Br. J. Pharmac. (1987), **91**, 475–479

Failure of the calcium channel activator, Bay K 8644, to increase the release of acetylcholine from nerve terminals in brain and diaphragm

Vladimír Doležal & Stanislav Tuček

Institute of Physiology, Czechoslovak Academy of Sciences, 14220 Prague, Czechoslovakia

1 The calcium channel activator Bay K 8644 did not increase the release of acetylcholine from rat brain cortex prisms incubated in the presence of 3 mmol l^{-1} or 25 mmol l^{-1} K^+ nor from rat diaphragms incubated in the presence of 5 mmol l^{-1} or 25 mmol l^{-1} K^+. It also did not influence the release of acetylcholine from cortex prisms incubated in the presence of 25 mmol l^{-1} K^+ and of lowered concentrations of Ca^{2+} ions.

2 It is concluded that the voltage-dependent Ca^{2+} channels in the nerve terminals, responsible for the depolarization-induced influx of Ca^{2+} ions into the nerve terminals and thus for the depolarization-evoked release of acetylcholine from the nerve terminals, are different from the voltage-dependent Ca^{2+} channels in the heart and smooth muscle cells.

Introduction

The release of acetylcholine (ACh) from nerve terminals evoked by the action potentials invading them depends on the opening of the voltage-dependent calcium channels and the influx of Ca^{2+} ions into the terminals (reviews: Reichardt & Kelly, 1983; Silinsky, 1985). In biochemical and pharmacological experiments, the nerve impulse can be replaced by a depolarization of the nerve terminals with a high concentration of K^+ ions, which is also accompanied by a calcium-dependent release of ACh (review: MacIntosh & Collier, 1976). Although the influx of Ca^{2+} is a well-defined phenomenon and is absolutely necessary for the impulse-evoked release of ACh to occur, no pharmacological manipulations of the release of ACh based on changes of Ca^{2+} influx have yet been devised that would find successful application in clinical pharmacology.

The advent of calcium channel activators, a novel class of drugs that increase the mean open time of the voltage-dependent Ca^{2+} channels in the heart and smooth muscle (Schramm *et al.*, 1983; reviews: Schramm & Towart, 1985, Stanfield, 1986), raised new hopes that a drug might become available which could be used to increase the amount of ACh released from the nerve terminals during their activation. In this paper, we describe the results of experiments with the calcium channel activator Bay K 8644, a drug closely related to the dihydropyridine calcium antagonist, nifedipine (Schramm *et al.*, 1983). We measured the release of ACh from prisms of rat brain cortex and from rat phrenic nerve-diaphragm preparations incubated *in vitro* in a physiological solution containing Bay K 8644 and either a physiological or an increased concentration of K^+ ions.

Methods

Wistar-type male rats weighing 167–207 g were used; they were killed by cervical dislocation and decapitation without anaesthesia.

Experiments with brain cortex prisms

The prisms were prepared by slicing the cortex in two directions at a 90° angle with a McIlwain tissue chopper (McIlwain & Rodnight, 1962) set at a slice thickness of 0.3 mm. They were then washed three times in a volume of the preincubation medium A corresponding to ten times their weight, preincubated 60 min in the same volume of medium A, sedimented by centrifugation and resuspended in the incubation medium B with KCl either 3 or 25 mmol l^{-1}. One ml portions of the suspension were placed in incubation vials, Bay K 8644 was added to them in appropriately diluted 10 µl portions, and the prisms were incubated for 30 min at 38°C. The incubation was stopped by transfer to an ice-cold bath and the medium was separated from the tissue by centrifugation.

Experiments with diaphragms

Hemidiaphragms were incubated for 120 min at 38°C in 1 ml of incubation medium C containing KCl either 5 or 25 mmol l^{-1} (Doležal & Tuček, 1983a).

Estimation of acetylcholine and protein

The concentration of ACh in the incubation media was determined with a radioenzymatic method converting ACh to choline and then to radioactive phosphorylcholine (Goldberg & McCaman, 1973; Doleăal & Tuček, 1983a).

The concentration of protein in cortical prisms was measured according to Peterson (1977) with human serum albumin as standard.

