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# Effect of the dihydropyridine Bay K 8644 on the release of [$^3$H]-noradrenaline from the rat isolated vas deferens

Valentin Ceña, Antonio G. García[2], Mohammad A. Khoyi[3], Mercedes Salaices & Pedro Sanchez-García[1]

Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma, C/ Arzobispo Morcillo, 4, 28029-Madrid, Spain.

**1** The effects of Bay K 8644 on the release of [$^3$H]-noradrenaline evoked by potassium, electrical stimulation or tyramine from the rat isolated vas deferens labelled with [$^3$H]-noradrenaline were investigated.

**2** Bay K 8644 (1 μM) by itself did not affect the spontaneous release of tritium from the rat isolated vas deferens. However, it increased the calcium-dependent release of tritium elicited by both high potassium (59 mM) and electrical field stimulation.

**3** The exposure of rat vas deferens to phentolamine (10 μM) increased the release of tritium induced by potassium (59 mM) and electrical field stimulation. Bay K 8644 (1 μM) failed to increase further the release of tritium elicited by both stimuli in preparations previously treated with phentolamine (10 μM).

**4** The calcium-independent release of [$^3$H]-noradrenaline evoked by tyramine (10 μM) was not affected by Bay K 8644 (1 μM).

**5** The results of our study support the view that $\alpha_2$-adrenoceptors modulate noradrenaline release by restricting calcium influx into sympathetic nerve terminals through voltage-dependent channels.

## Introduction

It is now well established that the release of noradrenaline is regulated by a negative feed back system involving $\alpha_2$-adrenoceptors (for reviews see, Kirpekar, 1975; Starke, 1977; Langer, 1981). The mechanism by which $\alpha_2$-adrenoceptors regulate noradrenaline release is a matter of controversy. However, the fact that such modulation can only be demonstrated for the calcium-dependent processes of transmitter release suggests the possibility that, $\alpha_2$-adrenoceptor agonists inhibit stimulation-evoked release by reducing the availability of $Ca^{2+}$ for the excitation-secretion coupling involved in the exocytotic release of noradrenaline (De Langen & Mulder, 1980; Alberts *et al.*, 1981; Langer, 1981). In this context, it is important to note that Horn & McAfee (1980) have shown that noradrenaline decreases a calcium current in postganglionic sympathetic neurones which is also blocked by cobalt and magnesium.

Schramm *et al.* (1983) have recently described a novel dihydropyridine derivative, Bay K 8644, that in contrast to the $Ca^{2+}$ channel blocking agents stimulated cardiac and vascular smooth muscle contractility. This compound can be considered as the first agonist for voltage-dependent calcium channels. García *et al.* (1984) have recently demonstrated that Bay K 8644 markedly enhances the catecholamine release and $^{45}Ca^{2+}$-uptake evoked by moderately high concentrations of potassium in the chromaffin cell of cat adrenal glands.

The aim of this work was to investigate the effects of Bay K 8644 on noradrenaline release, evoked by calcium and non calcium-dependent procedures, from sympathetic nerve terminals of rat isolated vasa deferentia, both in the presence and absence of phentolamine. The results show that Bay K 8644 potentiates the release of noradrenaline evoked by

[1] To whom correspondence should be addressed.
[2] Present address: Departamento de Farmacología, Facultad de Medicina, Alicante, Spain.
[3] Present address: Pharmaceutical Research Center, 18 km, Karaj Road Darou-Pakhsh Ave, Tehran, Iran.

both potassium and electrical field stimulation, but not that induced by tyramine, suggesting that $\alpha_2$-adrenoceptor modulation of noradrenaline release could be mediated through voltage-dependent calcium channels.

## Methods

### *Preparation of tissues*

Male Albino rats weighing 300–450 g were killed by a blow on the head and exsanguinated. Vasa deferentia were quickly removed and cleaned of vascular and connective tissue and then mounted in a holder between two platinum electrodes. Tissues were incubated in Krebs-Bicarbonate solution of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ $7H_2O$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, glucose 11, disodium salt of ethylenediaminetetraacetic acid (EDTA) 0.03 and ascorbic acid 0.1 mg $ml^{-1}$. This solution was bubbled continuously with a mixture of 95% $O_2$ and 5% $CO_2$, the final pH being 7.4 and maintained at 37°C. When external potassium was raised, NaCl was reduced to maintain the isotonicity of the medium.

