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Short communication

# Frequency-dependent inhibition of neurotransmitter release by besipirdine and HP 184

Lei Tang, Sathapana Kongsamut *

*Neuroscience Therapeutic Domain, Hoechst Marion Roussel, P.O. Box 2500, Somerville, NJ 08876, USA*

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**Abstract**

We have described the interaction of besipirdine (HP 749, *N*-(*n*-propyl)-*N*-(4-pyridinyl)-1*H*-indol-1-amine hydrochloride) with voltage-dependent $Na^+$ channels (Tang et al., 1995, Br. J. Pharmacol. 116, 2468). Here we describe studies with besipirdine and a related compound, HP 184 (*N*-(*n*-propyl)-3-fluoro-4-pyridinyl)-1*H*-3-methylindol-1-amine hydrochloride), showing that this interaction is voltage-dependent and leads to frequency-dependent inhibition of electrically stimulated neurotransmitter release. Thus, the inhibition of veratridine-induced increases in intracellular $Ca^{2+}$ was enhanced by depolarization with KCl ($IC_{50}$ shifted from 23.8 ± 1.4 μM in 5 mM KCl to 7.3 ± 1.2 μM in 15 mM KCl for besipirdine and from 58.2 ± 1.3 μM to 14.1 ± 1.3 μM for HP 184). Moreover, the enhancement of electrically stimulated [$^3$H]norepinephrine release by besipirdine was diminished at higher frequencies of stimulation. As has been previously suggested for such compounds, we predict that besipirdine would act as a filter in the brain allowing signalling at low frequencies but blocking transmission at high frequencies.

*Keywords:* $Na^+$ channel, voltage-dependent; Anticonvulsant

## 1. Introduction

Besipirdine (HP 749; *N*-(*n*-propyl)-*N*-(4-pyridinyl)-1*H*-indol-1-amine hydrochloride) is a compound that has been studied in clinical trials for efficacy in treating Alzheimer's disease, a progressive neurological disorder affecting an increasing number of people worldwide (Evans et al., 1989). Besipirdine and the related compound HP 184 (*N*-(*n*-propyl)-3-fluoro-4-pyridinyl)-1*H*-3-methylindol-1-amine hydrochloride) reverse memory deficits in animal behavioral models (Santucci et al., 1991; unpublished data). Other reports allude to their in vitro and in vivo pharmacological properties on multiple neurotransmitter systems (Cornfeldt et al., 1990; Huger et al., 1990; Zaczek et al., 1993; Smith et al., 1993, 1994).

Interactions of besipirdine with voltage-dependent $Na^+$ and $K^+$ channels have also been described (Huger et al., submitted; Tang et al., 1995). Besipirdine, at concentrations of 10–100 μM, inhibits both inward and outward currents and the pharmacological and electrophysiological properties of besipirdine and those of the $K^+$ channel inhibitor 4-aminopyridine have been compared (Huger et al., submitted). [$^3$H]Norepinephrine release induced by the $Na^+$ channel activator veratridine is inhibited by besipirdine (Tang et al., 1995). Furthermore, increases in intracellular $Na^+$ and $Ca^{2+}$ ($[Ca^{2+}]_i$) induced by veratridine are inhibited by besipirdine (Tang et al., 1995). Besipirdine also inhibits the binding of [$^3$H]batrachotoxin, a $Na^+$ channel activator. These observations point to an interaction of besipirdine with voltage-dependent $Na^+$ channels.

The functional consequences of binding to voltage-dependent $Na^+$ channels have not yet been fully explored. The inhibition of inward ($Na^+$) currents by besipirdine is voltage-dependent (Huger et al., submitted) with greater inhibition being seen at more depolarized potentials. On the other hand, besipirdine enhances the electrically stimulated release of [$^3$H]norepinephrine due to antagonism of $\alpha_2$-adrenoceptors and inhibition of norepinephrine reuptake (Smith et al., 1994). In contrast, HP 184 does not bind to $\alpha_2$-adrenoceptors and does not inhibit norepinephrine reuptake (Smith et al., 1993). Thus HP 184 does not enhance electrically stimulated release (Smith et al., 1993). However, HP 184 does inhibit [$^3$H]batrachotoxin binding, indicating similar interaction with the voltage-dependent

* Corresponding author. Tel.: 908-231-3072; fax: 908-231-2413; e-mail: KONGSAM1@brwhcc3.hcc.com.


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$Na^+$ channel (Tang et al., 1995). We anticipated from these observations that the effects of besipirdine and HP 184 on [$^3$H]norepinephrine release would be frequency-dependent; that is, at higher frequencies of stimulation, besipirdine and HP 184 would be more likely to bind to voltage-dependent $Na^+$ channels and inhibit their activity and consequently inhibit action potential propagation.