Bay K 8644

Methyl-1,4-dihydro-2, 6-dimethyl-3-nitro -4-(2-trifluoromethylphenyl) -pyridine-5-carboxylate (Bay K 8644) was provided by Dr M. Schramm (Bayer AG, Wuppertal, F.R.G.) and was dissolved freshly for each experiment in ethanol or dimethylsulphoxide (DMSO) as indicated. All work involving Bay K 8644 was performed in a dark room illuminated by a red photographic lamp.

Incubation media

Princubation medium A (used for preincubation of brain prisms) had the following composition (mmol l^{-1}): NaCl 125, KCl 3, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 5. Incubation medium B (used for incubation of brain prisms) differed from the preincubation medium A in that it contained choline (10 μmol l^{-1}) and the cholinesterase inhibitor paraoxon (58 μmol l^{-1}) and that the concentration of KCl was either 3 mmol l^{-1} or 25 mmol l^{-1} (in which case the concentration of NaCl was lowered to 103 mmol l^{-1}). Variations in the concentration of Ca^{2+} are indicated in the text. Incubation medium C (used for incubation of the diaphragms) had the same composition as medium B, but the concentration of KCl used was either 5 or 25 mmol l^{-1} (with NaCl 123 or 103 mmol l^{-1}). All media were saturated with 95% O_2 and 5% CO_2 and the preincubations and incubations were performed in this atmosphere.

Results

In experiments with cortical prisms incubated in the presence of K^+ ions 3 mmol l^{-1} (Table 1), the amount of ACh released per mg protein during the 30 min incubation appeared increased in the presence of Bay K 8644 10^{-6} mol l^{-1} and 10^{-5} mol l^{-1}, but the same increase was also obtained in control incubations with corresponding concentrations (0.1% and 1.0%, v/v) of DMSO, the solvent in which Bay K 8644 had been dissolved.

In experiments with cortical prisms incubated in the presence of K^+ ions 25 mmol l^{-1}, the addition of Bay K 8644 produced no change in the amount of ACh released into the medium (Table 1). In these experiments, Bay K 8644 was dissolved in ethanol; it was found in independent experiments that ethanol alone at final concentrations of 0.01% and 0.1% (v/v) (i.e., the highest concentrations that were present during incubations with Bay K 8644) did not influence the release of ACh from the prisms.

Table 1 The release of acetylcholine (pmol ACh mg^{-1} protein $30\ min^{-1}$) from brain cortex prisms during incubation with different concentrations of K^+ and Bay K 8644

		K^+ concentration	
		3 mmol l^{-1}	25 mmol l^{-1}
Control		123.5 ± 14.3 (3)	574.2 ± 37.2 (3)
Bay K 8644	10^{-9} mol l^{-1}		652.6 ± 89.1 (3)
	10^{-8} mol l^{-1}		636.4 ± 107.9 (3)
	10^{-7} mol l^{-1}	116.1 ± 37.1 (3)	466.3 ± 33.2 (3)
	10^{-6} mol l^{-1}	140.9 ± 23.5 (6)	550.7 ± 42.1 (3)
	10^{-5} mol l^{-1}	164.7 ± 15.2 (3)	500.7 ± 32.2 (3)
DMSO 0.1%		145.5 ± 26.9 (6)	
DMSO 1.0%		179.9 ± 3.0 (3)	

Data are means ± s.d. of the number of observations indicated in parentheses. In experiments with K^+ 3 mmol l^{-1}, Bay K 8644 was dissolved in dimethylsulphoxide (DMSO), the final concentrations of which were 0.01%, 0.1% and 1.0% during the incubations with Bay K 8644 10^{-7}, 10^{-6} and 10^{-5} mol $l,^{-1}$ respectively. In experiments with K^+ 25 mmol l^{-1}, Bay K 8644 was dissolved in ethanol, the final concentrations of which were, respectively, 0.00001–0.1% (v/v) at Bay K 8644 concentrations of 10^{-9}–10^{-5} mol l^{-1}.

