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Effect of (+)-Sparteine on Nicotinic Acetylcholine Receptors in the Neurons of Rat Superior Cervical Ganglion

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SUMMARY

The effects of (+)-sparteine, a ganglionic blocking agent, on acetylcholine (ACh)-induced membrane currents and on fast excitatory postsynaptic currents (EPSCs) were studied in the neurons of rat isolated superior cervical ganglion, with the whole-cell patch-clamp recording method and the two-electrode voltage-clamp method, respectively. (+)-Sparteine (2 μM) reduced the ACh-induced current caused by activation of nicotinic ACh receptors (AChRs) in a voltage-independent manner at membrane potentials of −50 mV to +30 mV, whereas its blocking effect increased at more negative membrane potentials. The dose-response relationship for ACh was modified by 2 μM (+)-sparteine at −50 mV and at −90 mV in a fashion typical for competitive rather than noncompetitive antagonists. The apparent mean open time of the AChR channel, as estimated from the power density spectrum of the ACh-induced current fluctuations at −90 mV, was not decreased by 2 μM (+)-sparteine, in contrast to what was observed with hexamethonium, the well known open-channel blocker for ganglionic AChRs. At higher concentrations, i.e., 5 μM and 10 μM (lower concentrations were not effective), (+)-sparteine reduced the amplitude of the EPSC and the time constant of the EPSC decay. The former effect was voltage independent, whereas the latter effect was voltage independent at membrane potentials of −70 mV and more positive and increased at membrane potentials of −90 and −110 mV. These results suggest that (+)-sparteine produces in ganglionic AChRs a competitive blocking effect and, in addition, an open-channel blockade. The latter component probably provides a smaller contribution than does the former to the blockade by (+)-sparteine of the ACh-induced current. Conformational analysis of the (+)-sparteine molecule was performed, and the dimensions of the molecule were measured. Minimum dimensions of the space-filling profile for two conformers, high and low populated, were found to be 7.3 × 7.9 Å and 6.8 × 7.5 Å, respectively. Both profiles are larger than the channel profile at which the open-channel blockers have been suggested to bind, which may explain comparatively low open-channel-blocking activity of (+)-sparteine.

Two main mechanisms, a competitive block and an open-channel block, have been identified in the effects produced by selective ganglionic blocking agents on nicotinic AChRs in the neurons of autonomic ganglia. The competitive blocking agents are less numerous than the channel-blocking ones. Examples of the former are trimethaphan (1, 2), surugatoxin (3), and II-S1 component of snake venom (see Ref. 4). Further investigation of the ligand structures responsible for the competitive and the open-channel blockades is desirable as an approach to elucidating the distinctions between the two corresponding binding sites in the AChR molecule.

In contrast to the open-channel blockers like hexamethonium, pempidine, and their derivatives (4), trimethaphan is too large a molecule to reach a site in the AChR channel where the open channel blockers are presumed to bind (5) (see below). Another comparatively large nicotinic antagonist, (+)-tubocurarine, is known to cause in the end-plate AChRs competitive as well as open-channel blockade (6), whereas only the latter type of blockade by (+)-tubocurarine has been observed in the AChRs of parasympathetic ganglion neurons (1).

(+)-Sparteine (also known as pachicarpine) (7), a ganglionic blocking agent with additional oxytocic action (8, 9), is similar to the aforementioned antagonists in possessing a positive charge and is somewhat larger than the open channel blockers. The mechanisms of the blocking effect of (+)-sparteine on ganglionic AChRs have not been studied. Their examination was the goal of the present work.

It has been commonly accepted that competitive block, in contrast to open-channel block, is voltage independent (1, 10), decreases with increased agonist doses, and is not followed by a decrease in mean channel open time (see, e.g., Ref. 6). All three criteria were used in the present work to identify the type of the (+)-sparteine-induced blockade of ganglionic AChRs.

The effects of (+)-sparteine on the amplitude and fluctua-

ABBREVIATIONS: AChR, acetylcholine receptor; EPSC, excitatory postsynaptic current; ACh, acetylcholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid.

tions of ACh-induced current, as well as on the EPSC recorded from the neurons of rat superior cervical ganglion, were examined. It was found that (+)-sparteine had a competitive blocking action and additional weak open-channel-blocking action. In addition, the size of the (+)-sparteine molecule was measured by using special computer programs. The results suggest that at least one of the factors responsible for a comparatively weak open-channel-blocking activity of (+)-sparteine is a larger molecular size, compared with the size of the AChR channel profile. A short communication on these results was published elsewhere (11).

Materials and Methods

Experimental procedures. Rat superior cervical ganglion was removed from the animal, with ether anesthesia, and was put in a salt solution of the following composition (in mM): NaCl, 133; KCl, 5.9; $CaCl_2$, 2.5; Tris hydrochloride, 10; glucose, 11; pH 7.4. For investigation of ACh-induced currents, the ganglion was desheathed and incubated with 0.4% collagenase (Sigma type 1A) at 37° for about 1 hr, to clean the nerve cell surface. ACh was applied to a nerve cell ionophoretically, through a micropipette containing 1.5 M ACh, with a resistance of about 50 mΩ. The ionophoretic current was 2–200 nA. The ACh-induced currents were recorded with a whole-cell patch-clamp method at 20–24°. The recording micropipette contained a solution of the following composition (in mM): KCl, 140; EGTA-KOH, 11; HEPES-KOH, 10; pH 7.2. (+)-Sparteine was applied by perfusion.