We report here that the inhibition of veratridine-induced increases in $[Ca^{2+}]_i$ by besipirdine and HP 184 is more potent under depolarizing conditions and that neurotransmitter release is inhibited by these compounds at higher frequencies of stimulation. As previously suggested (Huger et al., submitted), the predicted functional consequence of these properties is that besipirdine and HP 184 should act as a low-pass filter for neuronal signals. This is similar to what has been seen with anticonvulsants such as diphenylhydantoin and carbamazepine (Willow et al., 1985; Catterall, 1987).

## 2. Materials and methods

Besipirdine and HP 184 were synthesized according to Effland et al. (1990). All other compounds were obtained from commercial sources. Studies in this report were carried out in accordance with the Declaration of Helsinki and with the guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

### 2.1. Measurements of intracellular calcium

Whole brain (minus cerebellum) synaptosomes were prepared from male Wistar rats by a Percoll gradient method described by Kongsamut and Nachshen (1988). Following the Percoll gradient centrifugation, synaptosomes were resuspended in Hepes-buffered saline (HBS, composition in mM: NaCl, 124; KCl, 5; $CaCl_2$, 1; $NaHCO_3$, 25.9; $KH_2PO_4$, 1.2; Hepes, 10; glucose 10; pH to 7.4 with NaOH) and were incubated with 10 $\mu$M fura2/AM for 60 min at 30°C, then centrifuged to wash out excess fura2/AM (Molecular Probes, Eugene, OR, USA). After a 15 min period to allow the fura2/AM taken up to be hydrolyzed, experiments were begun. Aliquots (100 $\mu$l) of synaptosomes were spun down in a microcentrifuge, resuspended and placed into 2 ml of buffer with 5 mM KCl (composition in mM: NaCl, 104; KCl, 5; choline Cl, 20; $CaCl_2$, 1; $NaHCO_3$, 25.9; $KH_2PO_4$, 1.2; Hepes, 10; glucose 10; pH to 7.4 with NaOH) or 15 mM KCl (composition in mM: NaCl, 104; KCl, 15; choline Cl, 10; $CaCl_2$, 1; $NaHCO_3$, 25.9; $KH_2PO_4$, 1.2; Hepes, 10; glucose 10; pH to 7.4 with NaOH) in a cuvette in a spectrofluorometer (PTI, South Brunswick, NJ, USA) at room temperature (~ 22°C). This would set the calculated membrane potential at −70 and −40 mV respectively. Fluorescence was monitored with excitation wavelengths alternating between 340 nm and 380 nm and emission set at 510 nm. A 20-s baseline was collected before besipirdine or HP 184 was added, allowing drug to bind under two KCl conditions; 50 s later, veratridine (25 $\mu$M; Sigma, St. Louis, MO, USA) was added. The ratios of the signals at 340 and 380 nm were calculated and normalized to percent inhibition of the response to veratridine in the absence of drug.

### 2.2. Measurement of neurotransmitter release

[$^3$H]Norepinephrine (35 Ci/mmol, DuPont-NEN) release was measured using a previously described method (Smith et al., 1994). Briefly, cortical slices (0.4 mm) were pre-incubated in Krebs buffer (composition in mM: NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; $KH_2PO_4$, 2.2; $NaHCO_3$, 24.9; $CaCl_2$, 1.3; dextrose, 11.1; saturated with 95% $O_2$/5% $CO_2$, pH 7.4) for 30 min at 35°C and then incubated in fresh Krebs buffer containing 25 nM [$^3$H]norepinephrine for 30 min at 35°C. The slices were then placed in glass superfusion chambers (Zahniser et al., 1986) and perfused at 0.75 ml/min at 35°C; fractions were collected at 7 min intervals. Electrical stimulation (parameters: 40 V, 15–30 mA (depending on the resistance of individual chambers), 5, 10 or 20 Hz, for 1 min) was applied at fraction 3. Besipirdine (10 and 30 $\mu$M) or HP 184 (10 $\mu$M) was introduced after fraction 10 and a second electrical stimulus was applied at fraction 13. The fractions collected were counted in 10 ml of Liquiscint (National Diagnostics, Atlanta, GA, USA) scintillation fluid and corrected for quench. Tissue slices were dissolved overnight in 0.5 ml of Protosol (DuPont-NEN), buffered with 1 ml of Tris HCl (Sigma), and counted. Percent fractional release (%FR) was defined as the ratio of tritium released versus the amount present in the tissue. The ratio of the %FR at the second vs. the first stimulation period (S1/S2) was calculated.

