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Characterization of the binding of [^{3}H]-SB-204269, a radiolabelled form of the new anticonvulsant SB-204269, to a novel binding site in rat brain membranes

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1 SB-204269 (*trans*-(+)-6-acetyl-4**S**-(4-fluorobenzoylamino)-3,4-dihydro-2,2-dimethyl-2*H*-benzol[*b*]pyran-3**R**-ol, hemihydrate) shows potent anticonvulsant activity in a range of animal seizure models, with a lack of neurological or cardiovascular side-effects. The profile of the compound suggests that it may have a novel mechanism of action. This study describes the characteristics of a binding site for [^{3}H]-SB-204269 in rat forebrain membranes.

2 Specific [^{3}H]-SB-204269 binding was saturable and analysis indicated binding to a homogenoeous population of non-interacting binding sites with a dissociation constant (K_D) of 32 ± 1 nM and a maximum binding capacity (B_{max}) of 253 ± 18 fmol mg^{-1} protein. Kinetic studies indicated monophasic association and dissociation. Binding was similar in HEPES or Tris-HCl buffers and was unaffected by Na^+, K^+, Ca^{2+} or Mg^{2+} ions. Specific binding was widely distributed in brain, but was minimal in a range of peripheral tissues.

3 Specific [^{3}H]-SB-204269 binding was highly stereoselective, with a 1000 fold difference between the affinities of SB-204269 and its enantiomer SB-204268 for the binding site. The affinities of analogues of SB-204269 for binding can be related to their activities in the mouse maximal electroshock seizure threshold (MEST) test of anticonvulsant action.

4 None of the standard anticonvulsant drugs, phenobarbitone, phenytoin, sodium valproate, carbamazepine, diazepam and ethosuximide, or the newer anticonvulsants, lamotrigine, vigabatrin, gabapentin and levetiracetam, showed any affinity for the [^{3}H]-SB-204269 binding site. A wide range of drugs active at amino acid receptors, Na^+ or K^+ channels or various other receptors did not demonstrate any affinity for the binding site.

5 These studies indicate that SB-204269 possesses a specific CNS binding site which may mediate its anticonvulsant activity. This binding site does not appear to be directly related to the sites of action of other known anticonvulsant agents, but may have an important role in regulating neuronal excitability.

Keywords: [^{3}H]-SB-204269; SB-204269; SB-204268; fluorobenzoylamino benzopyran; anticonvulsant; novel binding site; rat brain; stereospecific binding

Introduction

Studies on a series of 4-(benzoylamino)-benzopyran compounds related to the adenosine 5′-triphosphate (ATP)-sensitive potassium channel opener cromakalim have led to the discovery of SB-240269 (*trans*-(+)-6-acetyl-4**S**-(4-fluorobenzoylamino)-3,4-dihydro-2,2-dimethyl-2*H*- benzol[*b*]pyran - 3**R** - ol, hemihydrate), which has potent anticonvulsant activity in the mouse maximal electroshock seizure threshold (MEST) test (Chan *et al*., 1996). In the accompanying paper (Upton *et al*., 1997), we describe the anticonvulsant properties of SB-204269 in a range of animal seizure models, together with other aspects of the *in vivo* and *in vitro* pharmacological activity profile of the compound. These results indicate that SB-204269 possesses potent anticonvulsant activity in a variety of models, with a very high therapeutic index and a lack of neurological or cardiovascular side-effects. The compound shows no activity in a wide range of receptor binding or functional assays *in vitro*, suggesting that it may have a novel mechanism of action.

In a preliminary communication (Herdon *et al*., 1996), we also described the discovery of a stereospecific binding site for [^{3}H]-SB-204269 in mouse forebrain membranes, which may mediate the anticonvulsant action of the compound. In this paper, we describe the characteristics of the binding site for [^{3}H]-SB-240269 in rat brain membranes. The identification of this novel stereospecific CNS binding site makes the anticonvulsant properties of the compound of particular interest, since it suggests that this activity may be mediated by a unique site not shared by other currently known anticonvulsant drugs.

