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European Journal of Pharmacology 478 (2003) 11–19
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Localization and photoaffinity labelling of the levetiracetam binding site in rat brain and certain cell lines

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Received 3 April 2003; received in revised form 11 August 2003; accepted 19 August 2003

Abstract

Levetiracetam (2*S*-(2-oxo-1-pyrrolidinyl)butanamide, KEPPRA®), a novel antiepileptic drug, has been shown to bind to a specific binding site located in the brain (Eur. J. Pharmacol. 286 (1995) 137). To identify the protein constituent of the levetiracetam binding site in situ, we synthesized the photoaffinity label [^{3}H]ucb 30889 ((2*S*)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide), a levetiracetam analog with higher affinity for the levetiracetam binding site. This radioligand was used to map the levetiracetam binding site within the brain and to study its cellular and subcellular distribution. Autoradiography experiments using [^{3}H]ucb 30889 in rat brain revealed a unique distribution profile that did not match that of classical receptors known to be involved in the generation of epileptic seizures. There was a high level of binding in the dentate gyrus, the superior colliculus, several thalamic nuclei, the molecular layer of the cerebellum and to a lesser extent in the cerebral cortex, the striatum and the hypothalamus. The levetiracetam binding site was restricted to neuronal cell types, undifferentiated PC12 cells and was highly enriched in synaptic vesicles. [^{3}H]ucb 30889 was also used in photoaffinity labelling studies and shown to bind covalently to a membrane protein with a molecular weight of approximately 90 kDa.

Keywords: Anticonvulsant; Binding; Brain; Levetiracetam; Photoaffinity labelling

1. Introduction

Levetiracetam (2*S*-(2-oxo-1-pyrrolidinyl)butanamide, KEPPRA®) is a novel potent antiepileptic drug efficacious as adjunctive therapy to partial onset seizures in adults (Hovinga, 2001; Nash and Sangha, 2001). It displays a pharmacological profile distinct to that of established antiepileptic drugs. Although the mechanism of action for levetiracetam is not completely understood, it is thought that levetiracetam exerts its anticonvulsant action via multiple mechanisms, including suppression of inhibition of γ-aminobutyric acid (GABA)- and glycine-gated currents by Zn^{2+} and β-carbolines and partial inhibition of N-type voltage-gated Ca^{2+} channels (Margineanu and Klitgaard, 2002). A specific [^{3}H]levetiracetam binding site, which is saturable, reversible and stereoselective, has been found in rat brain (Noyer et al., 1995). The levetiracetam binding sites are preferentially located in the cortex, the hippocampus, the striatum and the cerebellum, but not detected in peripheral tissues. Levetiracetam does not displace radioligands for a variety of receptors and channels and more than 50 reference drugs were shown to lack affinity for this site. [^{3}H]levetiracetam was found to label a single site with micromolar affinity making it unsuitable for detailed characterization of the levetiracetam binding site. In a companion paper by Gillard et al. (2003), we describe the binding properties of [^{3}H]ucb 30889, (2*S*)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide, a novel and more potent analog of levetiracetam. [^{3}H]ucb 30889 displays 30-fold higher affinity for the levetiracetam binding site than [^{3}H]levetiracetam. Competition binding curves and tissue distribution of specific binding confirmed that [^{3}H]ucb 30889 and [^{3}H]levetiracetam bind to the same sites.

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doi:10.1016/j.ejphar.2003.08.033

Here we used this novel radioligand to map the levetiracetam binding site within the rat brain and study its cellular and subcellular distribution. In parallel, we used [^{3}H]ucb 30889 for photoaffinity labelling experiments in an attempt to identify the protein constituent of the levetiracetam binding site in situ.

2. Materials and methods

2.1. Drugs and radioligands

Levetiracetam was synthesized at UCB (Braine-l'Alleud, Belgium). [^{3}H]ucb 30889 (32 Ci/mmol) was custom labelled by Amersham Biosciences (Roosendaal, The Netherlands). [^{3}H]PK11195 (85.5 Ci/mmol) and [^{3}H]MK801 (28.9 Ci/mmol) were purchased from Perkin Elmer Life Sciences (Zaventem, Belgium) and [^{3}H]*N*-methylscopolamine (78 Ci/mmol) was purchased from Amersham Biosciences.

2.2. Autoradiography

Intact brains from three male Sprague–Dawley rats (250–300 g) were removed and rapidly frozen in isopentane (−20 °C). Brains were sectioned in the coronal plane (25 μm), mounted onto gelatine-coated microscope slides and stored at −20 °C until used. Slide-mounted brain sections were brought to room temperature and incubated twice for 10 min at room temperature in 50 mM Tris–HCl buffer (pH 7.4) containing 0.5% bovine serum albumin. The brain sections were incubated for 120 min at 4 °C in 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 0.05% bacitracine, 2 mM EGTA, 0.5% bovine serum albumin and 1.3 nM of [^{3}H]ucb 30889. Brain sections were evaluated for nonspecific binding by incubation under identical conditions in the presence of 1 mM levetiracetam. Following further slide washings (2 × 10 min) in ice-cold Tris–HCl buffer and in ice-cold water, sections were dried and apposed to [^{3}H]-sensitive Hyperfilm (Amersham Biosciences). After 3 weeks at −20 °C, the films were developed.

