Peripheral benzodiazepine receptors in platelets of epileptic patients

J. G. LARKIN, P. J. W. MCKEE, G. G. THOMPSON & M. J. BRODIE

Epilepsy Research Unit, University Department of Medicine and Therapeutics, Western Infirmary, Glasgow, Scotland

Tolerance to the anticonvulsant effect of benzodiazepines is likely to involve changes at the central benzodiazepine-GABA receptor complex. Peripheral benzodiazepine receptors (pBZRs), which can be measured in platelets, may also be involved. Using a binding assay with [³H]-PK 11195 as radioligand, pBZRs were assayed in platelets of patients taking a variety of antiepileptic drugs (AEDs). Comparisons were made with untreated patients. pBZR receptor density (mean $\pm$ s.e. mean) was increased *vs* controls (8083 ± 557 fmol mg⁻¹ protein) in the platelets of patients taking a polypharmacy regime including the benzodiazepine clobazam (12661 ± 1011 fmol mg⁻¹ protein, $P < 0.005$) and also in those receiving sodium valproate as monotherapy (15003 ± 1756 fmol mg⁻¹ protein, $P < 0.01$). The significance of these findings is unclear, but the use of a specific pBZR antagonist may be a promising avenue for investigating the mechanism of BZ tolerance and its prevention.

Keywords antiepileptic drugs benzodiazepines epilepsy receptors tolerance

Introduction

Benzodiazepines (BZs) are effective antiepileptic drugs [1], but tolerance to their anticonvulsant efficacy is a major drawback to their use [2]. Insight into the mechanism responsible for the development of tolerance might benefit from improved knowledge of the effects of these drugs on BZ receptors (BZRs). A variety of receptors have been identified [3]. These include a high affinity 'central' BZR (cBZR), which is linked to the gamma-aminobutyric acid (GABA) receptor/Cl⁻ ionophore complex in the brain. Affinity to this receptor seems to correlate with the anticonvulsant and anxiolytic actions of many BZs [4,5]. More recently a 'peripheral' BZR (pBZR) has been identified. This occurs in various tissues, including the brain and platelets [6,7], the latter giving an accessible medium for study in man.

Tolerance would logically concern the cBZR, most likely via changes in GABA sensitivity [8,9]. However, there is some evidence that the pBZR may also be involved. The anticonvulsant effects of carbamazepine include pBZR-mediated actions [10], while this drug exhibits cross-tolerance with pBZR ligands [11]. Whether changes in pBZRs in platelets reflect changes in brain pBZRs is doubtful [12], but numbers in platelets have been shown to increase with diazepam therapy for depression [13] and with carbamazepine treatment for

epilepsy [14]. If tolerance to BZs or other anticonvulsants does involve the pBZR, changes in numbers or affinity of these receptors might occur in patients taking long-term therapy with such drugs. In the present study, pBZR affinity and numbers were measured in the platelets of epileptic patients taking various antiepileptic drugs, including the BZ clobazam. Untreated epileptic patients were utilised as comparative controls, as the disorder itself may induce changes [14].

Methods

Patients

Eighty-nine patients attending the epilepsy clinic at the Western Infirmary in Glasgow agreed to take part in the study, which was approved by the local ethics committee. Blood was obtained for platelet receptor analysis and anticonvulsant concentration assay at their routine clinic visit. Clinical details were noted at the same time (Table 1). Patients in the untreated group were either recently diagnosed as having epilepsy or had been withdrawn from treatment following a prolonged seizure-free period. No patient had suffered a

Table 1 Clinical details of patients in each treatment group. Values are mean (range) $\pm$ s.d.

	n	Male:Female	Age (years)	Daily dose (mg)	Concentration (mg l ⁻¹)
Untreated	17	7:10	27 (16–45)	—	—
Sodium valproate	7	1:6	31 (25–42)	1850 (1000–2500) $\pm$ 470	86 (60–131) $\pm$ 26.9
Carbamazepine	17	6:11	33 (15–52)	1040 (400–1400) $\pm$ 320	9.5 (3.7–15.4) $\pm$ 3.2
Phenytoin	15	8:7	40 (25–65)	396 (200–500) $\pm$ 128	21.6 (8.8–45) $\pm$ 11.9
Polypharmacy without clobazam	14	6:8	28 (15–43)	—	—
Polypharmacy with clobazam	19	12:7	36 (21–54)	23.7 (10–40) $\pm$ 10.1	

seizure in the week prior to entering the study. The 19 patients, who were receiving clobazam, took this drug in addition to a range of other anticonvulsants (10 carbamazepine, two sodium valproate, one phenytoin, one phenobarbitone, five polypharmacy including sodium valproate). Blood was drawn between 13.30 h and 15.30 h by standard venepuncture and collected in 2.2% sodium citrate/1.2% citric acid for receptor assay. Anticonvulsant concentrations were measured on site at the clinic [15] by enzyme immunoassay (Emit, Syva, Palo Alto, USA).

