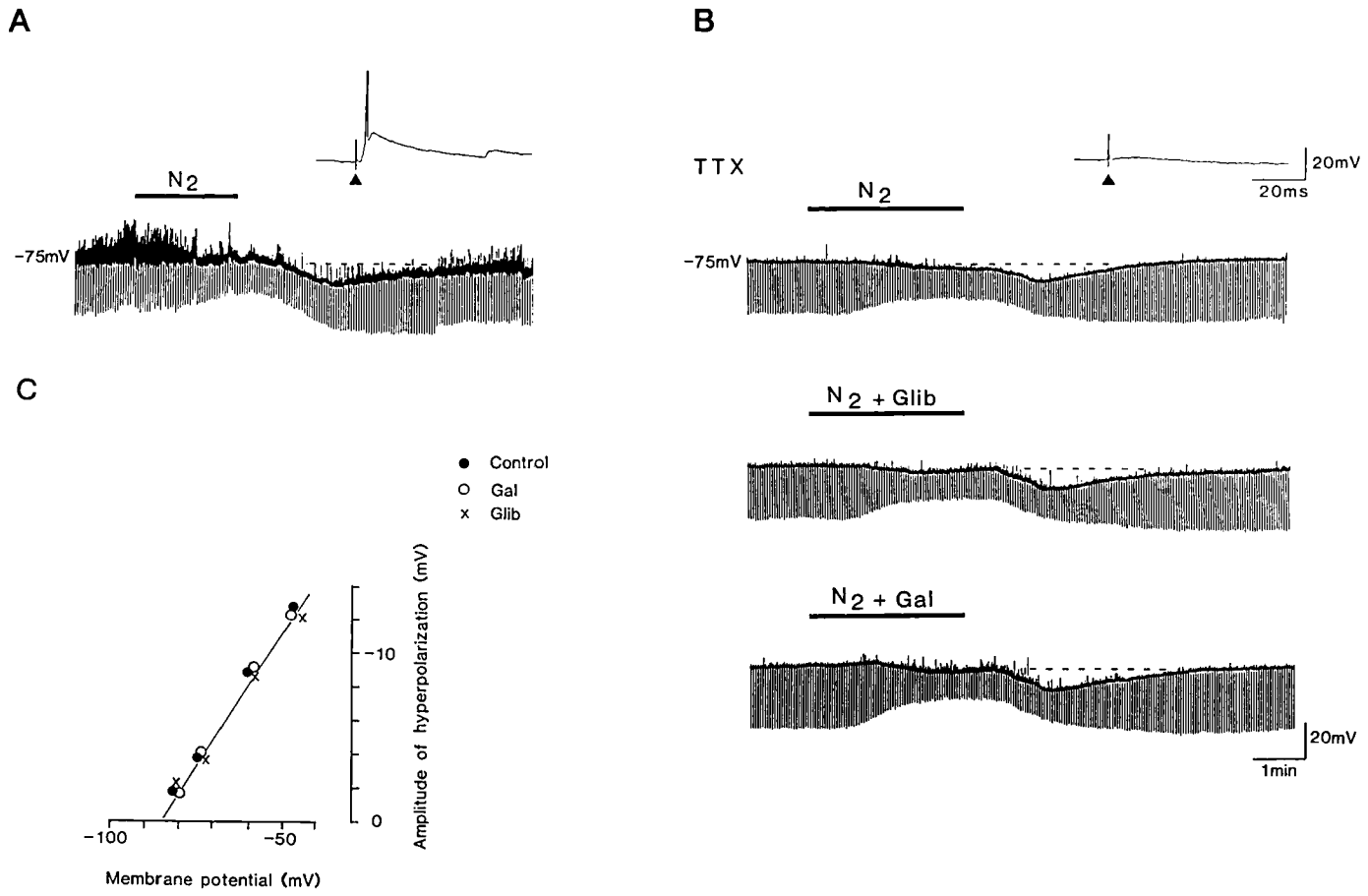

FIG. 3. Effects of galanin and glibenclamide on the reversal potential of the anoxia induced hyperpolarization in the presence of TTX.