## 3. Results

Fig. 1A and B shows the voltage-dependent effects of besipirdine and HP 184. Depolarization of the membrane by elevation of the extracellular $K^+$ concentration from 5 mM to 15 mM produces a calculated change in resting membrane potential from −70 mV to −40 mV. This change in $[K^+]$ leads to a shift to the left of the dose-response curve for both compounds. The $IC_{50}$ values for inhibition of veratridine-induced increases in $[Ca^{2+}]_i$ shifted from 23.8 ± 1.4 $\mu$M to 7.3 ± 1.2 $\mu$M for besipirdine, and from 58.2 ± 1.3 $\mu$M to 14.1 ± 1.3 $\mu$M for HP 184.

Table 1 shows the frequency- and concentration-dependent effects of besipirdine and HP 184 on electrically stimulated [$^3$H]norepinephrine release. As can be seen and as has been previously reported (Smith et al., 1994),

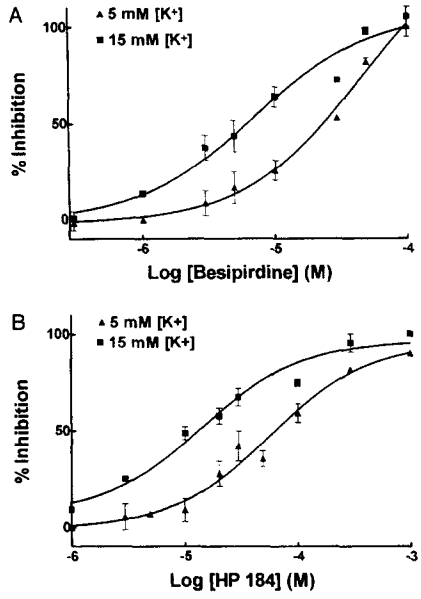


Fig. 1. The inhibitory effects of both besipirdine and HP 184 on veratridine-stimulated increases in $[Ca^{2+}]_i$ are membrane potential dependent. Rat brain synaptosomes were prepared as described in Materials and methods. They were then loaded with 10 $\mu$M fura2/AM for 60 min at 30°C. Aliquots (100 $\mu$l) of synaptosomes were washed once and placed into 2 ml of Hepes-buffered saline with 5 mM or 15 mM KCl for fluorescence measurements. A 30 s baseline was collected before the besipirdine was added; 90 s later, veratridine (25 $\mu$M) was added. The ratios of the fluorescence signals from excitation at 340 and 380 nm were calculated and analyzed. Data are means ± S.E. ($n = 3$). (▲) 5 mM KCl and (■) 15 mM KCl. A: Besipirdine. B: HP 184.

besipirdine enhanced the release of [$^3$H]norepinephrine evoked by 5 Hz electrical stimulation. This is due to its properties as an $\alpha_2$-adrenergic (autoreceptor) antagonist and as an inhibitor of norepinephrine reuptake (Smith et al., 1994). At the 5 Hz frequency of stimulation, the enhancement was larger at 30 $\mu$M than it was at 10 $\mu$M (Smith et al., 1994). When the frequency of stimulation was raised to 10 Hz, the enhancement at 10 $\mu$M was reduced, and at 30 $\mu$M, release was significantly inhibited by besipirdine (S2/S1 = 0.77). At 20 Hz stimulation, both concentrations of besipirdine significantly inhibited release, and greater inhibition was seen at 30 $\mu$M.

HP 184 does not have the effects on $\alpha_2$-adrenoceptors and norepinephrine reuptake that besipirdine has (Smith et al., 1993) and it did not enhance release evoked by 5 or 10 Hz electrical stimulation (Table 1). At a higher frequency of stimulation (20 Hz), HP 184 significantly inhibited release.

## 4. Discussion

We have demonstrated that the ability of besipirdine and HP 184 to inhibit veratridine-induced increases in $[Ca^{2+}]_i$ is voltage-dependent. This higher affinity at a depolarized membrane potential is expected to translate to a greater inhibition of voltage-dependent $Na^+$ channels when the membrane is depolarized, as for example during an action potential. During electrically stimulated release, when the frequency of stimulation is increased, the membrane is, on average, depolarized for a longer period of time. Thus, at higher frequencies of stimulation, besipirdine would be expected to inhibit voltage-dependent $Na^+$ channels leading to an inhibition of release. This is exactly what has been observed. We thus believe that the voltage-dependent effects of besipirdine and HP 184 translate to the frequency-dependent effects observed, and that besipirdine would act as a low-pass filter in the nervous system, inhibiting high-frequency signals but allowing low-frequency transmission. This mechanism has been proposed for anticonvulsants such as diphenylhydantoin and carbamazepine (Willow et al., 1985; Catterall, 1987).