Tissue preparation

The assay largely followed the methods of Gavish *et al.* [16]. Briefly, anticoagulated blood was centrifuged immediately after collection at 900 rev min⁻¹; 15 min; 23° C. The platelet-rich supernatant was collected and centrifuged at 3000 rev min⁻¹; 15 min; 23° C. The resultant platelet pellet was immediately frozen (–20° C) for batch analysis within 4 days. Reconstitution was by thawing and homogenising in 50 mM Tris HCl buffer pH 7.4 followed by centrifugation at (49000 g); 15 min; 4° C. The last two procedures were repeated once, before homogenisation of the pellet in Tris HCl buffer, measurement of protein [17] and adjustment of protein concentration to 0.7–1 mg ml⁻¹ (final assay concentration 0.4–0.6 mg ml⁻¹) using further buffer.

Binding assay

Tritiated PK11195 ([³H]-PK11195) was used for the assay. Platelet solution, (200 μ l) prepared as above, were incubated with 50 μ l [³H]-PK11195 together with 50 μ l buffer for total binding or 50 μ l 'cold' ligand (Ro 5–4864, final assay concentration 1 μ M) for nonspecific binding. Incubations at six different concentrations of [³H]-PK11195 (0.55–27.75 nM) were performed in

duplicate for estimation of dissociation constant (K_d) and numbers (B_{max}) of the peripheral BZR. Assays were incubated for 60 min at 0° C and the reaction was terminated by vacuum filtration through GF-B filters using a Brandel Cell Harvester (3–4 washes with 5 ml 50 mM Tris HCl buffer pH 7.4). The filters were dried overnight and then placed in plastic phials with Scintillin. Binding was counted and analysed using a Packard 2000CA scintillation counter with Combicept 1.1 computer programme.

K_d and B_{max} results were estimated using both Scatchard and saturation isotherm analyses. Assays were considered satisfactory if the correlation coefficient on the Scatchard line was >0.875 and the Scatchard and saturation isotherm calculations did not differ by >25%. The mean of these two calculations was recorded as the assay result. Preliminary experiments in 10 control subjects revealed a mean (s.d.) K_d of 2.92 (0.897) nM and a B_{max} of 9729 (4749) fmol mg⁻¹ protein. Coefficient of variance in repeated assay of single samples was approximately 14% for K_d and 10% for B_{max} .

Statistics

Comparisons of the results in the different treatment groups were performed using initial analysis of variance with subsequent *t*-testing against controls, if the former was found to be significant. Correlations quoted are Pearson's moment correlations.

Results

The results (mean $\pm$ s.d.) in men did not differ significantly from those in women for either K_d (3.25 $\pm$ 1.3 vs 3.51 $\pm$ 1.87 nM) or B_{max} (9854 $\pm$ 3845 vs 10392 $\pm$ 4765 fmol mg⁻¹ protein) and no sex difference was seen

Figure 1 Peripheral benzodiazepine receptor K_d and B_{max} in platelets of untreated epileptic patients and patients taking antiepileptic drugs (VPA sodium valproate, CBZ carbamazepine, PHT phenytoin, poly polypharmacy without clobazam, clobazam polypharmacy with clobazam). Receptors were measured by binding assay using [³H]-PK 11195 (0.55–27.75 nM) with Scatchard and isotherm analyses. Results are shown as mean $\pm$ s.e. mean.
* $P < 0.01$ ** $P < 0.0005$ vs untreated group.

within any of the treatment groups including the untreated 'controls'. Age showed no significant correlation with K_d or B_{max} either overall ($r = 0.036$ and -0.068 respectively) or within the treatment groups.

Mean ($\pm$ s.e. mean) results in the six treatment groups are compared in Figure 1. Analysis of variance did not confirm an abnormal distribution of K_d between the groups, and the apparently raised K_d with sodium valproate did not reach conventional levels of significance. However, the B_{max} was shown to be affected by therapy ($P < 0.02$); specifically, polypharmacy with clobazam ($P < 0.0005$) and sodium valproate monotherapy ($P < 0.01$) appeared to increase pBZRs on platelets. The apparent increases with carbamazepine and polypharmacy without clobazam were not significant, although circulating concentrations of carbamazepine were shown to correlate with both K_d ($r = 0.446$, $P < 0.05$) and B_{max} ($r = 0.544$, $P < 0.02$).