2.3. [^{3}H]ucb 30889 binding to whole cells

Cerebellar granule neurons were prepared from 7-day-old rat pups and cells were diluted to 2.5×10^6 cells/ml (Leprince et al., 1989). Fifty microliters of the cell suspension was plated into 24-well plastic culture dishes, and were used after 2 days of culture. The cell suspension from P0 mouse cortical astrocytes (Leprince et al., 1989) was grown and transferred, at a density of 1×10^5 cells per well, into 24-well plastic culture dishes. Other cell lines (PC12, SK-N-SH, NG108-15, N1E-115, HCN-la, CHO-K1, COS-7) were grown in appropriate media in 24-well plates at confluency for binding assays. Cells were slowly cooled to 4 °C and rinsed with cold phosphate buffered saline. For saturation studies, [^{3}H]ucb 30889 was used at concentrations ranging from 1 to 30 nM and higher concentrations were obtained by isotopic dilutions with cold ucb 30889. For all experiments, nonspecific binding was measured in the presence of 1 mM levetiracetam. Cells were incubated with the radioligand for 120 min at 4 °C and then rapidly washed three times with cold phosphate buffered saline before measurement of radioactivity by beta counting.

2.4. Preparation of subcellular fractions

The subcellular fractionation was prepared according to Lee et al. (2001) with slight modifications. Adult Sprague–Dawley rats were decapitated and the whole brains were removed. Brains were homogenized by disruption (10 strokes at 1,100 rpm using a motor-driven glass/teflon Potter homogenizer) in 0.32 M sucrose, 20 mM Tris–HCl pH 7.4, 2 mM EDTA containing protease inhibitors (Complete, Roche) and 0.25 mM phenylmethanesulfonyl fluoride. The homogenates were centrifuged at $1{,}000 \times g$ for 10 min to remove nuclei and large debris. The pellets (P1) were washed three times. The supernatants (S1) were pooled, centrifuged at $30{,}000 \times g$ for 20 min to obtain a crude synaptosomal fraction (P2) and a supernatant (S2). Subsequently, the P2 fraction was lysed hypo-osmotically (20 mM Tris–HCl, pH 7.4) and centrifuged at $25{,}000 \times g$ for 60 min to obtain a pellet (LP1) and a supernatant (LS1). The supernatant (S2) was spun down at $165{,}000 \times g$ for 60 min to obtain the pellet (P3) containing microsomal membranes and a supernatant (S3). The LS1 supernatant was centrifuged at $165{,}000 \times g$ for 60 min yielding a final pellet (LP2) enriched in synaptic vesicles. In order to isolate mitochondria and synaptic plasma membranes, the P2 fraction from a separate experiment was layered on top of a discontinuous gradient of sucrose (0.8, 1, 1.2 M) and centrifuged at $100{,}000 \times g$ for 120 min in a swing-out rotor. The membrane fractions were characterized by binding experiments with [^{3}H]ucb 30889, [^{3}H]*N*-methylscopolamine, [^{3}H]MK801 and [^{3}H]PK11195 (Wong et al., 1986; Noyer et al., 1995; Gillard et al., 2003).

2.5. Photoaffinity labelling

For the investigation of irreversible binding to proteins, P2 membranes were resuspended in 50 mM Tris–HCl pH 7.4, 2 mM EDTA containing protease inhibitors (Complete) and incubated for 120 min at 4 °C in the dark with 40 nM [^{3}H]ucb 30889 in the absence or presence of 1 mM levetiracetam. The samples were washed three times with 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$ at $30{,}000 \times g$ for 5 min at 4 °C. Aliquots of 5 mg were placed in 3-ml quartz cuvettes (1-cm light path) and irradiated with a Spectroline UV light (model CM-10) for 30 min at 254 nm and at 4 °C in the dark. The membranes were washed five times in the same buffer and stored at −70 °C. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gels (Laemmli, 1970). For the analysis of

the incorporated radioactivity, proteins were stained with Coomassie brilliant blue R-250 and gel slices were cut out and dissolved overnight in 3.5-fold diluted SOLUENE 350 (Packard Bioscience) followed by determination of the incorporated radioactivity by liquid scintillation counting. In a few experiments, photolabelled membranes were digested in 0.25 M sodium phosphate (pH 8.0), 10 mM β-mercaptoethanol, 1 mM pefablock (Roche), 5 mM EDTA using 90 milliunits/ml of *N*-glycosidase F (Roche). Incubations were performed for 18 h at 37 °C and terminated by the addition of SDS-PAGE sample buffer.