### *Release of [$^3$H]-noradrenaline*

After an initial 30 min equilibration period, vasa deferentia were incubated for an additional period of 30 min in 5 ml Krebs solution containing 1 $\mu$Ci $ml^{-1}$ of [$^3$H]-(±)-noradrenaline ([$^3$H]-(±)-NA; New England Nuclear, specific activity 15 Ci $mmol^{-1}$). Then tissues were washed every 10 min for a 90 min period. At the end of the washout period, a 5 min sample was taken to measure spontaneous release of $^3$H. The tissues were then either electrically stimulated (600 pulses, 2 Hz, 100 V, 1 ms), exposed to high potassium (59 mM for 5 min) or tyramine (10 $\mu$M for 10 min). The electrical stimulation and the exposure to potassium or tyramine were repeated twice in each tissue 30 min apart ($S_1$ and $S_2$). During the stimulation periods ($S_1$ and $S_2$) 5 min samples were collected to measure evoked $^3$H release. Drugs were usually present 10 min before and during $S_2$. Aliquots of 0.4 ml of each sample were added to 2.5 ml of Instagel and their radioactivity content was measured in a Nuclear Chicago liquid scintillation counter Isocap 300; quenching was corrected for using an automatic external standard.

At the end of the experiment, tissues were placed in a vial containing 0.25 ml of Soluene 100 and dissolved by heating at 55°C overnight and their radioactivity content determined the following day. [$^3$H]-noradrenaline release was expressed as fractional release (% of the total tissue radioactivity content at the beginning of $S_1$).

### *Drugs used*

The following drugs were used: phentolamine hydrochloride (Ciba-Geigy), tyramine hydrochloride (Sigma), and Bay K 8644 (methyl 1,4 - dihydro - 2,6 - dimethyl - 3 - nitro - 4 - (2 - trifluoromethylphenyl) - pyridine - 5 - carboxylate) generously supplied by Prof. F. Hoffmeister, Bayer, A.G., Wuppertal, F.R.G.).

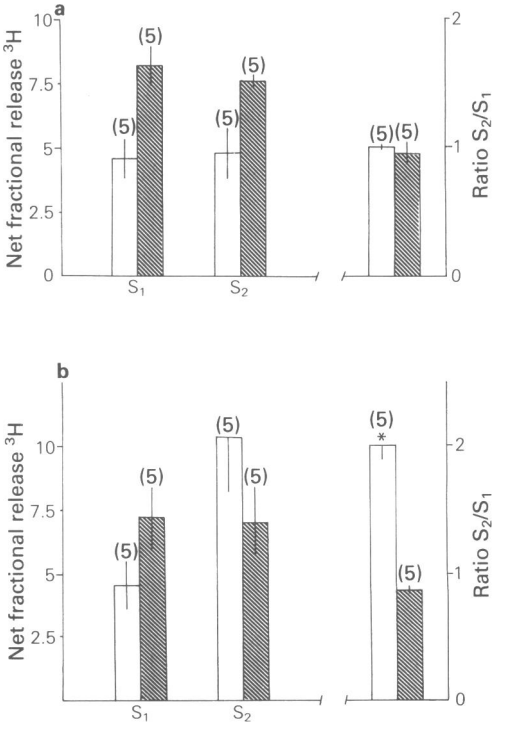


**Figure 1** Effect of Bay K 8644 on the release of tritium ($^3$H) elicited by potassium in control (open columns) and phentolamine-treated (hatched columns) rat vasa deferentia previously labelled with [$^3$H]-noradrenaline (see Methods). Left ordinate scale indicates the net fractional release of tritium by two consecutive pulses of potassium (59 mM) with 30 min interval, ($S_1$ and $S_2$). Right ordinate scale shows ratios between net release obtained during $S_1$ and $S_2$. Each column represents the mean and vertical lines s.e.mean, of the number of experiments shown in parentheses. (a) Net fractional release of $^3$H-evoked by potassium, and $S_2/S_1$ ratio, in control and phentolamine (10 $\mu$M) treated preparations. (b) As in (a), but Bay K 8644 (1 $\mu$M) was present in the medium 10 min before and during $S_2$ both in control and phentolamine (10 $\mu$M)-treated preparations. * $P < 0.001$, compared to control in (a).