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Methods

Tissue preparation

Adult male Sprague-Dawley rats (Charles River, U.K.) were killed by stunning and cervical dislocation followed by decapitation. Forebrain or peripheral tissues were quickly removed and dissected on ice; subsequent procedures were performed at 4°C. The tissues were homogenized in 15 vol (w/v) of either 50 mM HEPES or 50 mM Tris-HCl buffer (pH 7.4) by an Ultra-Turrax homogenizer. The homogenates were centrifuged at 48,000 g for 15 min. The resultant pellets were resuspended in 30 volumes of the same buffer and stored at −70°C until used. Protein content was assayed by the Bradford method (Bradford, 1976).

[^{3}H]-SB-204269 binding assays

Tissue membrane preparations (0.5–1 mg protein) were incubated with [^{3}H]-SB-204269 in buffer as above in a total volume of 0.5 ml, at 25°C for 60 min. The incubation was

terminated by rapid filtration through Whatman GF/B glass fibre filters, followed by rapid washing of the filters with four 1 ml aliquots of ice-cold buffer. The radioactivity present on the filters was determined by liquid scintillation counting. Non-specific binding was defined as that remaining in the presence of unlabelled SB-240269 (3 μM). Saturation studies were performed by incubating forebrain membranes with a range of concentrations of [^{3}H]-SB-204269 (5–200 nM). Specific binding data were analysed by the programme Ligand (Biosoft) to provide estimates of K_D and maximum binding capacity (B_{max}) values. Competition studies were performed by incubating forebrain membranes with [^{3}H]-SB-240269 (20 nM) and a range of concentrations of the compound being studied. Competition curves were analysed by non-linear least-squares fitting to a four-parameter logistic equation by Microsoft Excel in order to determine IC_{50} values (Bowen & Jerman, 1995). Calculation of K_i values from these IC_{50} values was then performed by use of a K_D value derived from saturation analysis (Cheng & Prussoff, 1973). Association kinetic studies were performed by measuring specific binding of [^{3}H]-SB-204269 (20 nM) to forebrain membranes at 0.5–80 min after membrane addition. For dissociation studies, forebrain membranes were first incubated with [^{3}H]-SB-204269 (20 nM) for 60 min. Specific binding was then measured at 0.5–60 min after addition of SB-204269 (3 μM). Association and dissociation data were analysed by the programme GraFit (Erithacus Software) to provide estimates of K_{on} and K_{off} values. Results are given as means (±s.e.mean where appropriate) of 3–6 experiments.

Materials

SB-204269 (*trans*-(+)-6-acetyl-4**S**-(4-fluorobenzoylamino)-3,4-dihydro-2,2-dimethyl-2*H*-benzol[*b*]pyran-3**R**-ol, hemihydrate) and SB-204268 (*trans*-(−)-6-acetyl-4**R**-(4-fluorobenzoylamino)-3,4-dihydro-2,2-dimethyl-2*H*-benzol[*b*]pyran-3**S**-ol, hemihydrate) were synthesized by SmithKline Beecham Pharmaceuticals. Other drugs and chemicals were purchased from RBI (Natick, MA, U.S.A.). Tocris Cookson (Bristol, U.K.), Sigma (Poole, U.K.) or Fisons (Loughborough, U.K.).

[^{3}H]-SB-204269 (Figure 1; specific activity 21.5 Ci mmol^{-1}) was synthesized by SmithKline Beecham Pharmaceuticals.

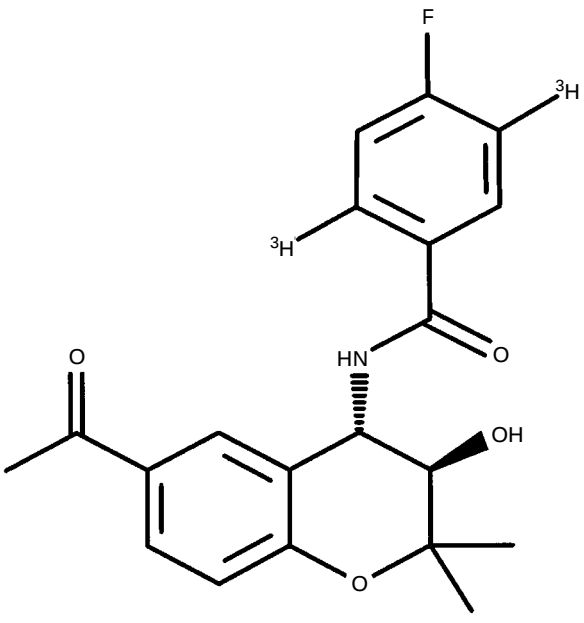

Figure 1 Chemical structure of [^{3}H]-SB-204269.