In subsequent experiments, we tested the effect of Bay K 8644 on the release of ACh from cortical prisms incubated in the presence of K^+ (25 mmol l^{-1}) and different concentrations of Ca^{2+}. The two experiments summarized in Table 2 were separated by a time interval of more than 1 year and we do not know the reason of the difference in the amounts of ACh released under control conditions. It is apparent, however, that no increase in the release of ACh was produced by Bay K 8644 10^{-7} or 10^{-6} mol l^{-1} in the presence of Ca^{2+} 2.5, 1.0, 0.2 mmol l^{-1} or after Ca^{2+} had been replaced with EGTA 1 mmol l^{-1}. It is also apparent from experiment B that approximately 60% of the release was Ca^{2+}-dependent. The proportion of Ca^{2+}-independent release may seem rather high, but it should be noted that it would diminish at higher K^+ concentrations (producing more complete depolarization and higher ACh release; see Doležal & Tuček, 1983b).

Bay K 8644 also had no effect on the amount of ACh released from hemidiaphragms incubated with Ca^{2+} 2.5 mM and K^+ 5 or 25 mmol l^{-1} (Table 3).

In a control experiment on rat heart ventricles perfused via the aorta and paced electrically at 350 contractions min^{-1} as described by Tuček *et al.* (1987), our preparation of Bay K 8644 was found to have a strong positive inotropic effect when added to the perfusion fluid at a final concentration of approximately 1 μmol l^{-1}.

Discussion

Bay K 8644 has been shown to increase the mean Ca^{2+} channel opening time in heart myocytes (Kokubun & Reuter, 1984; Hess *et al.*, 1984; Reuter *et al.*, 1986) and in neurones of spinal ganglia (Nowycky *et al.*, 1985) and its pharmacological effects on smooth muscle have been found to depend on an increase of Ca^{2+} influx into the cells via the voltage-dependent Ca^{2+} channels (Kanmura *et al.*, 1984; Schramm *et al.*, 1985; Schramm & Toward, 1985). In most of our incubations, we used K^+ 25 mmol l^{-1} in order to achieve a partial depolarization of the nerve terminals, associated with an increased opening of the voltage-dependent Ca^{2+} channels and with an increase in the release of ACh. Under the conditions of our experiments, this concentration of K^+ was found to produce only about 60% of the maximum possible stimulation of ACh release, while concentrations of K^+ close to 50 mmol l^{-1} were necessary for the maximum release (Doležal & Tuček, 1983b); it appears, however, that the effects of Bay K 8644 are easier to demonstrate when a partial rather than the maximum possible activation of Ca^{2+} channels is achieved (Schramm *et al.*, 1983; Kanmura *et al.*, 1984; Middlemiss & Spedding, 1985; Kendall & Nahorski, 1985; Thomas *et al.*, 1985).

Bay K 8644 produced no increase in the release of ACh from either the cortical prisms or the motor nerve terminals in the diaphragm incubated either at resting (3 or 5 mmol l^{-1}) or partially depolarizing (25 mmol l^{-1}) concentrations of K^+. It may be noted that in 3 independent experiments (Tables 1 and 2) on cortical prisms incubated with Ca^{2+} 2.5 mmol l^{-1} the amounts of ACh released in the presence of 1 μmol l^{-1} Bay K 8644 corresponded to 96%, 107% and 99% of the amounts released in its absence. Our results suggest that the voltage-dependent Ca^{2+} channels in the nerve terminals are not affected by Bay K 8644 and, consequently, that they are different from the bulk of voltage-dependent Ca^{2+} channels in the heart and smooth muscle cells. Such a conclusion may not be surprising. Whereas the function of the voltage-dependent Ca^{2+} channels in the heart and smooth muscles has been amply shown to be affected by

Table 2 The release of acetylcholine (pmol ACh mg^{-1} protein 30 min^{-1}) from brain cortical prisms during incubations with K^+ 25 mmol l^{-1} and with different concentrations of Ca^{2+} and Bay K 8644

