ELSEVIER

European Journal of Pharmacology 287 (1995) 197–200

ejp

Short communication

Interactions of *N*-ethylmaleimide and aluminium fluoride with $GABA_B$ receptor function in rat neocortical slices

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Received 13 September 1995; accepted 10 October 1995

Abstract

Interactions of *N*-ethylmaleimide and aluminium fluoride (AlF_4^-) with $GABA_B$ receptors have been examined using spontaneously discharging rat neocortical slices. The suppression of discharges by the $GABA_B$ receptor agonist baclofen (5–10 μM) was irreversibly prevented by *N*-ethylmaleimide (10–50 μM) and its analog *N*-phenylmaleimide (10–50 μM), whilst superfusion of slices with NaF (10 mM) and $AlCl_3$ (100 μM) to form a fluoroaluminate (AlF_4^-) complex markedly potentiated the action of baclofen. The lipoxygenase inhibitors, nordihydroguaiaretic acid (10–50 μM) and eicosatetraynoic acid (10–50 μM) or the phospholipase A_2 inhibitor bromophenacylbromide (50–100 μM) did not affect the response to baclofen. The depressant action of baclofen is evidently mediated through G-proteins, but is not dependent on arachidonic acid metabolites.

Keywords: $GABA_B$ receptor; Baclofen; Neocortical slice, rat; *N*-Ethylmaleimide; Aluminum fluoride; G-protein

1. Introduction

$GABA_B$ receptors are a distinct subclass of receptors for the major inhibitory transmitter 4-aminobutanoic acid (GABA) that mediate depression of synaptic transmission. Baclofen, a β-*p*-chlorophenyl derivative of GABA, is a well characterised specific agonist for bicuculline-insensitive $GABA_B$ receptors (Bowery, 1993). The latter are known to be coupled to a variety of biochemical pathways, and can either inhibit adenylate cyclase activity or potentiate cyclic AMP formation induced by other neurotransmitters, as well as stimulate GTPase activity (for review see Bowery, 1993). $GABA_B$ receptors are also linked to either Ca^{2+} or K^+ channels, through guanosine triphosphate (GTP)-binding regulatory proteins (G-proteins) (Dolphin and Scott, 1987; Dutar and Nicoll, 1988), and activation of these receptors can reduce voltage-dependent Ca^{2+} conductance in presynaptic nerve terminals (Dolphin and Scott, 1987), or hyperpolarise postsynaptic neurones by increasing K^+ permeability (Dutar and Nicoll, 1988). The $GABA_B$ receptor-mediated inhibitory postsynaptic potentials are mimicked by the GTP analog GTPγS, are blocked by pertussis toxin, and are antagonised by the hydrolysis-resistant GDP analog, GDPβS (Nicoll et al., 1990). In addition, agonist binding to the $GABA_B$ receptor in brain membrane preparations is inhibited by guanine nucleotide (Asano et al., 1985), all of which indicates the involvement of G_i/G_o-proteins in $GABA_B$ receptor function.

N-Ethylmaleimide, a sulfhydryl alkylating agent which selectively inactivates pertussis toxin-sensitive G-proteins (Jakobs et al., 1982; Shapiro et al., 1994), is known to inhibit GABA binding to $GABA_B$ receptors in bovine cerebral cortex by modifying the inhibitory GTP-binding proteins (G_i-proteins) linked to these receptors (Asano and Ogasawara, 1986). On the other hand, fluoride ions can activate G-proteins directly, an effect that may be mediated by the fluoroaluminate complex, AlF_4^- (Bigay et al., 1987; Sondek et al., 1994; Sternweis and Gilman, 1982), but so far, it is not known if *N*-ethylmaleimide or AlF_4^- will modify functional responses to $GABA_B$ receptor activation in the isolated neocortical slice. It has also been proposed that neural responses to activation of $GABA_B$ receptors may involve an arachidonic acid cascade (Gage, 1992). Using AlF_4^- and *N*-ethylmaleimide, the present study aims to determine whether the $GABA_B$ receptor-mediated responses in the spontaneously active neocortical slice preparation might be mediated

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SSDI 0014-2999(95)00659-1

through G_i/G_o-proteins, and if this action requires any involvement of arachidonic acid metabolites.