Results

Saturation and kinetic studies

Specific binding of [^{3}H]-SB-240269 was saturable, whereas non-specific binding increased linearly with radioligand concentration (Figure 2). Analysis of binding data indicated that [^{3}H]-SB-204269 bound to a homogeneous population of non-interacting binding sites in rat forebrain, with a K_D of 32±1 nM, a Hill coefficient of 0.98±0.01 and a B_{max} of 253±18 fmol mg^{-1} protein. Specific binding increased linearly with increasing protein concentration in the range 0.2–5 mg ml^{-1} (data not shown).

Association curves were monophasic, and indicated that equilibrium was reached within 60 min (Figure 3). Dissociation upon addition of excess non-radioative SB-240269 was slow, with approximately 50% of specific binding being dissociated after 15 min (Figure 4). Analysis of these results gave a K_{obs} of 0.091±0.003 min^{-1} and a K_{off} of 0.065±0.013 min^{-1}, leading to a calculated K_{on} of 1.45±0.27 min^{-1} μM^{-1}. The K_D value derived from these data was 44 nM, which is in good agreement with the K_D derived from saturation studies.

Effects of buffers, pH, ions and GTPγS

The binding characteristics of [^{3}H]-SB-204269 were very similar in either 50 mM HEPES or 50 mM Tris-HCl buffer (pH 7.4). Optimal levels of binding were observed at pH 6–8 (data not shown). Binding was unaltered by the presence of 125 mM Na^+, 125 mM K^+, 2.5 mM Ca^{2+} or 10 mM Mg^{2+} ions. The presence of guanosine-5′-*O*-(γ-thio-triphosphate) (GTPγS; 1–100 μM) also had no effect on the capacity or affinity of [^{3}H]-SB-204269 binding (data not shown).

Tissue distribution

The binding of [^{3}H]-SB-204269 to membrane preparations from rat brain regions and a range of peripheral tissues was examined. The B_{max} value of specific binding in hippocampus (221±21 fmol mg^{-1} protein) was similar to that in forebrain, and B_{max} values in cerebral cortex, hypothalamus and striatum were also similar (data not shown). However, the B_{max} value in cerebellum was somewhat higher (372±13 fmol mg^{-1} pro-

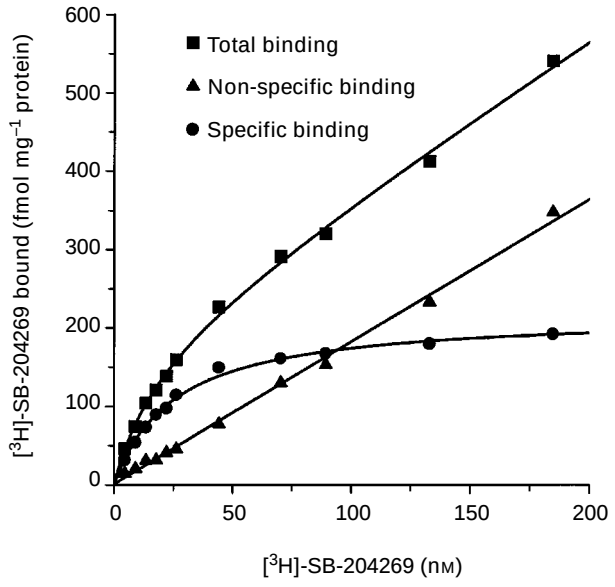

Figure 2 Total, non-specific and specific binding of [^{3}H]-SB-204269 to rat forebrain membranes with increasing concentrations of [^{3}H]-SB-204269. Non-specific binding was defined as that remaining in the presence of 3 μM SB-204269. Data shown are from a single experiment (each point determined in duplicate), which was replicated six times with similar results.

tein). In contrast, virtually no specific binding (<10% of total) could be detected in membrane preparations from rat liver, heart or kidney.