	Control	*Bay K 8644* 10^{-7} mol l^{-1}	*Bay K 8644* 10^{-6} mol l^{-1}
Experiment A			
Ca^{2+} 2.5 mmol l^{-1}	853.2 ± 19.5	—	917.1 ± 105.0
Ca^{2+} 1.0 mmol l^{-1}	875.5 ± 172.7	896.6 ± 99.8	773.8 ± 115.7
Ca^{2+} 0.2 mmol l^{-1}	530.8 ± 51.7	595.2 ± 26.4	583.8 ± 44.5
Experiment B			
Ca^{2+} 2.5 mmol l^{-1}	536.2 ± 40.2	501.7 ± 131.8	532.2 ± 128.5
EGTA 1 mmol l^{-1} (no Ca^{2+})	214.1 ± 30.3	237.0 ± 34.0	199.3 ± 59.8

Data are means ± s.d. of 3 observations. Final concentrations of dimethylsulphoxide were 0.01% and 0.1% in experiment A and 0.0005% and 0.005% in experiment B during incubations with Bay K 8644 10^{-7} and 10^{-6} mmol l^{-1}, respectively.

Table 3 The release of acetylcholine (nmol g^{-1} 120 min^{-1}) from isolated hemidiaphragms during incubation with different concentrations of K^+ and Bay K 8644 and with the solvent dimethylsulphoxide (DMSO)

		K^+ concentration	
		5 mmol l^{-1}	25 mmol l^{-1}
Control		5.87 ± 1.14	8.70 ± 2.90
Bay K 8644	10^{-6} mol l^{-1}	5.15 ± 0.65	7.77 ± 1.02
	10^{-5} mol l^{-1}	5.05 ± 1.16	7.38 ± 0.76
DMSO 0.1%		5.05 ± 1.13	8.27 ± 1.98

Data are means ± s.d. of 4 observations. Bay K 8644 was dissolved in DMSO, the final concentrations of which were 0.01% and 0.1% during the incubations with Bay K 8644 10^{-6} and 10^{-5} mol l^{-1}, respectively.

dihydropyridine calcium channel inhibitors closely related to Bay K 8644, with which they compete for the binding sites (Rogg *et al.*, 1985; Finet *et al.*, 1985), and by other organic Ca^{2+} channel inhibitors (reviews: Schwartz & Triggle, 1984; Fleckenstein, 1985), no consistent effects of Ca^{2+} channel blocking drugs on the release of ACh have been reported. The uptake of Ca^{2+} into brain synaptosomes, induced by brief depolarizations, was also found to be unaffected by low concentrations of dihydropyridine calcium channel inhibitors (Daniell *et al.*, 1983; Wei & Chiang, 1985; Adam-Vizi & Ligheti, 1986; Carvalho *et al.*, 1986).

Our negative result is at variance with recent reports describing positive effects of Bay K 8644 on the evoked release of noradrenaline from the vas deferens (Ceña *et al.*, 1985), cultured sympathetic neurones (Perney *et al.*, 1986), chromaffin tissue (Albus *et al.*, 1984) and adrenal glands (Artalejo & Garcia, 1986), of 5-hydroxytryptamine (5-HT) from brain slices (Middlemiss & Spedding, 1985), of ACh and noradrenaline from brain slices (Middlemiss, 1985) and of dopamine from striatal synaptosomes (Woodward & Leslie, 1986). Most of these studies have been performed with a different technique involving preincubation of the tissue with a labelled transmitter (or a labelled precursor, in the case of ACh) and then measurement of the stimulated release of radioactivity. Although this difference in methods might be of importance for the interpretation of the difference in results, it seems more interesting to note that the depolarization-evoked release of 5-HT (Middlemiss & Spedding, 1985), ACh and noradrenaline (Middlemiss, 1985; Perney *et al.*, 1986) and dopamine (Woodward & Leslie, 1986) was not sensitive to dihydropyridine calcium channel inhibitors, while the component of the release for which Bay K 8644 was responsible could be inhibited by them. It appears, therefore, that in the studies cited in this paragraph, Bay K 8644 affected the release of transmitters by acting on a specific group of dihydropyridine-sensitive neuronal Ca^{2+} channels which are ordinarily not involved in depolarization-induced transmitter release but which can be activated by Bay K 8644 and then inhibited by calcium antagonists in depolarized tissue. Such channels appear unlikely to play a role in the physiological process of transmitter release in the nerve terminals; perhaps they are located in neuronal cell bodies rather than in the nerve endings. The presence of Bay K 8644-sensitive Ca^{2+} channels in the brain has been indirectly demonstrated by Kendall & Nahorski (1985).