3. Results

3.1. Regional distribution in rat brain

[^{3}H]ucb 30889 binding sites exhibited a widespread distribution in the brain and the cerebellum. The white matter including corpus callosum, anterior commissure (Fig. 1A), internal capsule (Fig. 1C), mammillotegmental and mammillothalamic tractus (Fig. 1E) was devoid of labelling. The labelling was also absent in the choroïd plexus. Marked [^{3}H]ucb 30889 binding was observed in the cerebral cortex (Fig. 1A–E). More precisely, the labelling was the most prominent in the piriform cortex (Fig. 1B). Within the cerebral cortex, the labelling did not show a distinct laminar distribution. In the cerebellum, the molecular layer displayed high [^{3}H]ucb 30889 binding with low binding in the granule cell layer (Fig. 1F). A high localization of binding sites was evident in the hippocampus (Fig. 1B–D) but labelling was less pronounced in the neuronal cell bodies. No difference in labelling density was seen between the CA1–CA3 pyramidal cell layers but labelling was strong in the dentate gyrus. The superior colliculus (Fig. 1E), the central gray in the mesencephalon (Fig. 1E) and the caudate putamen (Fig. 1A) showed high labelling. The highest binding density was observed in the superior colliculus and the dentate gyrus. Most thalamic nuclei were labelled (Fig. 1C) with the lowest level in the paraventricular nucleus and the highest in the dorsomedial and geniculate nuclei (Fig. 1C–E). Other brain structures were also labelled, with medium density in the septum (Fig. 1A), high density in the anterior and posterior hypothalamic areas (Fig. 1D) and low density in the habenula and the hypothalamic paraventricular nucleus (Fig. 1C).

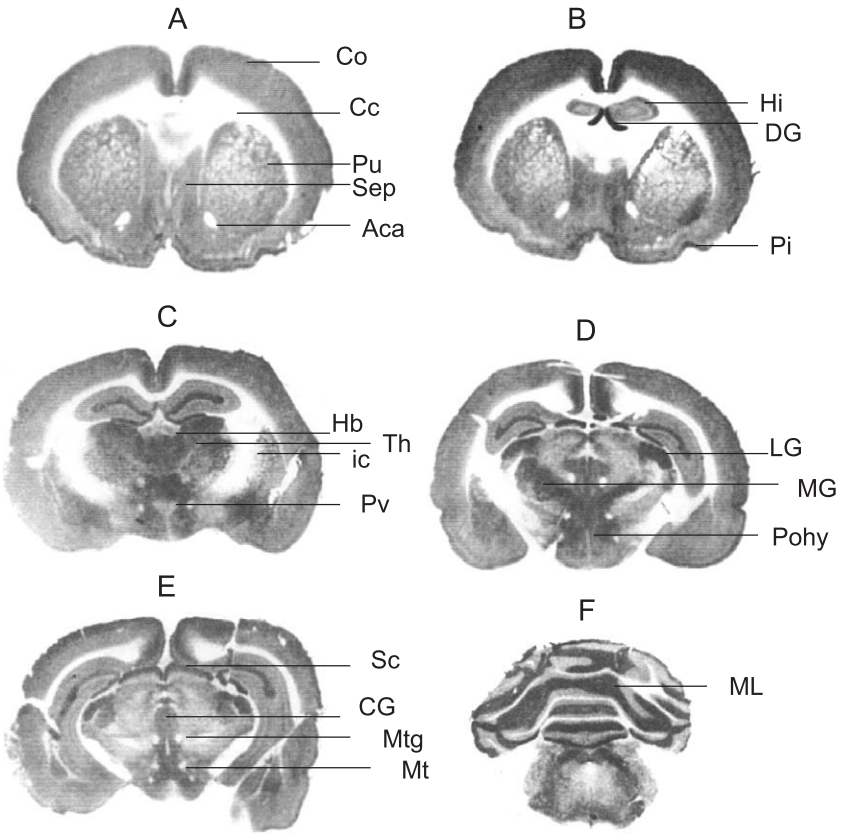

Fig. 1. Autoradiographs of [^{3}H]ucb 30889 binding to coronal sections of rat brain. Nonspecific binding represented less than 10% of the total binding and was indistinguishable from background. Abbreviations: Co, cerebral cortex; Cc, corpus callosum; Aca, anterior commissure; ic, internal capsule; Mtg, mamillotegmental tractus; Mt, mammillothalamic tractus; ML, molecular layer; Hi, hippocampus; DG, dentate gyrus; Sc, superior colliculus; CG, central grey; Pu, caudate putamen; Pv, paraventricular hypothalamic nucleus; MG, medial geniculate nuclei; LG, lateral geniculate nucleus; Pohy, posterior hypothalamic areas; Hb, habenula; Pi, piriform cortex; Th, thalamus; Sep, septum.

Table 1
Density and affinity of [^{3}H]ucb 30889 binding in various cell types

Cell type	B_{max} (pmol/mg prot)	K_d (nM)
Rat cerebellar granule neurons	0.7	59
Mouse cortical neurons	1.4	34
Mouse cortical astrocytes	nd	nd
PC12	1.4	40
SK-N-SH	nd	nd
NG108-15	nd	nd
N1E-115	nd	nd
HCN-1a	nd	nd
CHO-K1	nd	nd
COS-7	nd	nd

nd: not detected.

3.2. Cellular distribution

Saturation experiments with intact cells aimed at characterizing the binding of [^{3}H]ucb 30889 in different cell types in culture indicated a single binding site in rat cerebellar granule neurons and mouse cortical neurons (Table 1). Saturable specific binding was also observed on the clonal undifferentiated PC12 cells (rat phaeochromocytoma cells not treated with nerve growth factor (NGF)). The K_d values in these cells were similar to the value measured in rat cerebral cortex (30 nM; see companion paper by Gillard et al., 2003). In contrast, no specific binding was detected in primary astrocytes or in a range of nervous system-related cell lines, such as SK-N-SH (human neuroblastoma), NG108-15 (murine neuroblastomaxrat glioma hybrid), N1E-115 (murine neuroblastoma), HCN-1a (human cortical neurons) or peripheral cell lines like COS (monkey kidney fibroplast) and CHO (Chinese hamster ovary).