## Results

*Potassium-evoked release of tritium in control and phentolamine-treated rat vasa deferentia*

Rat vasa deferentia preloaded with [$^3$H]-noradrenaline were exposed to potassium-enriched (59 mM) Krebs solution for 5 min twice ($S_1$ and $S_2$) 30 min apart. Figure 1a shows that the net fractional release of tritium was $4.5 \pm 0.7$ and $4.75 \pm 1.09$ during $S_1$ and $S_2$, respectively. The mean $S_2/S_1$ ratio was $1.01 \pm 0.02$.

In a second group of similar experiments phentolamine (10 $\mu$M) was present 10 min before and during the stimulation periods $S_1$ and $S_2$. Under these experimental conditions, the potassium-evoked $^3$H release was $8.2 \pm 0.8$ and $7.5 \pm 0.3$ in $S_1$ and $S_2$ respectively (Figure 1a). Even though the net fractional release of $^3$H evoked by potassium in phentolamine-treated preparations was markedly increased the $S_2/S_1$ ratios were similar in both control and phentolamine-treated preparations.

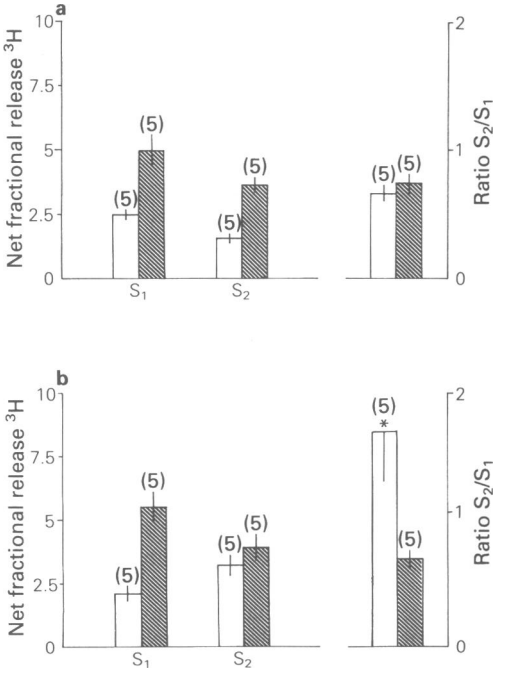


**Figure 2** Effect of Bay K 8644 on the release of tritium ($^3$H) evoked by electrical stimulation in control (open columns) and phentolamine-treated (hatched columns) rat vasa deferentia preloaded with [$^3$H]-noradrenaline. (a) Net fractional release of tritium evoked by two consecutive periods of stimulation and $S_2/S_1$ ratios in control and phentolamine (10 $\mu$M)-treated preparations. (b) As in (a), but Bay K 8644 (1 $\mu$M) was present in the medium, 10 min before and during $S_2$ both in control and phentolamine (10 $\mu$M)-treated preparations. Each column represents the mean and vertical lines s.e.means, of the number of experiments shown in parentheses. * $P < 0.05$, compared to control in (a).

*The effect of Bay K 8644 on the potassium-evoked release of tritium in control and phentolamine-treated vasa deferentia*

Since the release of noradrenaline evoked by potassium is due to activation of voltage-dependent calcium channels and the novel dihydropyridine, Bay K 8644, seems to activate such channels it was of interest to explore its effects on this release of $^3$H. Bay K 8644 (1 $\mu$M) was present in the medium 10 min before and during $S_2$ and was found not to alter the spontaneous release of tritium. Figure 1b shows the results and indicates that the net fractional $^3$H release evoked by potassium was $4.5 \pm 0.9$ and $10.3 \pm 2.1$ in $S_1$ and $S_2$, respectively; the ratio of $S_2/S_1$ was $2.3 \pm 0.14$ ($P < 0.001$, compared to controls).