Pharmacological specificity

The binding of [3H]-SB-204269 was highly stereoselective, since SB-204269 itself was 1000 fold more potent than SB-204268, its 3**S**,4**R** enantiomer, in competing for the binding of [3H]-SB-204269 (Figure 5). The K_i value for SB-204269 (48±4 nM) is in good agreement with K_D values for [3H]-SB-204269 derived from saturation and kinetic studies (see above). Comparison of

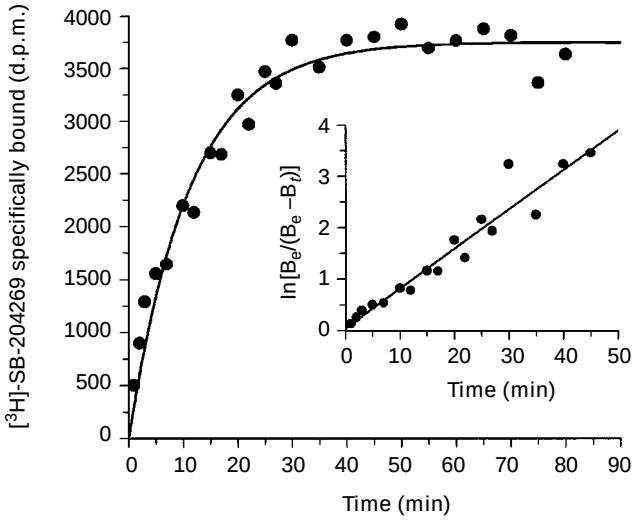

Figure 3 Time course of the association of [3H]-SB-204269 binding to rat forebrain membranes. Specific binding of 20 nM [3H]-SB-204269 was determined at various time intervals. Data shown are from a single experiment, which was replicated three times with similar results. The inset shows the data transformed as a semi-log plot, where B_t is the specific binding at time t and B_e is the specific binding measured at equilibrium. The linear nature of the plot ($r=0.96$) indicates pseudo-first order kinetics for the rate of association.

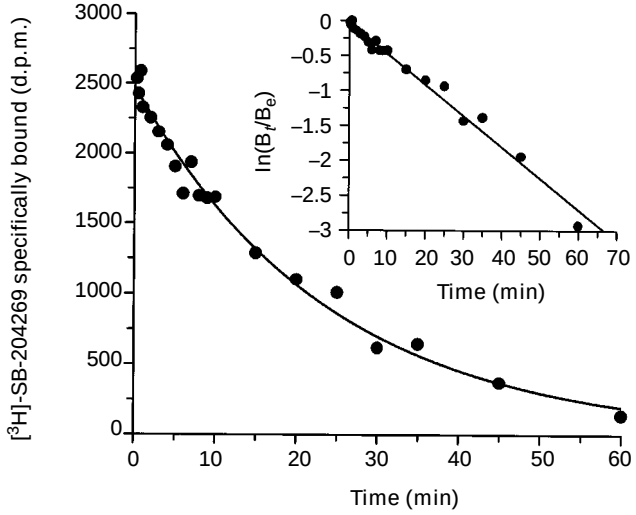

Figure 4 Time course of the dissociation of [3H]-SB-204269 binding from rat forebrain membranes. [3H]-SB-204269 (20 nM) was incubated for 60 min to achieve equilibrium. Dissociation was then induced by addition of 3 μM unlabelled SB-204269 and the amount of specifically bound [3H]-SB-204269 was determined at various time intervals. Data shown are from a single experiment, which was replicated three times with similar results. The inset shows the data transformed as a semi-log plot, where B_t is the specific binding at time t and B_e is the specific binding measured at equilibrium. The linear nature of the plot ($r=0.99$) suggests dissociation from a single site.

pK_i values for SB-204269 and eleven of its analogues (see Chan *et al*., 1996) with the activities of these compounds in the mouse MEST test (assessed as % increase in seizure threshold at 10 mg kg^{-1}, p.o.) showed a clear relationship between the two parameters (Figure 6).

A range of known anticonvulsant drugs was tested in the binding assay. None of the standard anticonvulsants, phenobarbitone, phenytoin, sodium valproate, carbamazepine, diazepam and ethosuximide, or the newer anticonvulsants, lamotrigine, vigabatrin, gabapentin and levetiracetam, showed any affinity for the [3H]-SB-240269 binding site (IC_{50} values >100 μM).