A distinctive characteristic of PC12 cells is the dramatic change in phenotype upon exposure to NGF. This treatment induces differentiation and acquisition of properties characteristic of sympathetic neurons, including the growth of long neurites and electrical excitability. We tested pre-treatment of these cells for 48 h with a number of stimulants, growth and neurotrophic factors including NGF for their ability to modulate the expression of the levetiracetam binding site. Since G-protein-coupled receptors agonists induce down-regulation of receptors after prolonged treatment, we also measured the effect of levetiracetam exposure on binding. None of the above factors had a significant effect on the level of the levetiracetam binding site (data not shown).

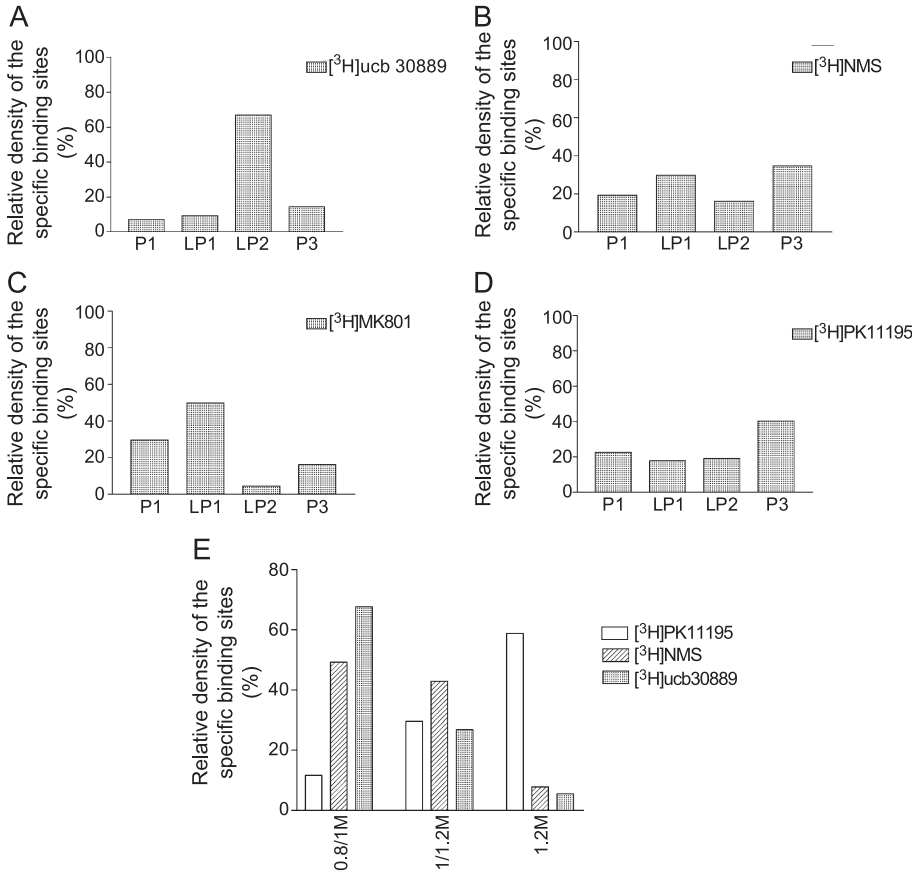

Fig. 2. Subcellular distribution of [^{3}H]ucb 30889 binding. Rat brain membranes were separated by differential centrifugation and binding to the levetiracetam binding site (A), muscarinic (B), NMDA (C) and peripheral benzodiazepine (D) receptors was determined using [^{3}H]ucb 30889, [^{3}H]*N*-methylscopolamine, [^{3}H]MK801 or [^{3}H]PK11195, respectively. A subfractionation by isopycnic centrifugation in sucrose gradient of the crude synaptosomal membranes is shown in (E). Data are representative of two independent experiments. The density of the specific binding sites is expressed in dpm per mg of protein.

3.3. Subcellular distribution

We examined the localization of the levetiracetam binding site in the brain by subcellular fractionation. The distribution of [^{3}H]ucb 30889 binding indicated that the levetiracetam binding site was present in crude synaptosomal (LP1) and microsomal membranes (P3) (Fig. 2A). However, [^{3}H]ucb 30889 binding site was enriched in synaptic vesicle (LP2) fractions. A saturation binding study using synaptic vesicles indicated that the levetiracetam binding site was extremely abundant in this fraction with a density of 60 pmol/mg protein (data not shown) compared to 5 pmol/mg protein measured in cortical membranes (Noyer et al., 1995; Gillard et al., 2003). In contrast, the other receptors studied (muscarinic, *N*-methyl-D-aspartate (NMDA) and peripheral benzodiazepine) determined using [^{3}H]NMS, [^{3}H]MK801 and [^{3}H]PK11195, respectively, were equally or less abundant in the synaptic vesicle (LP2) fractions compared to other fractions. This is in accordance with their preferential location in plasma membranes (muscarinic and NMDA receptors) and mitochondria (peripheral benzodiazepine receptor). In order to isolate mitochondria and synaptic plasma membranes, we loaded synaptosomal membranes onto a sucrose gradient (Fig. 2E). The effectiveness of the fractionation was evaluated by the use of binding assays for markers of mitochondria ([^{3}H]PK11195) and plasma membranes ([^{3}H]NMS). As expected, based on a previous study (Noyer et al., 1995), the levetiracetam binding site was found in synaptic plasma membranes and was not present in the 1.2 M sucrose pellet containing purified mitochondrial fraction.