In a second group of experiments, phentolamine (10 $\mu$M) was present 10 min before and during $S_1$ and $S_2$ and additionally, Bay K 8644 (1 $\mu$M) was added to the medium 10 min before and during the second stimulation period ($S_2$) with potassium. The results are shown in Figure 1b and indicate that under these experimental conditions the net fractional release of $^3$H evoked by potassium was $7.2 \pm 1.2$ and $7.0 \pm 1.4$ in the absence and presence of Bay K 8644, respectively. The $S_2/S_1$ ratio amounted to $0.78 \pm 0.01$, a value not significantly different from that found in phentolamine-treated preparations in the absence of Bay K 8644.

*The effect of Bay K 8644 on the release of tritium evoked by electrical field stimulation in control and phentolamine-treated vasa deferentia*

Vasa deferentia labelled with [$^3$H]-noradrenaline were stimulated electrically twice ($S_1$ and $S_2$) with a 30 min interval. Under these circumstances the net fractional release was $2.5 \pm 0.2$ and $1.5 \pm 0.3$ in $S_1$ and $S_2$, respectively. The $S_2/S_1$ ratio was $0.66 \pm 0.06$ (Figure 2a). In a second group of experiments Bay K 8644 (1 $\mu$M) was present in the medium 10 min before and during $S_2$. The results are shown in Figure 2b and indicate that Bay K 8644 increases the release of $^3$H evoked by electrical stimulation during $S_2$. The ratio $S_2/S_1$ in this case was $1.7 \pm 0.45$, a value significantly different from the control value, obtained in preparations in the absence of Bay K 8644.

In a group of similar experiments, phentolamine (10 $\mu$M) was present in the medium 10 min before and during $S_1$ and $S_2$. Under these experimental conditions, even though the net fractional release of tritium markedly increased ($4.95 \pm 0.6$ and $3.5 \pm 0.3$ for $S_1$ and $S_2$, respectively), the $S_2/S_1$ ratio ($0.74 \pm 0.05$), was

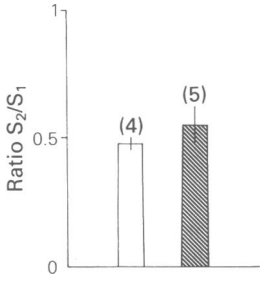


**Figure 3** Inability of Bay K 8644 to facilitate the release of tritium evoked by two consecutive pulses ($S_1$ and $S_2$) of tyramine (10 μM, 10 min with a 30 min interval) in the rat vas deferens preloaded with [$^3$H]-noradrenaline. Ordinate scale shows ratios between the net fractional release of $^3$H obtained in $S_2$ and $S_1$, in control (open columns) and Bay K 8644 (1 μM)-treated (hatched columns) preparations. Bay K 8644 was present 10 min before and during $S_2$. Each column represents the mean, and vertical lines s.e.mean, of the number of experiments shown in parentheses.

not significantly different from the control (not treated with phentolamine) ratio (Figure 2a).

Bay K 8644 (1 μM), when it was applied 10 min before and during $S_2$, did not further increase the release of tritium evoked by electrical stimulation of phentolamine (10 μM)-treated preparations (Figure 2b).

*The effect of Bay K 8644 on the release of tritium induced by tyramine in the rat vas deferens*

Since tyramine-evoked release of noradrenaline is not a calcium-dependent process (Lindmar *et al.*, 1967) one would not expect Bay K 8644 to affect the release of tritium induced by this sympathomimetic agent. In order to substantiate this, vasa deferentia labelled with [$^3$H]-noradrenaline were exposed twice to tyramine (10 μM for 5 min) with a 30 min interval. The net fractional release of $^3$H induced by tyramine was $6.42 \pm 0.67$ and $3.09 \pm 0.7$ in $S_1$ and $S_2$, respectively; the mean $S_2/S_1$ ratio was $0.48 \pm 0.02$ (Figure 3).

When Bay K 8644 (1 μM) was present 10 min before and during $S_2$ no differences in tritium release were found. The net fractional release in $S_1$ and $S_2$ was respectively $6.57 \pm 0.77$ and $3.59 \pm 0.44$. The $S_2/S_1$ ratio under these conditions ($0.55 \pm 0.08$) was not significantly modified compared to controls (Figure 3).