We thank Dr M. Schramm (Bayer A.G., Wuppertal) for the kind gift of Bay K 8644 and Dr Ann Silver and the Wellcome Trust for assistance in providing reagents.

References

ALBUS, U., HABERMANN, E., PERRY, D.R. & GLASSMANN, H. (1984). Novel 1,4-dihydropyridine (Bay K 8644) facilitates calcium-dependent [^{3}H]-noradrenaline release from PC 12 cells. *J. Neurochem.*, **42**, 1186–1189.

ADAM-VIZI, V. & LIGETI, E. (1986). Calcium uptake of rat brain synaptosomes as a function of membrane potential under different depolarizing conditions. *J. Physiol.*, **372**, 363–377.

ARTALEJO, C.R. & GARCÍA, A.G. (1986). Effects of Bay K 8644 on cat adrenal catecholamine secretory responses to A 23187 or ouabain. *Br. J. Pharmac.*, **88**, 757–766.

CARVALHO, C.A.M., COUTINHO, O. & CARVALHO, A.P. (1986). Effects of Ca^{2+} channel blockers on Ca^{2+} translocation across synaptosomal membranes. *J. Neurochem.*, **47**, 1774–1784.

CEÑA, V., GARCÍA, A.G., KHOYI, M.A., SALAICES, M. & SANCHEZ-GARCÍA, P. (1985). Effect of the dihydropyridine Bay K 8644 on the release of [^{3}H]-noradrenaline from the rat isolated vas deferens. *Br. J. Pharmac.*, **85**, 691–696.

DANIELL, L.C., BARR, E.M. & LESLIE, S.W. (1983). $^{45}Ca^{2+}$ uptake into rat whole brain synaptosomes unaltered by dihydropyridine calcium antagonists. *J. Neurochem.*, **41**, 1455–1459.

DOLEŽAL, V. & TUČEK, S. (1983a). The synthesis and release

of acetylcholine in normal and denervated rat diaphragms during incubation *in vitro*. *J. Physiol.*, **334,** 461–474.

DOLEŽAL, V. & TUČEK, S. (1983b). The effects of 4-aminopyridine and tetrodotoxin on the release of acetylcholine from rat striatal slices. *Naunyn-Schmiedebergs Arch. Pharmac.*, **323,** 90–95.

FINET, M., GODFRAIND, T. & KHOURY, G. (1985). The positive inotropic action of the nifedipine analogue, Bay K 8644, in guinea-pig and rat isolated cardiac preparations. *Br. J. Pharmac.*, **86,** 27–32.

FLECKENSTEIN, A. (1985). Calcium antagonism in heart and vascular smooth muscle. *Med. Res. Rev.*, **5,** 395–425.

GOLDBERG, A.M. & McCAMAN, R.E. (1973). The determination of picomole amounts of acetylcholine in mammalian brain. *J. Neurochem.*, **20,** 1–8.

HESS, P., LANSMAN, J.B. & TSIEN, R.W. (1984). Different modes of Ca channel gating behaviour favoured by dihydropyridine Ca agonists and antagonists. *Nature*, **311,** 538–544.

KANMURA, Y., ITOH, T. & KURIYAMA, H. (1984). Agonist actions of Bay K 8644, a dihydropyridine derivative, on the voltage-dependent calcium influx in smooth muscle cells of the rabbit mesenteric artery. *J. Pharmac. exp. Ther.*, **231,** 717–723.

KENDALL, D.A. & NAHORSKI, S.R. (1985). Dihydropyridine calcium channels activators and antagonists influence depolarization-evoked, inositol phospholipid hydrolysis in brain. *Eur. J. Pharmac.*, **115,** 31–36.

KOKUBUN, S. & REUTER, H. (1984). Dihydropyridine derivatives prolong the open state of Ca channels in cultured cardiac cells. *Proc. natn. Acad. Sci., U.S.A.*, **81,** 4824–4827.

MACINTOSH, F.C. & COLLIER, B. (1976). Neurochemistry of cholinergic terminals. In *Handbook of Experimental Pharmacology*, New Series, Vol. 42, *Neuromuscular Junction*, ed. Zaimis E. pp. 99–228. Berlin: Springer Verlag.