Voltage-dependent effects of besipirdine studied using electrophysiological methods have been previously reported (Huger et al., submitted). These studies showed that besipirdine inhibits both inward and outward currents and that the inhibition of inward currents, presumably carried

Table 1
Effects of besipirdine and HP 184 on electrically stimulated [$^3$H]norepinephrine release

| Drug | Concentration ($\mu$M) | Frequency (Hz) | S1/S2 | | |
|---|---|---|---|---|---|
| | | | Control | Drug | Drug/Control |
| Besipirdine | 10 | 5 | 1.26 ± 0.15 | 3.38 ± 0.18 [a] | 2.68 |
| | | 10 | 0.85 ± 0.03 | 1.83 ± 0.08 [a] | 2.15 [b] |
| | | 20 | 1.10 ± 0.06 | 0.86 ± 0.03 | 0.85 [b] |
| | 30 | 5 | 1.15 ± 0.05 | 4.87 ± 0.35 [a] | 4.22 |
| | | 10 | 1.06 ± 0.01 | 0.77 ± 0.08 | 0.72 [b] |
| | | 20 | 1.05 ± 0.02 | 0.69 ± 0.01 [a] | 0.65 [b] |
| HP 184 | 10 | 5 | 1.25 ± 0.04 | 1.39 ± 0.03 | 1.11 |
| | | 10 | 0.97 ± 0.04 | 1.09 ± 0.02 | 1.12 |
| | | 20 | 1.00 ± 0.01 | 0.67 ± 0.03 [a] | 0.67 [b] |

[$^3$H]Norepinephrine release was measured as described in Materials and methods. Electrical stimulation was applied at fraction 3. Besipirdine (10–100 $\mu$M) was introduced after fraction 10 and a second electrical stimulus was applied at fraction 13. The ratio of the %FR at the second vs. the first stimulation period (S1/S2) was calculated ($n = 3$). [a] Significantly different from control ($P < 0.05$). [b] Significantly different from 5 Hz stimulation group at the same concentration ($P < 0.05$).

by $Na^+$ channels, is voltage-dependent. The results reported here are consistent with and extend the electrophysiological data.

Besipirdine has previously been reported to enhance [$^3$H]norepinephrine release due to its inhibitory effects on norepinephrine reuptake and $\alpha_2$-adrenoceptors (Smith et al., 1994). Those studies were performed with an electrical stimulation frequency of 5 Hz. Our results also show that besipirdine produced a concentration-dependent enhancement of release at this frequency. It was only at the higher frequencies (10 and 20 Hz) that we observed a concentration-dependent *decrease* in release.

Enhancement of neurotransmitter release via autoreceptor and reuptake inhibition also show frequency dependence (see for example Wightman and Zimmerman, 1990). At high frequencies, reuptake has a diminishing effect on release because there is insufficient time between stimuli for the reuptake mechanisms to act (Wightman and Zimmerman, 1990). Similarly, the release-enhancing effects of autoreceptor inhibition also diminish at higher frequencies of stimulation, but an inhibition of release would not be expected (Wightman and Zimmerman, 1990). These frequency-dependent effects cannot explain the inhibition of release observed, however. We must thus invoke inhibition of voltage-dependent $Na^+$ channels to explain this effect. Indeed, HP 184, a structurally related compound which does not have the reuptake or autoreceptor inhibitory properties of besipirdine, did show frequency-dependent inhibition of release.

Although besipirdine has inhibitory effects on $K^+$ channels, we do not believe that these effects contribute to the observations we reported here. Inhibition of $K^+$ channels would be expected to *increase* $[Ca^{2+}]_i$ and neurotransmitter release. Since both besipirdine and HP 184 do not increase $[Ca^{2+}]_i$ by themselves at concentrations up to 100 $\mu$M (Tang et al., 1995; Tang and Kongsamut, unpublished data), or increase release at concentrations up to 30 $\mu$M (Smith et al., 1993, 1994), these effects on $K^+$ channels are apparently not predominant. Since $K^+$ channel inhibition would be expected to have opposite effects on release, we do not believe that what we observed was due to $K^+$ channel blockade.

We thus conclude that the voltage-dependent inhibition of besipirdine and HP 184 on voltage-dependent $Na^+$ channels leads to its frequency-dependent effects on neurotransmitter release which in turn selectively inhibits high-frequency neurotransmission. This property is consistent with besipirdine having anticonvulsant activity, as has been previously suggested (Tang et al., 1995).

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