(A) Anoxia induced a reduction in spontaneous activity and a post-anoxic hyperpolarization.

(B) TTX (1 μM) blocked the epsp (insert) and most of the synaptic noise; hypoxia now induced a small hyperpolarization. The second and third records are in the presence of glibenclamide (GLIB, 5 μM) and galanin (GAL, 1 μM) respectively.

(C) Amplitude of the hypoxia induced hyperpolarization versus membrane potential in control ACSF (closed circles) and in the presence of galanin (open circles) or glibenclamide (crosses). Note that the reversal potential was not changed by galanin or glibenclamide.

hyperpolarization in the presence of kynurenate (n=3) or TTX (n=3). Therefore, during oxygen deprivation GAL was likely to act presynaptically to reduce transmitter release.

Discussion

Anoxic hyperpolarization is due to activation of gKCa and not gK ATP

Earlier studies in CA1 hippocampal neurons have shown that anoxia induces a hyperpolarization which is clearly due to the activation of a K+ conductance (Hansen et al., 1982; Fujiwara et al., 1987; Krnjevic and Leblond, 1987). Two types of K+ conductances have been suggested to mediate the hyperpolarization: (i) a calcium dependent K+ conductance, which would be activated by an increase in $[Ca]_i$ secondary to a fall in intracellular ATP and thus the block of Ca++ extrusion (Krnjevic, 1975; Hansen et al., 1982); (ii) ATP sensitive K+ channels (K+ATP) directly activated by the fall in intracellular ATP produced by anoxia (Fujiwara et al., 1987). Experiments using intracellular injections of Ca++ chelators have provided controversial observations (Krnjevic and Leblond, 1989; Fujiwara et al., 1987) perhaps due to the difficulty in avoiding local changes in Ca++ at sites distal from the intracellular electrodes. However in both CA1 (Krnjevic and Leblond, 1988) and CA3 (present study) agents known to modulate the K+ATP channels such as the sulfonylureas have no effect on the hyperpolarization; therefore although the participation of other K+ conductances cannot be excluded, the present observations suggest that the anoxic hyperpolarization is more likely due to the calcium dependent K+ conductance than to K+ATP as initially suggested by Krnjevic (1975).

Upon return to oxygenated solution there was a delayed post-anoxic hyperpolarization. As in CA1 neurons (Hansen et al., 1982; Fujiwara et al., 1987; Krnjevic and Leblond, 1989) this is probably due to reactivation by oxygenation of an electrogenic (Na+K+) ATPase pump.

Anoxic depolarization is due to enhanced transmitter release notably glutamate

In addition to a direct postsynaptic effect, anoxia also induced a depolarization which was blocked by TTX. In fact the blockade of synaptic activity revealed more clearly the anoxic hyperpolarization