To exclude the possibility that the patients in the clobazam groups who were also taking sodium valproate had produced an apparent increase in B_{max} due to clobazam, B_{max} results (mean $\pm$ s.d.) in the other patients ($13,246 \pm 5107$ fmol mg⁻¹ protein) were separately compared with controls, and found to be increased ($P < 0.001$). Results in polypharmacy

patients taking sodium valproate did not differ significantly from the others in the group.

Discussion

The present study demonstrated an increase in pBZR numbers in the platelets of epileptic patients treated with polypharmacy regimens including clobazam and with sodium valproate monotherapy compared with epileptic patients on no therapy. Receptor affinities were essentially unchanged. The increase in pBZR numbers with certain anticonvulsants may be a move towards normal levels, as has been shown with diazepam in untreated depressed patients who have decreased receptor density [13]. Our untreated patients did have lower receptor numbers than healthy controls, but these assays were not performed simultaneously for direct comparison. The previously described upregulation with carbamazepine [14] could not be demonstrated directly in this study, although circulating concentrations of the drug were shown to correlate with K_d and B_{max} .

The significance of these changes is uncertain. We have previously found that a marked BZ-induced increase in platelet pBZR numbers in rats did not reflect changes in the brain cBZRs [12]. Although no association with brain pBZRs was shown, the small numbers failed to exclude this possibility. The present results, therefore, do not suggest similar occurrences in brain cBZRs, but pBZRs may be altered and this may be of significance. The proconvulsant and anticonvulsant actions of cBZR and pBZR ligands have been reviewed [6,7]. They appear complex and possibly interconnected. While cBZR agonists are usually anticonvulsants, agonism at the pBZR may be proconvulsant, and may mediate the epileptogenic effects of pyrethroid insecticides [18]. Specific pBZR agonists may induce convulsions which can be inhibited by pBZR antagonists [19] but also, surprisingly, by the cBZR antagonist flumazenil [20].

Peripheral BZRs may also be involved in the action of established antiepileptic drugs. The anticonvulsant action of carbamazepine can be blocked by a specific pBZR ligand [10]. Chronic dosing with carbamazepine can produce tolerance which is accompanied by tolerance to specific pBZR ligand effects — the actions of cBZR ligands remaining unaffected [11]. These findings might suggest a role for pBZRs in the mechanism of tolerance to CBZ. Tolerance to BZ effects might also involve the pBZR, as pBZR antagonists will inhibit tolerance to the anticonvulsant effects of diazepam and clonazepam [21]. It is worth noting that tolerance to diazepam seems to develop more quickly than with the latter drug [2]. Experimental studies show tolerance to diazepam at 5 days [22] similar to that with clobazam at 4 days [23], while clonazepam has been shown to cause markedly less and slower tolerance than clobazam [24]. The lack of an active metabolite with clonazepam is a suggested explanation but diazepam's increased affinity for the pBZR (K_i 0.013 μ M) vs clonazepam ($K_i > 10$ μ M) would be consistent with pBZRs being involved [6].

The cBZR receptor antagonist flumazenil (Ro 15-1788) has been suggested as a possible agent to 'reset' the central receptor and reduce BZ tolerance [25]. The dangers of inducing BZ withdrawal seizures are being assessed [26]. The use of specific pBZR antagonists

might be an alternative avenue for investigating the mechanism of BZ tolerance and its prevention.