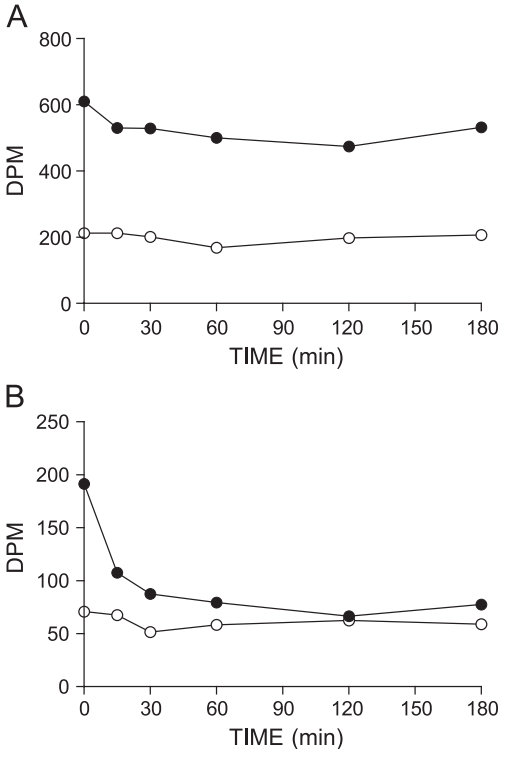

Fig. 3. Photolabelling of the levetiracetam binding site by [^{3}H]ucb 30889 and irreversibility of the complex. (A) Synaptic membranes (closed symbols) were preincubated with 40 nM [^{3}H]ucb 30889, irradiated with UV light and washed. At 0 min, 1 mM levetiracetam was added and aliquots were taken at the indicated time. Nonspecific binding (open circles) was determined using preincubation and incubation in 1 mM levetiracetam. (B) Same experiments as in A except the absence of UV light irradiation. The data were obtained from a single experiment performed in duplicate.

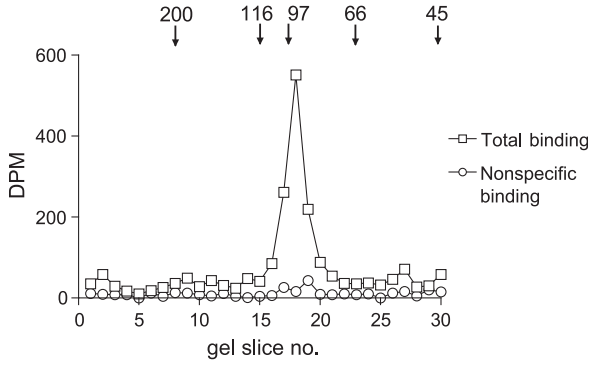

Fig. 4. Gel electrophoresis of membrane proteins labelled by [^{3}H]ucb 30889. Photoaffinity labelling was performed in the absence (squares) or in the presence of 1 mM levetiracetam (circles). The arrows represent the position of molecular weight markers and the numbers above indicate their molecular sizes.

3.4. Photoaffinity labelling

The novel radioligand was designed with an azidophenyl motif capable of forming a covalent complex with the protein after UV light irradiation. Several validation experiments with [^{3}H]ucb 30889 were performed to determine optimal photolabelling conditions. The time course of incorporation of [^{3}H]ucb 30889 indicated that 30 min of irradiation was necessary to achieve high yield of covalent

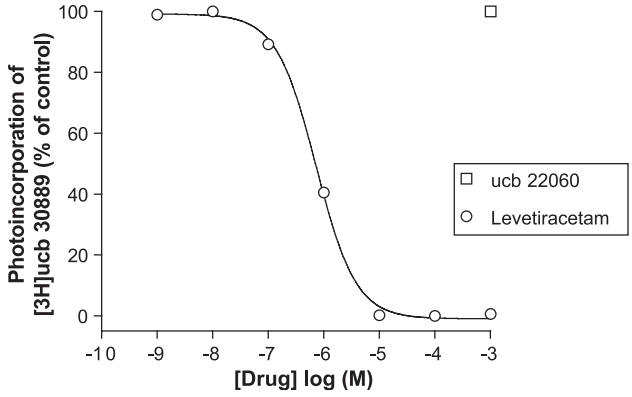

Fig. 5. Inhibition of photolabelling with levetiracetam and ucb 22060. Displacement potencies were determined by incubation of membranes for 120 min at 4 °C with [^{3}H]ucb 30889 in the presence of the indicated concentrations of the test drugs immediately followed by irradiation with the test drug in the assay buffer. After SDS-PAGE, the gels were sliced and the radioactivity content was determined in the 90-kDa protein. Nonspecific binding was measured in the presence of 1 mM levetiracetam. Results are expressed as percentages of specific [^{3}H] incorporated in the absence of the displacing drug.