2. Materials and methods

2.1. Rat neocortical slice preparations

Outbred male adult Sprague-Dawley rats (250–350 g) were decapitated, their brains rapidly removed and immersed for 15 min in ice-cold Krebs solution oxygenated with 95% O_2 and 5% CO_2. Cerebral cortical slices were prepared by cutting coronal sections approximately 400 μm thick using a vibraslice microtome (Campden Instruments, UK). Using a superfusion method based on a grease-gap system (Kerr et al., 1988), the neocortex was initially superfused with Mg^{2+}-containing Krebs medium at 28°C delivered by a peristaltic pump at 1 ml/min, and allowed to equilibrate for 30 min, followed by Mg^{2+}-free medium. The composition of the Krebs medium was as follows (mM): NaCl 118, KCl 2.1, KH_2PO_4 1.2, $CaCl_2$ 2.0, $NaHCO_3$ 25, glucose 11, $MgSO_4$ 1.3, pH 7.4. For the Mg^{2+}-free medium, $MgSO_4$ was omitted. DC potentials between the cingulate cortex and corpus callosum were monitored by Ag/AgCl electrodes via agar/saline bridges with a high-input impedance DC amplifier, and responses displayed on a chart recorder.

The neocortical slices developed spontaneous paroxysmal discharges after equilibration for 60 min under Mg^{2+}-free conditions, and control responses were established for 30 min before superfusion with any drugs. The $GABA_B$ receptor agonist, baclofen, was subsequently applied to the cortical side of the tissues for 2–5 min, usually at 30–60 min intervals depending on the recovery of the responses to control level. A dose of 10 μM baclofen was routinely used and challenged with various concentrations of the compounds tested. All other drugs were initially superfused over the slice for at least 10–30 min in order to test their effects on the spontaneous activity, before added together with baclofen. In fluoride-containing Krebs medium, NaF (10 mM) was substituted isosmotically for NaCl, and $AlCl_3$ (100 μM) was added to generate aluminium fluoride (AlF_4^- complex); the latter on its own did not significantly affect the amplitude or the rate of discharge. Each experiment was repeated on 6 slices from 3 different animals, and n represents the number of preparations which showed the effect of each drug studied.

2.2. Drugs

(±)-Baclofen was a gift from Ciba-Geigy (Basel, Switzerland). *N*-Ethylmaleimide, nordihydroguaiaretic acid, eicosatetraynoic acid and bromophenacylbromide were obtained from Sigma. *N*-Phenylmaleimide was a gift from Lancaster Chemicals. Stock solutions of drugs insoluble in distilled water were made either in dimethyl sulphoxide or ethanol and kept at −20°C; the final concentrations of dimethyl sulphoxide and ethanol never exceeded 0.1%. Dimethyl sulphoxide and ethanol by themselves did not affect the spontaneous discharges or responses to baclofen. Prior to use, they were added to the Krebs solution to achieve the required concentration.

3. Results

In the rat neocortical slices, removal of Mg^{2+} from the superfusion medium for 60 min resulted in the appearance of repetitive spontaneous discharges, in the form of depolarising potentials with afterdischarges. Such spontaneous discharges were consistently modified and reduced by baclofen in a dose-dependent and reversible manner (1–50 μM; Kerr et al., 1988), with recovery of the activity generally being observed within 30 min after drug wash-out. Due to the complex interactions between discharge amplitude, after-potential duration, and inter-discharge interval, we have found it difficult to construct quantitative dose-response curves for the depressant actions of baclofen. Nevertheless, the relative potency of baclofen under various conditions could be derived from the minimal dose causing total arrest of the discharges. In this study, baclofen alone (10 μM) applied to the slice abolished the spontaneous discharge within 5 min, and complete recovery from the effect occurred within 30 min after commencing wash-out. The response to baclofen was remarkably consistent, and reproducible, in each of the brain slices used.

N-Ethylmaleimide (10 μM) applied 30 min before baclofen (10 μM) prevented the depressant effect of baclofen on the spontaneous discharges. Such prolonged pretreatment was not, however, necessary for a block of baclofen to occur. When applied for 2 min, and then for a further 4 min together with baclofen, *N*-ethylmaleimide slightly increased the frequency of discharges, and reduced their amplitude, during which time the depressant action of baclofen was blocked (Fig. 1a, $n = 6$). Such blocking actions of *N*-ethylmaleimide were seen over a concentration range of 10–50 μM and persisted for at least 30 min after changing back to control Krebs medium, during which time baclofen (10 μM) remained ineffective on the discharges. The action of baclofen did not recover even after prolonged wash-out periods of up to 60 min, so that control responses could not be re-established over that period of time. Indeed, most often, prolonged application of *N*-ethylmaleimide eventually led to a deterioration of the slice, with a loss of spontaneous activity. *N*-Phenylmaleimide (5 μM), a more potent alkylating agent, induced similar effects to *N*-ethyl-

maleimide (10 μM), and was therefore at least twice more potent than the latter in blocking the depressant effect of baclofen (Fig. not shown; $n = 6$).