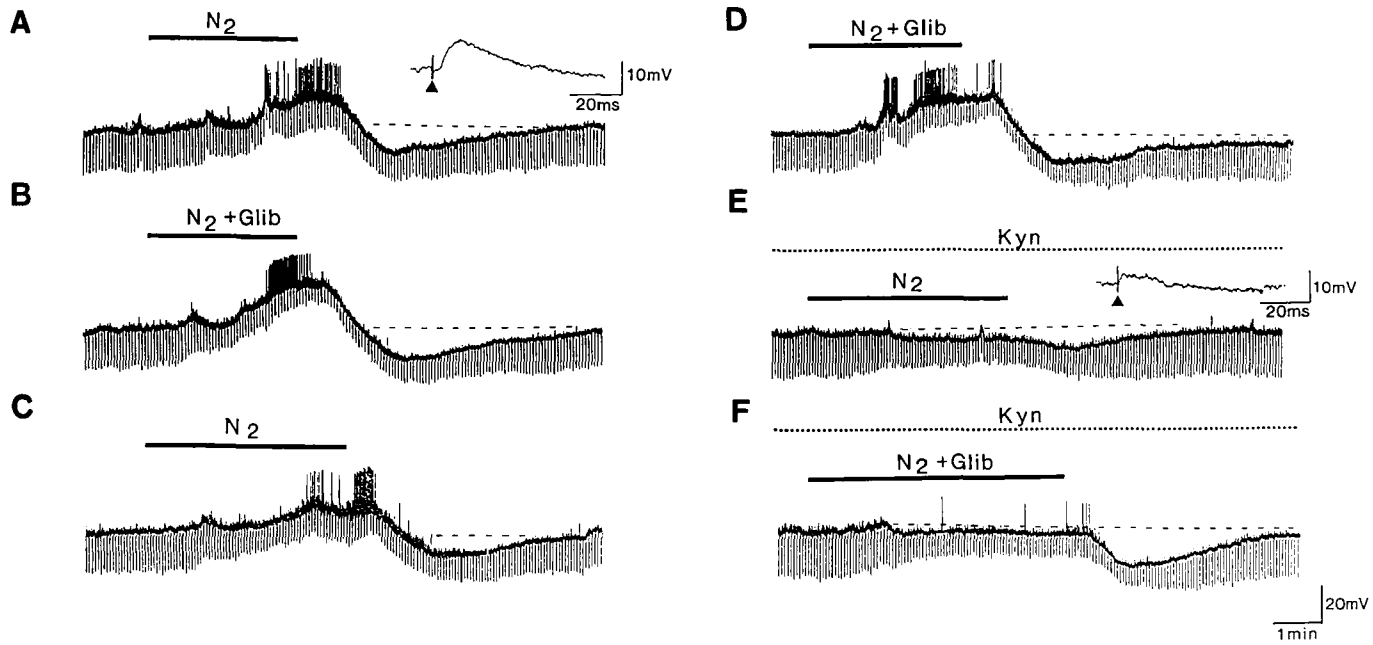

FIG. 4. Kynurenate blocks glibenclamide-induced increase in the anoxic depolarization.
(A and C) In control ACSF, anoxia (3 and 4 min respectively) induced a depolarization following reoxygenation by a post-anoxic hyperpolarization.
(B–D) Bath application of glibenclamide (GLIB, 5 μM) in the oxygen-deprived ACSF, augmented the anoxic depolarization.
(E and F) Bath application of kynurenate (1 mM) blocked the excitatory postsynaptic potential (epsp) evoked by electrical stimulation of the mossy fibres (e.g. inserts in A1 and E1). It also blocked the anoxic depolarization induced in the absence (E) and presence (F) of GLIB indicating that this was mediated by glutamate. In the presence of kynurenate, anoxia induced a hyperpolarization (E,F) which was masked in control ACSF by the anoxic depolarization.

suggesting that oxygen deprivation has both pre- and postsynaptic effects. Since the depolarization was also blocked by the broad spectrum antagonist kynurenate, it is likely that it is mediated by an excitatory amino acid although the type of receptor involved (i.e. NMDA or non-NMDA) has not been clarified. Studies *in vivo* have shown that anoxia induces an enhanced release of glutamate (Benveniste et al., 1984). In this laboratory, preliminary *in vitro* experiments confirm that brief anoxic episodes also release endogenous glutamate from hippocampal slices (V. Crepel, G. Charton, M. P. Roisin, and Y. Ben-Ari, unpublished observations).

Interestingly, in CA1 neurons the principal effect of anoxia was the activation of a K+ conductance, and a depolarization was only seen with relatively long periods of anoxia (>5 min, Fujiwara et al., 1987). The difference between the effects of anoxia on CA3 and CA1 neurons has been recently confirmed in this laboratory using the same (submerged) perfusion chamber (Cherubini et al., 1989). Further, GLIB and other sulfonylureas have no effect on the anoxic response of CA1 neurons (Krnjevic and Leblond, 1988). Therefore, regional differences are likely to occur with regard to the increase in transmitter release induced by anoxia (see below).