McILWAIN, H. & RODNIGHT, R. (1962). *Practical Neurochemistry*, pp. 126–129. London: J. & A. Churchill.

MIDDLEMISS, D.N. (1985). The calcium channel activator, Bay K 8644 enhances K^+-evoked efflux of acetylcholine and noradrenaline from rat brain slices. *Naunyn-Schmiedebergs Arch. Pharmac.*, **331,** 114–116.

MIDDLEMISS, D.N. & SPEDDING, M. (1985). A functional correlate for the dihydropyridine binding site in rat brain. *Nature*, **314,** 94–96.

NOWYCKY, M.C., FOX, A.P. & TSIEN, R.W. (1985). Long-opening mode of gating of neuronal calcium channels and its promotion by the dihydropyridine calcium agonist Bay K 8644. *Proc. natn. Acad. Sci., U.S.A.*, **82,** 2178–2182.

PERNEY, T.M., HIRNING, L.D., LEEMAN, S.E. & MILLER, R.J. (1986). Multiple calcium channels mediate neurotransmitter release from peripheral neurons. *Proc. natn. Acad. Sci., U.S.A.*, **83,** 6656–6659.

PETERSON, G.L. (1977). A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.*, **83,** 346–356.

REICHARDT, L.F. & KELLY, R.B. (1983). A molecular description of nerve terminal function. *A. Rev. Biochem.*, **52,** 871–926.

REUTER, H., KOKOBUN, S. & PROD'HOM, B. (1986). Properties and modulation of cardiac caclium channels. *J. exp. Biol.*, **124,** 191–201.

ROGG, H., CRISCIONE, L., TRUOG, A. & MEIER, M. (1985). In vitro comparative studies of the calcium-entry activators YC-170, CPG-28392 and Bay K 8644. *J. cardiovasc. Pharmac.*, **7,** S31–S37.

SCHRAMM, M., THOMAS, G., TOWART, R. & FRANCKOWIAK, G. (1983). Novel dihydropyridines with positive inotropic action through activation of Ca^{2+} channels. *Nature*, **303,** 535–537.

SCHRAMM, M. & TOWART, R. (1985). Modulation of calcium channel function by drugs. *Life Sci.*, **37,** 1843–1860.

SCHRAMM, M., TOWART, R., LAMP, B. & THOMAS, G. (1985). Modulation of calcium ion influx by the 1,4-dihydropyridines nifedipine and Bay K 8644. *J. cardiovasc. Pharmac.*, **7,** 493–496.

SCHWARTZ, A. & TRIGGLE, D.J. (1984). Cellular action of calcium blocking drugs. *A. Rev. Med.*, **35,** 325–339.

SILINSKY, E.M. (1985). The biophysical pharmacology of calcium-dependent acetylcholine secretion. *Pharmac. Rev.*, **37,** 81–132.

STANFIELD, P.R. (1986). Voltage-dependent calcium channels of excitable membranes. *Br. med. Bull.*, **42,** 359–367.

THOMAS, G., CHUNG, M. & COHEN, C.J. (1985). A dihydropyridine (Bay K 8644) that enhances calcium currents in guinea pig and calf myocardial cells. A new type of positive inotropic agent. *Circulation Res.*, **56,** 87–96.

TUČEK, S., DOLEŽAL, V., FOLBERGROVÁ, J., HYNIE, S., KOLÁŘ, F. & OŠŤÁDAL, B. (1987). Pertussis toxin inhibitis negative chronotropic muscarinic cholinergic effects on the heart. *Pflügers Arch.*, **408,** 167–172.

WEI, J.-W. & CHIANG, D.-H. (1985). Studies of [^{3}H]-nitrendipine binding and KCl-induced calcium uptake in rat cortical synaptosomes. *Gen. Pharmac.*, **16,** 211–216.

WOODWARD, J.J. & LESLIE, S.W. (1986). Bay K 8644 stimulation of calcium entry and endogenous dopamine release in rat striatal synaptosomes antagonized by nimodipine. *Brain Res.*, **370,** 397–400.

(Received July 15, 1986.
Revised March 1, 1987.
Accepted March 11, 1987.)