Superfusion of slices with NaF (10 mM) and $AlCl_3$ (100 μM) to form a fluoroaluminate (AlF_4^-) complex markedly potentiated the response to baclofen (5 μM) within 10 min, where there was a complete and prolonged suppression of the discharges. However, on its own, AlF_4^- when applied over a 10 min period, decreased the amplitude of the discharge without affecting its rate (Fig. not shown). In order to illustrate more effectively the enhancement of the $GABA_B$ receptor-mediated response to baclofen by AlF_4^-, a lower dose of baclofen (5 μM) that induced a less marked depressant effect was chosen (Fig. 1b; $n = 6$). This particular combination produced a total cessation of spontaneous discharges for 12 min, outlasting the period of application by 7 min. Such a significant potentiation of the baclofen response by AlF_4^- was seen over a concentration range of 5–10 μM baclofen. Upon wash-out of the fluoride medium, in general, complete return of the discharges to control levels occurred within 30 min, although in 50% of the slices tested, the rate of discharge remained slightly increased. Nonetheless, the responses to baclofen recovered within 30 min to levels similar to those seen before treatment with AlF_4^-. Control slices were exposed to 10 mM NaF without added Al^{3+} so that the effects of AlF_4^- could be separated from any action of fluoride alone. There was no discernible change in the spontaneous activity of the slices treated in this manner by NaF alone.

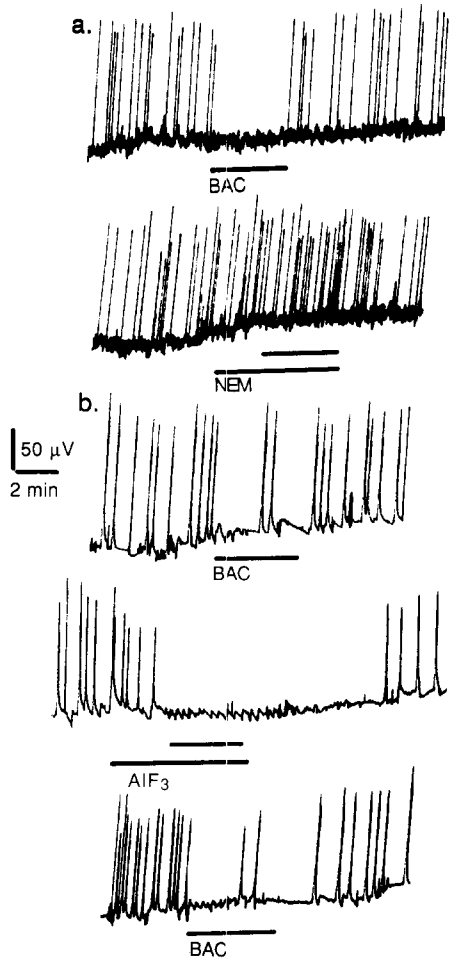

Fig. 1. In the rat spontaneously discharging neocortical slice, maintained in Mg^{2+}-free Krebs solution, (a) the depressant effect of baclofen (10 μM) on the spontaneous discharges was prevented by *N*-ethylmaleimide (NEM; 10 μM; $n = 6$). The action of baclofen did not recover even after prolonged wash-out periods of up to 60 min, so that control responses could not be re-established. (b) Superfusion of the slice with NaF (10 mM) and $AlCl_3$ (100 μM) to form a fluoroaluminate complex (AlF_3) markedly potentiated the response to baclofen (5 μM; $n = 6$), where there was a complete and prolonged suppression of the discharges, with a recovery of the spontaneous discharges after 7 min of drug wash-out. Following a further 30 min of drug wash-out, the depressant response to baclofen returned to control levels (bottom trace).

