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ENHANCEMENT OF DEPOLARIZATION-INDUCED SYNAPTOSOMAL CALCIUM UPTAKE AND NEUROTRANSMITTER RELEASE BY BAY K8644

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Abstract—Ca^{2+} uptake into submaximally stimulated synaptosomes was augmented by low concentrations of BAY K8644 (0 1–100 nM) Use of either veratrine or potassium as the depolarizing agent produced similar effects. Stimulated release of aspartate, glutamate and GABA was enhanced over a similar concentration range of BAY K8644. The extent of synaptosomal depolarization induced by veratrine was not enhanced by BAY K8644. The results are discussed in relation to BAY K8644 activation of voltage-dependent Ca^{2+} channels in neural tissue This is the first report of BAY K8644 acting on synaptosomes at concentrations that correlate to dihydropyridine binding studies

The dihydropyridine, BAY K8644, has been shown to have positive inotropic and vasoconstrictor effects [1, 2] due to its augmentation of Ca^{2+} influx through the Ca^{2+} channel in isolated guinea pig heart and rabbit aortic strips respectively. Electrophysiological and $^{45}Ca^{2+}$ flux experiments have yielded results in excellent agreement with the contraction data in cardiac tissue [3–5]. Nifedipine and other dihydropyridine (DHP) Ca^{2+} antagonists appear to compete for the same site of action as BAY K8644, whereas verapamil and diltiazem (Ca^{2+} antagonists from different structural classes) cause a non-competitive inhibition of the effects of BAY K8644 [1, 2].

We report here that the DHP Ca^{2+} antagonist, nimodipine, is able to inhibit $^{45}Ca^{2+}$ accumulation by mammalian brain synaptosomes with an IC_{50} close to 1 μM. Others have been unable to observe any effects of DHPs on synaptosomal Ca^{2+} uptake using different incubation conditions [7–9]. However, radioligand binding studies with [^{3}H] nitrendipine and [^{3}H] nimodipine have identified a DHP binding site in brain membranes with a dissociation constant of 0 1–1.0 nM [10–12] which is similar to the K_d values for the binding of these drugs to cardiac and smooth muscle membranes. The correlation between binding and pharmacology for DHPs in smooth muscle is very good, but in cardiac [13] and neuronal tissue [14] there is a 100–1000-fold discrepancy between pharmacological activity and binding properties.

The present paper illustrates pharmacological activity of BAY K8644 at concentrations consistent with those required to displace [^{3}H] nitrendipine binding in guinea pig brain synaptosomes [9] and with the K_d of [^{3}H] BAY K8644 binding itself [15].

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MATERIALS AND METHODS

Synaptosomes prepared from rat cerebral cortex [16] were incubated in a shaking water bath at 37°, in Krebs Tris medium of the composition (mM): NaCl, 138; KCl, 5; $MgSO_4$, 1; $CaCl_2$, 1 2, NaH_2PO_4, 1.2; Tris, 20; glucose, 10 (pH 7.4), to give a final protein concentration of 2 mg/ml. Experiments were carried out in an atmosphere of pure O_2 supplied by a gassing line suspended above each incubation. The whole incubation procedure took place in a dark room illuminated by a Kodak safelight filter No. 1, to avoid degradation of the BAY K8644 solution by light of wavelength less than 450 nm.

After 15 min preincubation BAY K8644 or Nimopidine (dissolved in 96% ethanol) was added to the appropriate incubations at the relevant concentration, whilst the solvent alone was added to the control incubations The final concentration of ethanol was 0.3% (v/v).

Addition of $^{45}CaCl_2$ (Amersham International, final specific activity 0 33 Ci/mole) was made to each incubation 5 min after the addition of BAY K8644. Veratrine HCl or KCl was added 2 min later (to give a final concentration of 25 μM or 25 mM respectively) to those incubations requiring stimulation The final volume of each incubation was 1 ml. After a total period of incubation with ^{45}Ca of 10 min, the incubations were terminated by placing a 250 μl aliquot into 4 ml ice cold incubation medium The synaptosomes were then separated from the incubation medium by rapid filtration through Whatman GF/C filters. The filters were washed with 3 × 4 ml Krebs Tris and subsequently dried and placed in 5 ml liquid scintillation cocktail (Packard 199) for radioactive counting using a Packard 300CD Tricarb scintillation counter. Protein levels were measured by the method of Lowry *et al* [17]. Synaptosomes were incubated

in a similar manner when K^+ content and amino acid release were measured, but the addition of ^{45}Ca was omitted These incubations were terminated by sedimentation of a 500 μl aliquot of the synaptosomes in an Eppendorf microcentrifuge for 2 min. The pellets were solubilized in 1 ml 1 M NaOH for 30 min at 50° K^+ was measured in these pellets using a SB900 Atomic Absorption Spectrophotometer (G B C Scientific Equipment, Australia) The supernatants were prepared for amino acid analysis and the amino acids were measured with a Chromospek J180 autoanalyser with fluorimeter J143 (Rank-Hilger, Margate, U K) as described previously [18]