complex. Irradiation of the membrane preparation at 254 nm for 30 min prior to incubation with [^{3}H]ucb 30889 did not change the capacity of the membranes for binding indicating that UV irradiation per se was not harmful for the levetiracetam binding site. In addition, three washing steps of 5 min at 4 °C prior UV light irradiation decreased the nonspecific labelling of [^{3}H]ucb 30889 from 24% to 6% as measured by filtration and scintillation counting of an aliquot of the reaction mixture. This treatment did not eliminate the relevant [^{3}H]ucb 30889 binding protein since a protein of the same molecular weight (see below) was identified by SDS-PAGE when the membranes were not washed before UV irradiation. To show the irreversibility of the [^{3}H]ucb 30889-binding site complex, synaptosomal membranes were incubated with the radioligand and irradiated for 30 min. At zero time, 1 mM levetiracetam was added to the reaction mixture and aliquots were counted at the indicated time intervals (Fig. 3A). The results demonstrated that after UV irradiation the radioligand remains bound to the protein despite addition of levetiracetam indicating covalent binding to this protein. As a control, the same experiment was conducted without irradiation (Fig. 3B) and it was observed that the radioligand dissociates completely upon addition of levetiracetam.

Since the experiments presented above indicated that the levetiracetam binding site was cross-linked to a radioactive label, we sought to determine the molecular weight of this protein using gel electrophoresis. After loading irradiated samples onto SDS-PAGE, it was found that the radioactivity was irreversibly incorporated into a major protein with a

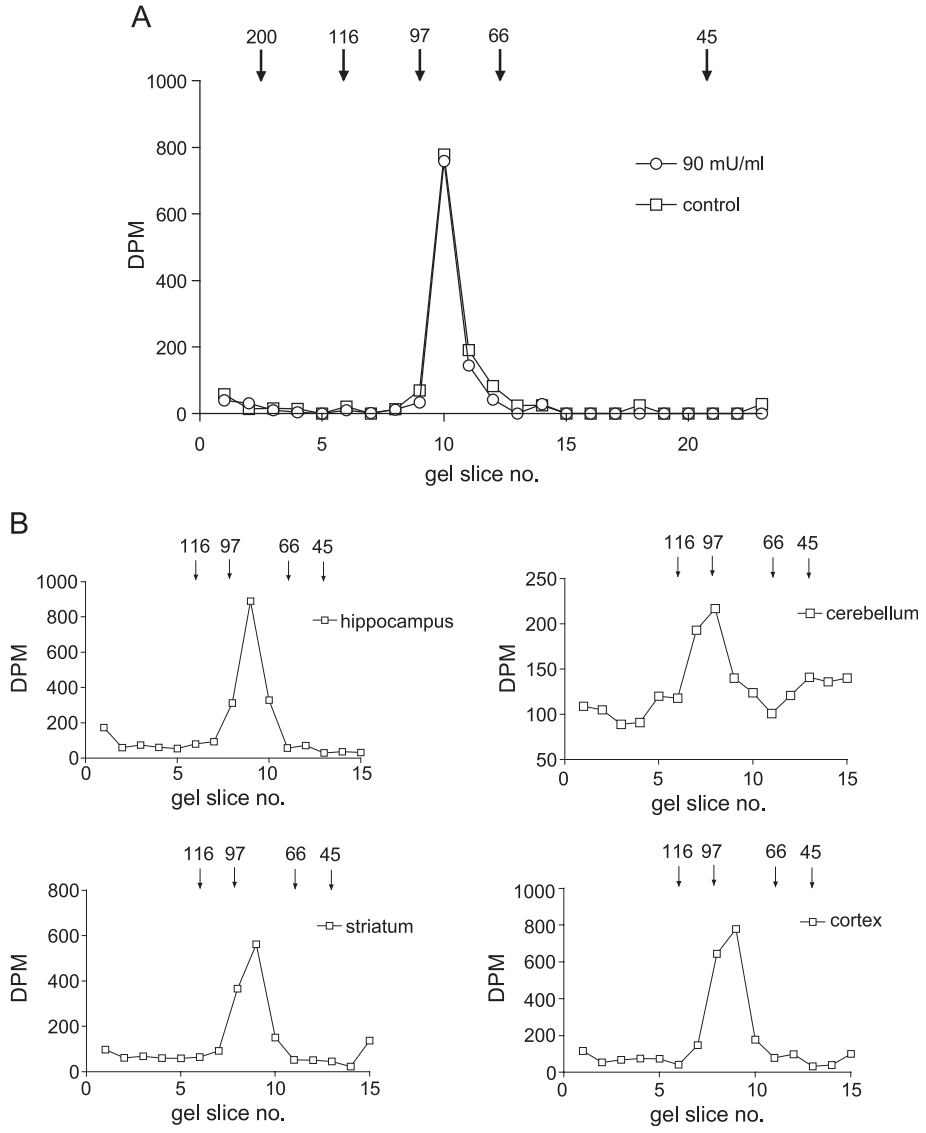

Fig. 6. (A) The photolabelled membranes were treated with peptide *N*-glycosidase F for 18 h at 37 °C (squares). The labelled protein with no treatment is compared (circles). The radioactivity associated to the protein was detected in each slice of the gel by scintillation counting. (B) Photolabelling in different brain regions. Striatal, cortical, cerebellar and hippocampal membranes were irradiated with [^{3}H]ucb 30889 under UV light. The molecular size of the levetiracetam binding site was calculated after counting the radioactivity in each gel slice.

molecular weight close to 90 kDa (Fig. 4). The amount of radioactivity incorporated into the 90-kDa band was quantified by measuring the radioactivity in individual gel slices by liquid scintillation counting. Photoincorporation of radioactivity into this protein was inhibited by the inclusion of levetiracetam (1 mM) in the binding assay indicating that labelling was specific. In this example, 821 fmol of binding sites (5705 dpm) per mg of protein was irreversibly labelled as measured by counting of the radioactivity in individual gel slices. Thus, 3% of specifically bound [^{3}H]ucb 30889 (182 894 dpm/mg) was irreversibly incorporated into the 90-kDa protein. These experiments were repeated several times and gave a molecular weight of 93±8 kDa (n=19) and similar efficiency of photolabelling.