A variety of other drugs with diverse pharmacological activity was tested in the binding assay (Table 1). A wide range of drugs known to be active at various glutamate, glycine or γ-aminobutyric acid (GABA) receptor-related sites did not demonstrate any affinity for the [3H]-SB-204269 binding site. The σ receptor agent 1,3-di(2-tolyl)guanidine (DTG) also showed no affinity. Table 1 also shows that a range of drugs known to act as Na^+ or K^+ channels did not show affinity for the [3H]-SB-240269 binding site. These include benzopyran K_{ATP}

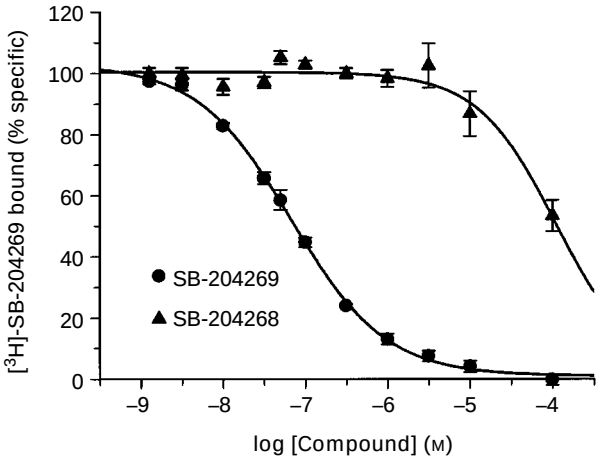

Figure 5 Competition for [3H]-SB-204269 binding to rat forebrain membranes by SB-204269 and SB-204268. [3H]-SB-204269 (20 nM) was incubated in the presence of increasing concentrations of the compounds. Data are means of three independent experiments; vertical lines show s.e.mean.

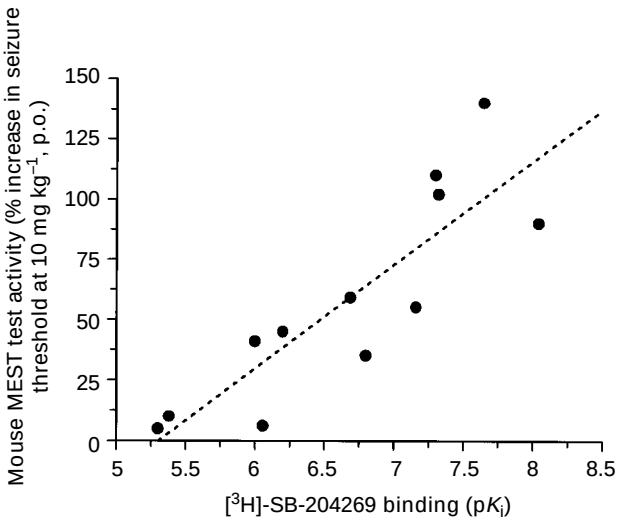

Figure 6 Comparison between the affinities of SB-204269 analogues for the [3H]-SB-204269 binding site and their anticonvulsant activity in the mouse MEST test. The data shown were taken from Chan *et al.* (1996) and are for the compounds referred to (in order of increasing pK_i values) as 5k, 5j, 5f, 5o, 5g, 5n, 5l, 5q, 5p, 5b (SB-204269), 5r, 5s in Table 1 of Chan *et al.* (1996).

Table 1 Affinities of a variety of drugs active at amino acid receptors, Na^+ or K^+ channels or other receptors for the [3H]-SB-204269 binding site

Drug *Amino acid-related*	IC_{50}(μM)	*Drug* *Na^+/K^+ channel-related*	IC_{50} (μM)
Glutamate	>100	Procaine	>100
AP-5	>10	Lidocaine	>100
MK-801	>10	Veratrine	>10
CPP	>10	Tetrodotoxin	>10
CNQX	>10	4-Aminopyridine	>100
Kainate	>10	Tetraethylammonium	>100
ACPD	>100	Quinine	>100
AP-3	>100	Apamin	>10
AP-4	>100	Levcromakalim	>10
Glycine	>100	Aprikalim	>100
Dichlorokynurenate	>10	Pinacidil	>10
Ifenprodil	>10	Niguldipine	>10
Spermine	>10	Diazoxide	>100
Strychnine	>10	Minoxidil	>100
GABA	>100		
Picrotoxin	>10	*Miscellaneous*	
Biculluline	>10	CPDPX	>10
Isoguvacine	>10	NECA	>10
Baclofen	>10	α,β-Methylene ATP	>100
Pregnenolone	>100	2-Methylthio-ATP	>100
Allopregnanolone	>10	PPADS	>100
5α-THDOC	>10	8-Hydroxy-DPAT	>10
PK 11195	>10	Sumatriptan	>100
4-Chlorodiazepam	>10	Methiothepin	>10
DTG	>10	Ketanserin	>10
		Substance P	>10
		Senktide	>10
		U-50488	>100