RESULTS

Ethanol (0 3% v/v) had no effect with respect to any of the parameters measured and was therefore considered to be a suitable solvent for BAY K8644 in these experiments The DHP itself had no significant effect on unstimulated Ca^{2+} uptake (at 10 μM or 1 nM) However, when synaptosomes were stimulated submaximally with either 25 μM veratrine or 25 mM K^+, an enhancement was seen in the levels of Ca^{2+} uptake and amino acid release. This enhancement did not occur when synaptosomes were maximally stimulated with 100 mM K^+

Over the range 0 1 nM to 100 nM BAY K8644 there was significant enhancement of K^+-stimulated Ca^{2+} uptake, with a maximal effect around 1 nM (130% increase, Fig 1) However, neither the lowest

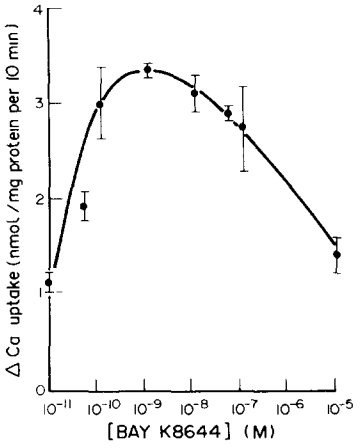

Fig 1 BAY K8644 enhancement of K^+ stimulated Ca^{2+} uptake into cerebrocortical synaptosomes Ca^{2+} values are the difference between each of the stimulated values and the unstimulated control In the presence of 25 mM K^+ alone Ca^{2+} uptake was 1 47 nmol/mg protein/10 min (N = 12) Unstimulated Ca^{2+} uptake was 4 38 ± 0 17 nmol/mg protein/10 min (N = 12) All points are mean ± S.E M (N = 6 for 1 nM and 10 nM BAY K8644, N = 3 for all other points) Significance of differences between conditions were compared to the stimulated control using Student's *t*-test, $P < 0\ 01$ for all points except 10 pM, 50 pM and 10 μM BAY K8644 In the presence of 10 μM and 1 nM BAY K8644 the unstimulated Ca^{2+} uptake was 4 20 ± 0 86 (N = 6), and 4 41 ± 0 33 (N = 3) nmol/mg protein/10 min respectively

Table 1 Synaptosomes were incubated as described in Materials and Methods The effect of BAY K8644 on the release of amino acids

Condition	Concentration of BAY K8644 (M)	Aspartate	Glutamate	GABA	Threonine	(N)
		(nmol/100 mg protein/10 min)				
	0	968	1469	137	610	(21)
	1 nM	1083	1563	164	594	(3)
Basal	10 μM	788	928	95	641	(6)
Stimulated with 25 mM K^+						
	0	1304	1779	496	614	(12)
	50 pM	1282	1734	593*	707	(6)
	100 pM	1355	1865	624*	714	(6)
	1 nM	1394	1964	681*	668	(9)
	10 nM	1891*	2091*	741*	696	(9)
	50 nM		2065*	730*	602	(6)
	100nM	1283	1974	550	704	(6)
	10 μM	1288	1818	421	708	(6)
Stimulated with 25 μM veratrine						
	0	1310	2146	602	623	(15)
	10 pM	1373	2107	573	684	(6)
	50 pM	1441*	2280	640	671	(6)
	100 pM	1495*	2263	632*	646	(6)
	1 nM	1508*	2333*	686	692	(6)
	10 nM	1506*	2408*	757*	659	(9)
	50 nM	1512*	2388	712*	616	(6)
	100 nM	1437	2318	657*	655	(9)
	10 μM	1250*	1998*	498*	578	(6)
Stimulated with 100 mM K^+						
	0	2006	2206	791	754	(3)
	1 nM	1947	2269	685	687	(3)

* All values are means with S D values less than 10% of the means Significance of differences between conditions were compared to the stimulated control using Student's *t*-test (* $P < 0\ 05$)

Table 2 The effect of nimodipine on $^{45}Ca^{2+}$ uptake, K^+ content and glutamate release