GAL reduces and GLIB augments anoxic depolarization

The principal conclusion of the present study is that GAL and GLIB modulate in an opposite manner the anoxic depolarization in CA3 neurons; that is, GAL reduces and GLIB augments the release of glutamate induced by anoxia. Since both agents had minimal effects in control oxygenated ACSF, it is likely that this action was mediated by K+ATP channels, since these would be opened during anoxia because of the reduction in intracellular ATP (e.g. Kass and Lipton, 1982). Other mechanisms however cannot be excluded. Thus, although

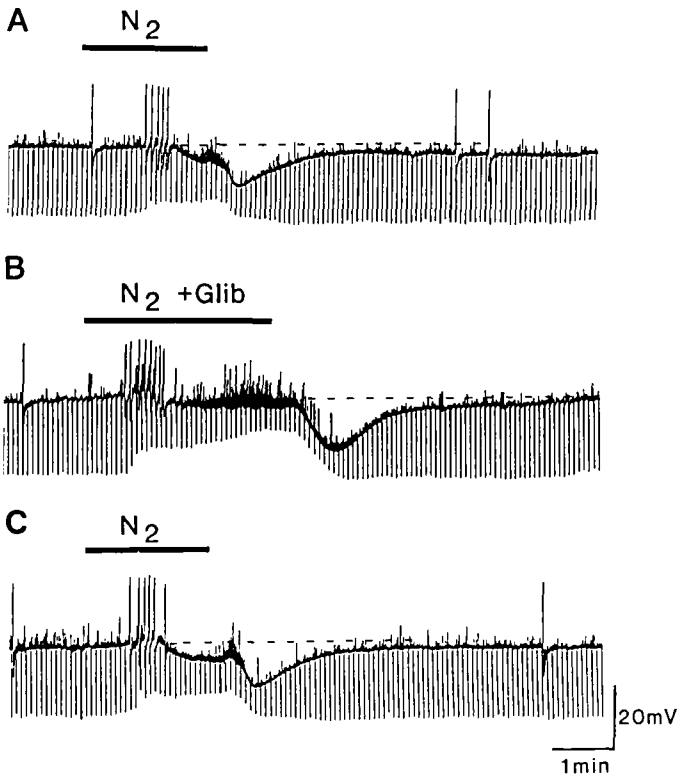

FIG. 5. (A,B) GLIB enhances synaptic noise during anoxia; in control conditions anoxic episode (A) induced a hyperpolarization which was masked by the increase in synaptic activity during GLIB (B).
(C) Recovery after wash of GLIB.