To definitively conclude that the 90-kDa protein is the levetiracetam binding site, inhibition of photoincorporation of [^{3}H]ucb 30889 by several concentrations of levetiracetam and the inactive enantiomer ucb 22060 was studied (Fig. 5). Inhibition of the irreversible specific binding by levetiracetam and ucb 22060 revealed pIC_{50}, s of 6.1 and below 3.0, respectively. These values are in the range of displacing potencies of these compounds reported by Noyer et al., 1995 and in the companion paper by Gillard et al. (2003) for reversible binding and confirmed that the 90-kDa protein is the levetiracetam binding site.

In a subsequent experiment, the glycosylation state of the levetiracetam binding site was examined using a specific endoglycosidase. After treatment of the photolabelled membranes with *N*-glycosidase F, the proteins were analyzed on SDS-PAGE (Fig. 6A). The absence of shift to a lower apparent molecular weight after the reaction suggests that the levetiracetam binding site does not contain *N*-(asparagine)-linked carbohydrate chains. However, this slicing method does not resolve very low ratios of carbohydrate to protein mass (<5 kDa) and the labelling of the levetiracetam binding site does not resist more harsh conditions used to remove strongly associated sugars. As a positive control for deglycosylation, we used the same membranes and observed a shift for the NMDA receptor probed with an anti-NR1 antibody (data not shown). Photolabelling experiments were also performed in different brain regions (Fig. 6B). Analysis of SDS-PAGE revealed only a single protein of 90 kDa, which was labelled in the striatum, cerebellum, cortex and hippocampus.

4. Discussion

In a companion paper (Gillard et al., 2003), we describe the binding characteristics of [^{3}H]ucb 30889 and show that this radioligand is a selective and high affinity label for the levetiracetam binding site. These characteristics made it possible to conduct autoradiography and photoaffinity labelling experiments. Upon irradiation with UV light, [^{3}H]ucb 30889 was able to irreversibly and specifically label a single polypeptide with an apparent molecular weight in the 90-kDa range. Peptide *N*-glycosidase F treatment of the labelled protein suggests that the levetiracetam binding site does not contain N (asparagine)-linked carbohydrates. Autoradiographic mapping revealed that [^{3}H]ucb 30889 binding sites were heterogeneously distributed throughout the rat brain and were absent in the white matter. The highest binding densities were observed in the dentate gyrus, the superior colliculus, most of the thalamic nuclei and in the molecular layer of the cerebellum. No differences were noted between the different pyramidal cell layers of the hippocampus and the various hypothalamic nuclei. Overall, the pattern of distribution of [^{3}H]ucb 30889 binding sites in rat brain correlates well to the high densities observed in membrane homogenates from the cortex, hippocampus and cerebellum (Gillard et al., 2003).

The brain distribution of the levetiracetam binding site does not appear to fit that of receptors involved in hyperexcitability or excitotoxicity. For instance, ligands of the kainate, AMPA and NMDA receptors are known to distinguish between CA1 and CA3 pyramidal cells or different layers of the cerebral cortex (Bailey et al., 2001; Mutel et al., 1998; Hawkins et al., 1995). AMPA sites are also abundant in areas only weakly labelled by [^{3}H]ucb 30889 such as the lateral nucleus of the septum. Like the levetiracetam binding site, NMDA and kainate receptors are highly expressed in hippocampus and cerebellum but in contrast to the levetiracetam binding site, they are preferentially expressed in the granular layer of the cerebellum (Jansen et al., 1990). Thus, despite their high molecular weight and density in brain membranes, similar to the levetiracetam binding site, the mapping of NMDA, kainate and AMPA receptors does not make them probable candidates for the levetiracetam binding site.

Metabotropic glutamate receptors are potential antiepileptic drug targets and some receptor subtypes have molecular weights in the 90-kDa range. However, the distribution of several of the metabotropic glutamate receptor subtypes does not fit that of [^{3}H]ucb 30889 (Hudtloff and Thomssen, 1998; Mutel et al., 1998). For instance, the subtype $mglu_5$ receptor presents a low expression level in the cerebellum and a higher density in the granular layer while $mglu_4$ receptor is weakly present in the superior colliculus. Only $mglu_3$ receptor presents a pattern similar to the levetiracetam binding site. Therefore, we tested agonists and antagonists for the mglu receptors including 3-HPG (hydroxyphenylglycine), 1*S*,3*R*-ACPD (aminocyclopentane dicarboxylic acid), (*S*)-AP-4 (aminophosphonobutyric acid), quisqualic acid, (2*S*,3*S*,4*S*)-CCG ((carboxycyclopropyl)glycine) and MPPG (methyl-phosphonophenylglycine) for their ability to displace [^{3}H]ucb 30889 or [^{3}H]levetiracetam binding in rat brain membranes. The weak affinity that these ligands have shown argues against the known members of the mglu receptor family as targets of levetiracetam (Lynch et al., 2002).