Nimodipine (μM)	Ca^{2+} uptake (% of control value *)	K^+ content (% of control value †)	Glutamate release (% of control value ‡)
0 5	90*	n d	75§
1 0	47	75§	63
10 0	30	89§	37
25 0	10	n d	0
50 0	12	67§	0

Synaptosomes were incubated as described in Materials and Methods, but nimodipine was employed in the place of BAY 8644, and veratrine (75 μM) was the stimulating agent Ca^{2+} uptake, K^+ content and glutamate release were calculated as the difference between basal and stimulated levels Nimodipine had no effect on the basal levels of any of the parameters measured Veratrine (75 μM) is present in each condition below All mean values (N = 6) are significantly different ($P < 0\ 05$ using Student's t-test) from the control value, unless indicated (§) n d = not determined Similar results were obtained for aspartate and GABA release

* Control Ca^{2+} Uptake is 4 80 ± 0 61 nmol/mg protein/10 min)

† Control K^+ Content is 21 34 ± 2 78 μ equiv/100 mg protein

‡ Control Glutamate Release is 1387 ± 163 nmol/100 mg protein

(0 05 nM) nor the highest (10 μM) concentration tested had any significant effect on the stimulated level. Similarly with 25 μM veratrine there was a bell-shaped dose response curve for the BAY K8644 augmentation of stimulated Ca^{2+} uptake, with a maximal effect around 1 nM (103% enhancement, Fig. 1).

K^+ efflux from synaptosomes can be taken as an index of the extent of membrane depolarization Exposure of synaptosomes to 25 μM veratrine significantly reduced potassium content by 25%. In the experiments reported here BAY K8644 (0.01 nM–10 μM) had no effect on either the extent of depolarization produced by veratrine or on basal potassium levels

The concentration range of BAY K8644 that enhanced submaximally stimulated amino acid release (0.1 nM–100 nM, Table 1) correlated well with the Ca^{2+} influx data. A significant inhibition of basal and veratrine-induced amino acid release was obtained with 10 μM BAY K8644 1 nM BAY K8644 had no significant effect on basal amino acid release Threonine release was not affected by BAY K8644 nor by the depolarizing agents used.

The extent of synaptosomal depolarization appeared to effect the degree of enhancement of Ca^{2+} influx by BAY K8644 in an inverse fashion Thus, whilst Ca^{2+} uptake stimulated by 25 μM veratrine (67% stimulation) was greater than that produced by 25 mM K^+ (34% stimulation), BAY K8644 enhancement is 100% and 130% respectively. Similarly, the smaller release of amino acids induced by 25 mM K^+ is enhanced by BAY K8644 to a greater degree than the larger release evoked by veratrine.

Relatively high concentrations of nimodipine (1–50 μM) were required to inhibit veratrine-stimulated Ca^{2+} uptake and amino acid release These concentrations of nimodipine had no effect on the extent of depolarization induced by veratrine (Table 2)

DISCUSSION

Partial depolarization of the synaptosomal plasma membrane was required for BAY K8644 to be effective. A similar effect was observed in smooth muscle where partial depolarization was necessary for BAY K8644 to produce contractions [1], and in primary cultured cardiac cells where depolarizing clamp pulses were required for BAY K8644 to produce Ca^{2+} channel openings, as recorded by the patch clamp method [4] Albus *et al.* [19], have reported an enhancement of K^+-evoked [^{3}H] noradrenaline release from PC12 cells (which are neuronal in origin) by BAY K8644 There was no augmentation of basal unstimulated noradrenaline release in these cells either. BAY K8644 will only augment [^{3}H] serotonin release from rat cerebrocortical slices in conditions of submaximal stimulation [20]

The inverse relationship between the extent of synaptosomal depolarization and the degree of enhancement of Ca^{2+} uptake by BAY K8644 suggests that the drug action is voltage-dependent, being more effective when the degree of depolarization is small

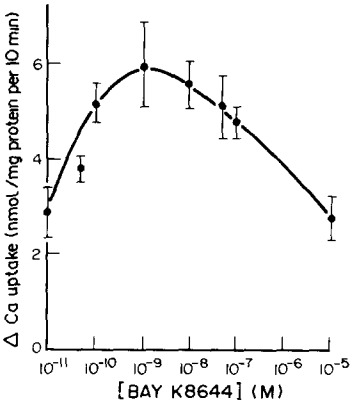

Fig 2 BAY K8644 enhancement of veratrine-stimulated Ca^{2+} uptake into cerebrocortical synaptosomes Ca^{2+} values are the difference between each of the stimulated values and the unstimulated control In the presence of 25 μM veratrine alone Ca^{2+} uptake was 2 94 nmol/mg protein/10 min (N = 12) Unstimulated Ca^{2+} uptake was 4 38 ± 0 17 nmol/mg protein/10 min (N = 12) All points are mean ± S E M (N = 6) Significance of differences between conditions were compared to the stimulated control using Student's t-test, $P < 0\ 05$ for all points except 10 pM, 50 pM and 10 μM BAY K8644