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References

- Robertson MM. Current status of the 1,4- and 1,5-benzodiazepines in the treatment of epilepsy: the place of clobazam. *Epilepsia* 1986; **27** (Suppl 1): S27-S41.
- Haigh JRM, Feely M. Tolerance to the anticonvulsant effect of benzodiazepines. *Trends pharmac Sci* 1988; **9**: 361-366.
- Meldrum BS, Chapman AG. Benzodiazepine receptors and their relationship to the treatment of epilepsy. *Epilepsia* 1986; **27** (Suppl 1): S3-S13.
- Colburn WA, Jack ML. Relationships between CSF drug concentrations, receptor binding characteristics and pharmacokinetic and pharmacokinetic properties of selected 1,4-substituted benzodiazepines. *Clin Pharmacokin* 1987; **13**: 179-190.
- Villar HO, Uyeno ET, Toll L, Polgar W, Davies MF, Loew GH. Molecular determinants of benzodiazepine receptor affinities and anticonvulsant activities. *Mol Pharmac* 1989; **36**: 589-600.
- Saano V, Rago L, Raty M. Peripheral benzodiazepine binding sites. *Pharmac Ther* 1989; **41**: 503-514.
- Verma A, Snyder SH. Peripheral type benzodiazepine receptors. *Ann Rev Pharmac Tox* 1989; **29**: 307-322.
- Hernandez TD, Rosen JB, Gallagher DW. Relationship of agonist efficacy to changes in GABA sensitivity and anticonvulsant tolerance following chronic benzodiazepine ligand exposure. *Eur J Pharmac* 1989; **170**: 145-155.
- Little HJ. The benzodiazepines: anxiolytic and withdrawal effects. *Neuropeptides*, 1991; **19** (Suppl.): 11-14.
- Weiss SRB, Post RM, Patel J & Marangos PJ. Differential mediation of the anticonvulsant effects of carbamazepine and diazepam. *Life Sci* 1985; **36**: 2413-2419.
- Weiss SRB, Post RM. Contingent tolerance to carbamazepine: a peripheral-type benzodiazepine mechanism. *Eur J Pharmac* 1991; **193**: 159-163.
- Larkin JG, Thompson GG, Scobie G, Brodie MJ. Central and peripheral benzodiazepine receptors in rat brain and platelets: effects of treatment with diazepam and clobazam. *Seizure* 1992; **1**: 177-182.
- Weizman R, Tanne Z, Graner M, Karp L, Golomb L, Tyano S *et al*. Peripheral benzodiazepine binding sites on platelet membranes are increased during diazepam treatment of anxious patients. *Eur J Pharmac* 1987; **138**: 289-292.
- Weizman A, Tanne Z, Karp L, Martfeld Y, Tyano S, Gavish M. Carbamazepine up-regulates the binding of [³H]PK1195 to platelets of epileptic patients. *Eur J Pharmac* 1987; **141**: 471-474.
- Larkin JG, Herrick AL, McGuire GM, Percy-Robb IW, Brodie MJ. Antiepileptic drug monitoring at the epilepsy clinic: a prospective evaluation. *Epilepsia* 1991; **32**: 89-95.
- Gavish M, Weizman A, Karp L, Tyano S, Tanne Z. Decreased peripheral benzodiazepine sites in platelets of neuroleptic-treated schizophrenics. *Eur J Pharmac* 1986; **121**: 275-279.
- Peterson GL. A simplification of the protein assay method of Lowry *et al* which is more generally applicable. *Anal Biochem* 1977; **83**: 346-356.
- Devaud L, Szot P, Murray TF. PK11195 antagonism of pyrethroid-induced proconvulsant activity. *Eur J Pharmac* 1986; **21**: 269-273.
- File SE. Pro- and anticonvulsant properties of PK11195, a ligand for benzodiazepine binding sites: development of tolerance. *Br J Pharmac* 1984; **83**: 471-476.
- Massotti M, Lucantoni D. The peripheral benzodiazepine receptor ligand Ro5-4864 induces supraspinal convulsions in rabbits. Reversal by the central benzodiazepine antagonist Ro 15-1788. *Psychopharmac* 1986; **88**: 336-340.
- Massotti M, Mele L, De Luca C. The involvement of 'peripheral' benzodiazepine receptor type (omega 3) in the tolerance to the electroencephalographic effects of benzodiazepines in rats: comparison of diazepam and clonazepam. *Pharmac Biochem Behav* 1990; **35**: 933-936.
- File SE. Tolerance to the anti-pentylenetetrazol effects of diazepam in the mouse. *Psychopharmac* 1983; **79**: 284-286.
- Haigh JRM, Pullar T, Gent JP, Feely M. *N*-Desemethylclobazam: a possible alternative to clobazam in the treatment of epilepsy? *Br J clin Pharmac* 1987; **23**: 213-218.
- Gent JP, Feely MP, Haigh JRM. Differences between the tolerance characteristics of two anticonvulsant benzodiazepines. *Life Sci* 1985; **37**: 849-856.
- Nutt D, Costello M. Rapid induction of lorazepam dependence and reversal with flumazenil. *Life Sci* 1988; **43**: 1045-1054.
- Savic I, Widen L, Stone-Elander S. Feasibility of reversing benzodiazepine tolerance with flumazenil. *Lancet* 1991; **337**: 133-137.

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