The pattern of distribution of the $GABA_A$ (γ-aminobutyric acid) receptor varies from one subunit composition

to another, making the link with the levetiracetam binding site very difficult. Most of the subunits have been found in the hypothalamic nuclei and some of them in the molecular layer of the cerebellum and the dentate gyrus like the [^{3}H]ucb 30889 binding site (Olsen et al., 1990). However, the molecular weight of the levetiracetam binding site close to 90 kDa definitively excludes the $GABA_A$ receptor complex as a candidate for the levetiracetam binding site since photolabelling experiments with a selective $GABA_A$ receptor ligand identified 50–60-kDa proteins (Sieghart et al., 1987). Indeed binding experiments with [^{3}H]ucb 30889 in the presence $GABA_A$ receptor ligands led to the same conclusion (Gillard et al., 2003).

Ion channels may also mediate the inhibitory effects of antiepileptic drugs. Channels with a size comparable to the levetiracetam binding site include the M-type K^+ channel which is a target of the anticonvulsant drug retigabine. Based on its distribution throughout the brain and its low membrane density, we can exclude this channel as the levetiracetam binding site (De Souza et al., 1992). Likewise, the density and distribution of the N-type Ca^{2+} channel do not correlate with that of the levetiracetam binding site. Indeed it has been shown that N-type Ca^{2+} channels have a low density in the molecular layer of the cerebellar cortex and the highest (0.2 pmol/mg) in the hippocampus (Whorlow et al., 1996). Although the voltage-sensitive Na^+ channel presents a distribution similar to the levetiracetam binding site (Mourre et al., 1988), the sizes of the α and β channel subunits are not comparable to that of the levetiracetam binding site. Furthermore, selective ligands for this channel did not displace binding to the levetiracetam binding site and levetiracetam did not affect the binding to the Na^+ channel (Noyer et al., 1995 and data not shown).

Support for a neuronal origin of the binding sites comes from the saturation binding studies with various cell types. The calculated K_d values in cerebellar and cortical neurons are similar to those obtained with rat brain membrane preparations (Gillard et al., 2003). The binding site is also present in the clonal cell line PC12 but at lower levels. Although isolated from a rat pheochromocytoma tumor, PC12 cells are considered to possess many neuronal-like properties. These cells synthesize and release catecholamines like their non-neoplastic counterparts, adrenal chromaffin cells, contain a high number of secretory vesicles and have routinely been used to examine the protein components of secretory vesicles and the release-uptake process (Chen et al., 2001).

The subcellular fractionation study showing that the levetiracetam binding site is mainly found in synaptic vesicles may explain this PC12-specific localization. With this cell type, we also observed no significant changes in binding after chronic levetiracetam treatment indicating that levetiracetam is probably not a G protein-coupled receptor agonist. In contrast to PC12, the other cell lines derived from neuronal tumors do not contain the [^{3}H]ucb 30889 binding site. The absence of the levetiracetam binding site or a low level of expression in these cells could be explained by a limited number of secretory vesicles and their smaller size compared to PC12 cells. Indeed it has been shown that the area of vesicular membrane per PC12 cell is five times larger than in NG108-15 cells (Cans et al., 2001). Similarly, the levetiracetam binding site was not detected in another PC12 clone which may have lower relative amounts of sites (data not shown). On the other hand, the absence of the levetiracetam binding site in peripherally derived cells correlates with the study of tissue distribution where no specific binding was detected in membrane preparations from rat liver, spleen, kidney, adrenal, heart and lung (Gillard et al., 2003).

It is tempting to speculate that the interaction of levetiracetam with a binding site found in synaptic vesicle is linked by an unknown pathway to its reported blockade of N-type Ca^{2+} channels (Lukyanetz et al., 2002). Indeed, it is well established that the release of synaptic vesicle containing neurotransmitters is triggered by depolarization of the presynaptic plasma membrane induced by an influx of Ca^{2+} mediated by N-type Ca^{2+} channels (Jarvis and Zamponi, 2001). Since there is clear correlation between affinities for the binding site and anti-seizure properties (Noyer et al., 1995), the inhibition of this current may contribute to the antiepileptic action of levetiracetam and related compounds. In contrast, the opposition of Zn^{2+} inhibition of GABA currents by levetiracetam (Rigo et al., 2002) is principally a postsynaptic event and can therefore not be assumed to be directly coupled to the modulation of the levetiracetam binding site. Obviously, this does not exclude that this action also contributes to the antiepileptic properties of levetiracetam.

In conclusion, the results presented in this paper indicate that the levetiracetam binding site has a molecular weight of approximately 90 kDa and appears to be selective for neurons and abundant in synaptic vesicles. Furthermore, its distribution differs to that of several classical receptors and ion channels associated with the action of other antiepileptic drugs. Further studies are ongoing to purify this novel binding site and to elucidate the molecular nature of the protein.

Acknowledgements

We thank Michaëlla Grossmann and Michel Legrand for their technical expertise and Dr. Roy Massingham for critically reading the manuscript.

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