IC_{50} values are means of 2–3 determinations obtained in rat forebrain membranes.

channel openers to which SB-204269 is chemically related. Drugs known to act as a range of other receptors which are thought to modulate neurotransmission did not demonstrate affinity for the [3H]-SB-204269 binding site.

Discussion

These results demonstrate that [3H]-SB-204269 labels a homogeneous population of binding sites in rat forebrain. The properties of [3H]-SB-204269 binding suggest that it labels a novel site which may be associated with the anticonvulsant effect of the compound. Thus, the affinity of the binding site for SB-204269 (48 nM) is similar to the brain concentration at which threshold anticonvulsant activity of the compound is observed in the rat MEST test (Upton *et al*., 1997). The stereoselectivity of the binding site also parallels the low *in vivo* anticonvulsant activity of SB-204268 (3**S**,4**R** enantiomer of SB-204269) in the rat MEST test and other seizure models (Upton *et al*., 1997). In addition, the relative affinities of a range of analogues of SB-204269 for the binding site show a clear relationship with their relative potencies in the mouse MEST test. The lack of effect of a wide range of pharmacological agents in competing for the binding of [3H]-SB-204269 is in agreement with the lack of effect of SB-204269 itself in a variety of receptor binding or functional assays (Upton *et al*., 1997). Taken together, these observations suggest that this specific CNS binding site for [3H]-SB-204269 may mediate the anticonvulsant activity of the compound.

The [3H]-SB-204269 binding site appears to be concentrated in the CNS, since little specific binding was observed in a range of peripheral tissues. Although binding was observed in brain areas known to be associated with seizure activity (e.g. hippocampus, cortex), the distribution in other areas (e.g. striatum, cerebellum) suggests that regional selectivity of binding cannot account for the lack of neurological side-effects of SB-204269 (see Upton *et al*., 1997). Autoradiographic and subcellular distribution studies of [3H]-SB-204269 binding are in progress.

The pharmacological nature of the binding site and its relationship to known mechanisms of anticonvulsant activity are of particular interest. The lack of activity of many other clinically-used anticonvulsant drugs in competing for [3H]-SB-204269 binding suggests that this binding site may not be related to the primary site of action of these drugs. Although specific CNS binding sites have been described for both gabapentin (Suman-Chauman *et al*., 1993) and levetiracetam (Noyer *et al*., 1995), neither agent showed activity at concentrations which would be expected to saturate their own specific sites. This finding is in agreement with the fact that the overall pharmacological profiles of these agents differ from that of SB-204269 (see Upton, 1994; Upton *et al*., 1997). The lack of effect on [3H]-SB-204269 binding of agents active at glutamate or GABA receptors, or Na^+ or K^+ channels, together with the lack of effect of SB-204269 in a wide range of standard receptor binding or functional assays (Upton *et al*., 1997), indicates that there may not be a direct association between the site of action of SB-204269 and well-known anticonvulsant mechanisms (Upton, 1994). However, it is possible that this novel binding site may mediate the effect of SB-204269 via an unknown molecular link to a known anticonvulsant mechanism. Although SB-204269 is chemically-related to ATP-sensitive K^+ channel openers such as cromakalim (Chan *et al*., 1996), there is no evidence that the compound is acting via this type of channel, though activity at a related K^+ channel cannot be ruled out at present.

In conclusion, the data presented in this study provide evidence for the existence of a specific CNS binding site for SB-204269, which may mediate the anticonvulsant effect of the compound. This site does not appear to be directly related to the sites of action of other known anticonvulsant agents, but may have an important role in regulating neuronal excitability.

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