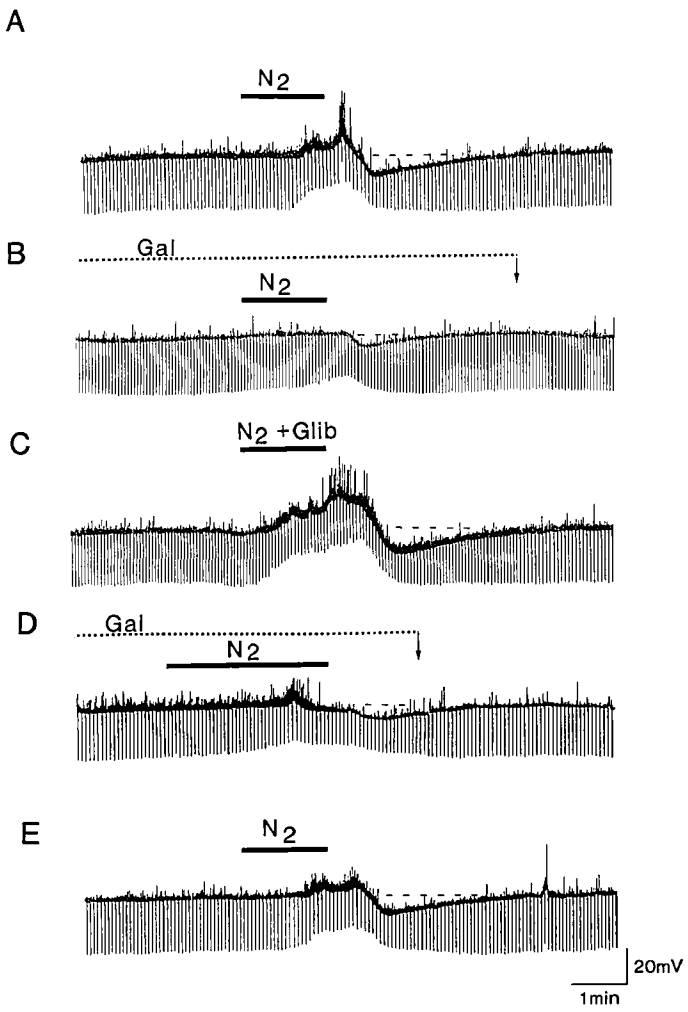

FIG. 6. Galanin blocks the depolarizing effects of anoxia.
(A) Anoxia (90 s) produced a depolarization and post-anoxic hyperpolarization.
(B) Galanin (1 μM), which in oxygenated ACSF produced no effect, in oxygen-deficient ACSF fully blocked the anoxic response.
(C) Glibenclamide (5 μM) increased anoxic depolarization.
(D) Second application of galanin reduces the anoxic depolarization.
(E) Partial recovery 15 min after wash in galanin.

GLIB does not reduce calcium currents in pancreatic B cells (Schmid-Antomarchi et al., 1987), GAL has been shown to decrease membrane excitability in mudpuppy cardiac ganglia (Konopka et al., 1989) and in myenteric neurons (Tamura et al., 1988) by opening K+ channels but also by a block of voltage-gated Ca++ channels.

Whatever the exact mechanism involved, the present observations suggest that there is in CA3 neurons a presynaptic modulation of transmitter release by agents known to act on K+ATP channels. Histological data support this view since the CA3 and hilar regions have a more extensive distribution of GAL immunoreactivity than other hippocampal regions (Skofitch and Jacobowitz, 1985; Melander et al., 1986) and the mossy fibre terminal region is particularly enriched with high affinity binding sites for GLIB (Mourre et al., 1989). The mossy fibres which originate in the granule cells of the fascia dentata and establish giant synaptic contacts with the apical dendrites of CA3 neurons are probably the sites where a K+ATP channel regulation of transmitter release is operated. The involvement of K+ATP channels in transmitter release from mossy fibres is probably a key factor in anoxic and epileptic brain damage since (i) the preferential destruction of CA1 neurons *in vivo* by anoxia-ischemia (Kirino, 1982; Benveniste et al., 1984; Pulsinelli et al., 1982; Smith et al., 1984) depends upon the integrity of the mossy fibre (Johansen et al., 1986) and the projections from CA3 to CA1 (Onodera et al., 1986), and (ii) mossy fibres have a central role in the preferential destruction of CA3 neurons seen in animal models of human temporal lobe epilepsy (refs in Ben-Ari, 1985). This is also suggested by preliminary observations that GLIB (Ben-Ari, unpublished observations), like repeated anoxic releases (Ben-Ari and Cherubini, 1988) or a variety of convulsant agents or procedures (Mody et al., 1987; Rutecki et al., 1985; Neuman et al., 1989; Ben-Ari and Gho, 1988), generate recurrent interictal discharge in CA3 neurons and produce a long-lasting change in synaptic activity of hippocampal circuitry (Ben-Ari and Gho, 1988).

To conclude, the present observations suggest that K+ATP channels modulate alterations in transmitter release which occur during anoxia. Interestingly, preliminary observations suggest that diazoxide, a well-established activator of K+ATP channels with little effect on other K+ channels (Trube et al., 1986; Ashcroft, 1988), also reduces anoxic depolarization in CA3 neurons (Ben-Ari and Krnjevic, unpublished observations). By preventing the excessive release of glutamate and other excitotoxic agents, GAL, diazoxide, and related agents may provide a tissue specific protection against anoxic damage since a fall in ATP is required to permit the action of K+ATP activators.

Acknowledgements

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Abbreviations

ACSF	artificial cerebrospinal fluid
CA1-CA1	hippocampal fields
GAL	galanin
GLIB	glibenclamide
K+ATP	ATP sensitive K+ channels
NMDA	N-methyl-D-aspartate
TTX	tetrodotoxin

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