## Discussion

In the present study the effect of the calcium channel agonist Bay K 8644 on the release of noradrenaline from sympathetic nerve terminals in the rat vas deferens, evoked by calcium-dependent and calcium-independent procedures, has been examined. The results show three main findings: (1) Bay K 8644 significantly potentiates the calcium-dependent release of tritium evoked by potassium and electrical stimulation, from vasa deferentia previously labelled with [$^3$H]-noradrenaline. (2) The facilitatory effect of Bay K 8644 on $^3$H release evoked by potassium and electrical stimulation was abolished in the presence of phentolamine. (3) The release of $^3$H induced by tyramine which is not a calcium-dependent process was unaffected by Bay K 8644.

Noradrenaline release evoked by electrical stimulation and high potassium is a calcium-dependent process triggered by an increase in the amount of calcium entering the cell through voltage-operated channels (Kirpekar & Misu, 1967; Kirpekar & Wakade, 1968; García *et al.*, 1976). Bay K 8644 is a dihydropyridine derivative that, in contrast to $Ca^{2+}$-channel blocking agents, (Ceña *et al.*, 1983) behaves as a $Ca^{2+}$ channel activator at the cat adrenal chromaffin cell membrane, increasing both calcium influx and catecholamine secretion evoked by moderately high $K^+$ concentrations (García *et al.*, 1984).

As in the adrenal medulla, Bay K 8644 did not modify the spontaneous release of tritium from vasa deferentia pre-labelled with [$^3$H]-noradrenaline but potentiated the release evoked by high potassium or electrical field stimulation. As suggested for cardiac cells (Hess *et al.*, 1984), Bay K 8644 might increase the probability of opening of voltage-dependent calcium channels located on the axolemma of sympathetic nerve terminals; this would result in more calcium entering the terminal varicosity and so enhance exocytotic release of the transmitter. This interpretation is supported by the observation that in the presence of phentolamine, a drug that removes the $\alpha_2$-adrenoceptor-mediated control of noradrenaline release (Kirpekar & Puig, 1971; Starke *et al.*, 1971; Enero *et al.*, 1972), Bay K 8644 did not further enhance transmitter release.

It has been suggested that activation of synaptic $\alpha_2$-adrenoceptors by physiologically released noradrenaline could act as a signal to decrease its further release by restricting the access of external calcium inside nerve terminals (Drew, 1978; Alberts *et al.*, 1981; Wakade & Wakade, 1983; but see opposing view by Kalsner, 1981). In fact, α-adrenoceptor agonists and antagonists modulate calcium currents in sympathetic neurones (Horn & MacAfee, 1980; McAfee *et al.*, 1981). When phentolamine is present in the medium the $\alpha_2$-adrenoceptor mediated negative feed back system controlling the release of neurotransmitter is removed, therefore, most of the calcium channels are either in their maximal state of activation or their rate of inactivation is so slow that Bay K 8644 can not

increase the probability of them opening or further delay their inactivation. Hence, under these circumstances, Bay K 8644 would be unable to increase further the release of neurotransmitter induced by depolarizing stimuli.

Since the release of noradrenaline evoked from adrenergic nerves by tyramine, in contrast to that induced by potassium and electrical stimulation, occurs in the absence of external calcium (Lindmar *et al.*, 1967) the presumed depolarization-dependent activation and inactivations of calcium channels should not interfere with the release of noradrenaline by this indirectly acting sympathomimetic amine. In accord with this reasoning our results showed that the release of $^3H$ evoked by tyramine was not affected by Bay K 8644. These experiments lend further support to the hypothesis that Bay K 8644 increases the release of noradrenaline, evoked by depolarizing stimuli, by acting on the voltage-dependent calcium channels to enhance the probability that the channels will show a mode of gating marked by long opening and brief closing events, as suggested by Hess *et al.* (1984).

In conclusion, our results show that Bay K 8644, a calcium-channel activator, increases the calcium-dependent release of [$^3H$]-noradrenaline evoked by potassium and electrical stimulation of adrenergic nerves. This facilitatory effect of Bay K 8644 on transmitter release is abolished in the presence of phentolamine. No facilitatory effect of Bay K 8644 on [$^3H$]-noradrenaline release induced by tyramine, a non-calcium-dependent secretagogue, was observed. Taken together, our data support the view that $\alpha_2$-adrenoceptors modulate noradrenaline release by limiting calcium entry through specific voltage-dependent channels present on the axolemma of sympathetic nerve terminals.

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