At relatively high concentrations of BAY K8644 (10 μM), the enhancement of stimulated Ca^{2+} uptake and amino acid release fell to zero. In addition, the spontaneous and veratrine-stimulated release of transmitter amino acids was significantly inhibited. This is possibly an anomalous effect due to the relatively high concentration of BAY K8644 employed. Also at these high concentrations BAY K8644 has a tendency to behave as a Ca^{2+} channel antagonist, since BAY K8644 is only a pure agonist over the nanomolar range, therefore BAY K8644 may be acting in a manner comparable to nimodipine [6] Similar bell-shaped curves were obtained for BAY K8644 for K^+-stimulated catecholamine release from chromaffin cells [21] and K^+-stimulated $^{45}Ca^{2+}$ uptake into NG108-15 cells [22]

The increased Ca^{2+} influx due to BAY K8644 was not a result of BAY K8644 mediated synaptosomal depolarization as indicated by the lack of action of the drug on the K^+ content of the synaptosomes. Veratrine-induced depolarization was not enhanced by BAY K8644 either These observations are in agreement with the proposition that the BAY K8644 binding site is closely coupled to the voltage-dependent Ca^{2+} channel itself, producing a direct effect on the channel. In this way the drug is able to mediate Ca^{2+}-dependent cellular events such as neurotransmitter release

The parallelism between the actions of BAY K8644 on stimulated Ca^{2+} uptake and release of aspartate, glutamate and GABA indicates a close link between Ca^{2+} entry into the nerve terminals via the voltage-dependent Ca^{2+} channel and neurotransmitter release.

The IC_{50} for nimodipine inhibition of veratrine-stimulated Ca^{2+} influx into synaptosomes is close to 1 μM (calculated from the values in Table 2) However, the K_d for [^{3}H] nimodipine and [^{3}H] nitrendipine binding in rat and guinea pig brain membranes has been found to be 0.1–1 0 nM [9–12]. Thus there is a 1000-fold discrepancy between the pharmacological activity and the binding of the DHP Ca^{2+} antagonists in mammalian brain Binding studies with [^{3}H] BAY K8644 in guinea-pig brain yield a high affinity binding site for BAY K8644 with a K_d of 2–3 nM [15] and BAY K8644 inhibition of [^{3}H] nitrendipine binding has a K_i of 2.3 nM [9] in guinea-pig cerebrocortical synaptosomes. This correlates closely with the maximal effect of BAY K8644 on stimulated synaptosomal Ca^{2+} accumulation which occurred at approx 1 nM, and suggests that the DHP binding site where BAY K8644 binds, in brain tissue, is the functional voltage dependent Ca^{2+} channel itself, or is closely linked to it

The discrepancy between DHP Ca^{2+} antagonist binding and pharmacological effect may be due to the state of the Ca^{2+} channel under these conditions. DHP Ca^{2+} antagonists, such as nimodipine, are thought to bind to the inactivated Ca^{2+} channel with high affinity [23] whereas DHP Ca^{2+} agonists bind to the open state [19] Hence the good correlation between BAY K8644 binding studies and pharmacology may indicate that the synaptosomal membrane potential in these experiments is such that the Ca^{2+}-channel remains in an open state for sufficient time for BAY K8644 to bind

These results compare well with BAY K8644 actions in the neuroblastoma-glioma cell line NG108–15 [22] where the enhancement of $^{45}Ca^{2+}$ uptake occurred over the range 1 nM–1 μM, and in rat clonal pheochromocytoma cells (PC12) where BAY K8644 potentiated K^+-evoked [^{3}H] noradrenaline release with an EC_{50} of 10 nM [19]

Although neuronal Ca^{2+} channels have only a low sensitivity to organic Ca^{2+} channel antagonists [6], the DHP binding properties of these channels are very similar to those in cardiac and smooth muscle Here we have shown that the DHP with Ca^{2+} agonist properties, BAY K8644, is able to activate Ca^{2+} channels in mammalian brain tissue at concentrations comparable to those at which it binds to this tissue and similar to the concentrations at which it acts as a stimulant in cardiac and smooth muscle Hence it appears that the BAY K8644 binding site may be on or closely associated with a functional voltage-dependent Ca^{2+} channel in neuronal tissue which can be activated by BAY K8644 and is involved in stimulus-secretion coupling.

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