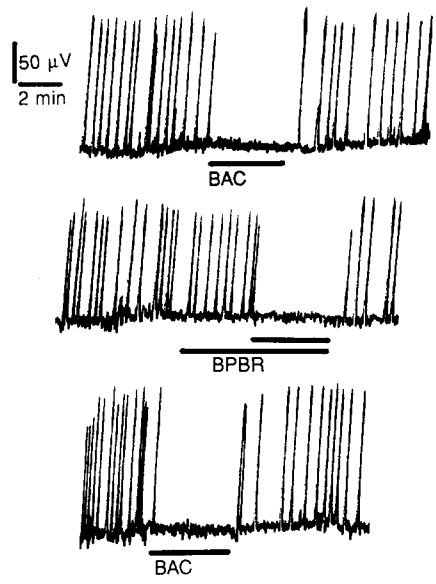

Fig. 2. Lack of effect of a phospholipase A_2 inhibitor, bromophenacylbromide (BPBR; 100 μM), on baclofen (10 μM)-induced suppression of spontaneous discharges. Bromophenacylbromide did not affect the spontaneous discharges themselves ($n = 4$).

In order to assess if arachidonic acid metabolites may be involved in the $GABA_B$ receptor-mediated action, nordihydroguaiaretic acid (10–50 μM), eicosatetraynoic acid (10–50 μM) and bromophenacylbromide (BPBR; 50–100 μM; Fig. 2) were tested against baclofen (10 μM) responses. None of these affected the spontaneous discharges, nor did they modify the responses to baclofen ($n = 4$).

4. Discussion

In rat neocortical slice preparations, the $GABA_B$ receptor agonist baclofen attenuates the amplitude and

rate of discharge of spontaneous activity occurring in Mg^{2+}-free Krebs medium. This action is mediated through $GABA_B$ receptor sites, and is sensitive to 2-hydroxysaclofen, a competitive $GABA_B$ receptor antagonist (Kerr et al., 1988). Apart from the knowledge that $GABA_B$ receptors belong to the superfamily of receptors coupled to GTP-binding proteins, the transduction pathways involved in their modulatory actions on synaptic transmission and neuronal transmission remain a matter of debate. Nevertheless, here, the abolition of the $GABA_B$ receptor-mediated response in the neocortical slice by *N*-ethylmaleimide and its more potent analog, *N*-phenylmaleimide, together with its marked potentiation by AlF_4^-, is consistent with the involvement of G_i/G_o-proteins in this particular action of baclofen. It has been suggested that $GABA_B$ receptor-mediated actions on presynaptic Ca^{2+} influx and postsynaptic inhibitory increase in K^+ conductance might involve arachidonic release or production of arachidonic acid metabolites (Gage, 1992). However, against this, neither bromophenacylbromide, a phospholipase A_2 inhibitor, nor the lipoxygenase inhibitors, nordihydroguaiaretic acid or eicosatetraynoic acid, had any influence on the depression of the spontaneous discharges by baclofen.

It has been reported that *N*-ethylmaleimide inactivates GTP-binding proteins, mainly pertussis toxin-sensitive G_i/G_o-proteins (Jakobs et al., 1982), uncoupling these from the $GABA_B$ receptors. This uncoupling of $GABA_B$ receptors from G-proteins by *N*-ethylmaleimide involves alkylation of a sulfhydryl group, where *N*-ethylmaleimide appears to interfere with the interaction between α and $\beta\gamma$ subunits of the G-proteins (Asano and Ogasawara, 1986). In cultured dorsal root ganglion neurons, $GABA_B$ receptor-mediated inhibition of N-type calcium channels occurs via G_o-proteins (Menon-Johansson et al., 1993), and a presynaptic action of this kind is likely involved in the arrest of neocortical spontaneous discharge by baclofen in the present study. By contrast, AlF_4^-, which activates members of the heterotrimeric G-protein family by binding near the site occupied by the γ-phosphate (Sondek et al., 1994), substantially enhanced the depressant action of baclofen in the slice, again reinforcing the notion that this response is mediated via G-proteins. Such effect of AlF_4^- in depressing synaptic transmission, in rat hippocampal slices, has been described previously (Breakwell and Publicover, 1994). Here, AlF_4^- on its own reduced the amplitude but did not completely halt the spontaneous discharges, their total abolition requiring the combined action of AlF_4^- and a low, partially effective, concentration of baclofen. Apart from reinforcing that baclofen responses mediated by $GABA_B$ receptors clearly involve G-protein activation, the effects of *N*-ethylmaleimide and AlF_4^- are of interest in themselves, as AlF_4^- provides a useful albeit non-physiological means for activating G-protein-coupled processes. The latter would be worth pursuing in greater detail in pre- and postsynaptic responses in hippocampal slice preparations.

Acknowledgements

We thank the Australian Research Council for the award of a Research Fellowship to J.O.

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