To discriminate between the ACh-induced current caused by activation of nicotinic AChRs and possible muscarinic current, ACh was applied ionophoretically with short (0.02–0.24-sec) pulses, in addition to the routine long ones (1 sec). The time between the onset of the ionophoretic pulse and the peak in the response to short ACh application was comparable to the reported latency of muscarinic response (about 0.1 sec) (12). Therefore, muscarinic current, if it occurred in our experiments, would not essentially interfere with the response to short ACh applications.

An independent test of whether muscarinic AChRs were involved was to compare the effects of (+)-sparteine on the ACh-induced currents in the absence and in the presence of 1 μM atropine.

The ACh-induced currents were recorded simultaneously with a low-gain amplifier (frequency band width, 0–4 kHz) and with a high-gain amplifier (frequency band width, 0–1 kHz) and were digitized at 0.1–1 kHz and at 0.5 kHz, respectively. The records obtained at high gain were used for analysis of ACh-induced current fluctuations. Their difference power spectra were obtained by subtraction of the control spectra recorded in the absence of (+)-sparteine from those recorded in the presence of (+)-sparteine.

For investigation of the EPSCs, the ganglion was isolated together with a piece of cervical sympathetic nerve (about 1.5 cm). The isolated ganglion was continuously perfused throughout the experiment, at 22°, with a salt solution of the following composition (in mM): NaCl, 140; KCl, 4.0; $CaCl_2$, 2.0; $MgCl_2$, 0.5; KH_2PO_3, 1.0; $NaHCO_3$, 12.0; glucose, 11.0; pH 7.2. The nerve was drawn into a sucking pipette electrode for stimulation. Membrane currents were evoked by single preganglionic stimuli applied at intervals of 14 sec. The currents were recorded by using a conventional two-electrode voltage-clamp technique. The intracellular electrodes were filled with 2.5 M KCl and had a resistance of 100–120 mΩ. All results (except molecular size and the size of channel profile) were expressed as mean ± standard error and were subjected to Student's *t* test for evaluation of statistical significance.

Chemical compounds. (+)-Sparteine (dodecahydro-7,14-methano-2*H*,6*H*-dihydro[1,2-*a*,1′,2′-*e*]diazocine iodide) was obtained as a gift from Professor I. V. Komissarov. Hexamethonium dibromide was synthesized by Dr. V. E. Gmiro in the Institute of Experimental Medicine, the USSR Academy of Medical Sciences, Leningrad. Atropine sulfate was obtained from the USSR Ministry of Health Industry.

Results

Measurement of the size of the (+)-sparteine molecule. The chemical structure and absolute configuration of (+)-sparteine, a tetracyclic compound, are shown in Fig. 1, *inset* (see Ref. 13). Two side cyclohexane rings and one piperidine ring of sparteine are in the most energetically preferable chair conformation, whereas another piperidine ring can convert from a chair into a boat form, with simultaneous inversion of a nitrogen atom (14, 15). Due to this interconversion, sparteine and its derivatives can exist, basically, in *trans*- and *cis*-conformations.

By using a universal program for calculations of molecular mechanics, as described elsewhere (5, 16), the energy minima corresponding to *trans*- and *cis*-conformations of protonated (+)-sparteine were found. According to this calculation, the population of the *cis*-conformer exceeded 99%. Minimum-energy conformers were visualized with the use of the MOLGRAPH molecular modeling package. Space-filling displays of *cis*- and *trans*-conformers of (+)-sparteine are shown in Fig. 1.

For estimation of minimal-profile dimensions of a conformer, its wire-frame display was turned about three orthogonal axes to fit a rectangle of minimal size (Fig. 2). The minimal wire-frame display of the *cis*-conformer was 4.9 Å in width and 5.5 Å in height, which yielded a space-filling profile of 7.3 × 7.9 Å after addition of two Van der Waals radii of hydrogen atoms. The minimal space-filling profile of the low-populated *trans*-conformer was 6.8 × 7.5 Å.

Effect of (+)-sparteine on the amplitude of the ACh-induced current. When applied at a concentration of 2 μM, (+)-sparteine decreased the amplitude of ACh-induced current to 43 ± 19% ($n = 7$) of its control value at −50 mV. The 2 μM concentration was chosen as being very close to the IC_{50} for reduction of the ACh-induced current by (+)-sparteine. The blocking effect (Fig. 3, A and B) usually developed for about 5 min. Voltage dependence of the ACh-induced current was not affected by (+)-sparteine at membrane potentials from −50 to +30 mV (Fig. 3C). In more strongly hyperpolarized cells, the blocking effect was increased. The current amplitude measured in the presence of the blocking agent at −90 mV was only 63% of that expected if the blocking effect was voltage independent; the difference was significant.

The results shown in Fig. 3A were obtained with the use of ACh ionophoretic pulses long enough (1 sec) to allow the response to reach a plateau level. As Fig. 3B illustrates, similar results were obtained with short applications of ACh (0.02–0.24 sec), which were used to minimize possible contributions of any muscarinic current. In addition, in two neurons atropine (1 μM) was added to the perfusion solution. This was not followed by any appreciable changes either in the degree of the blocking effect or in its voltage dependence.

These results suggest that 1) muscarinic AChRs do not contribute to the ACh-induced currents evoked by 1-sec applications of ACh, possibly because muscarinic AChRs are inactivated due to the lack of calcium ions and ATP inside the cell (see Ref. 4), and that 2) the responses to ACh, evoked by 1-sec ACh applications, are not essentially affected by desensitization.

To express the blocking effect of (+)-sparteine quantitatively, we calculated λ, the ratio of ACh-induced current in the absence of a blocker (I) to the current in the presence of a blocker (I_B). The effect of a blocker was expressed as (λ − 1)

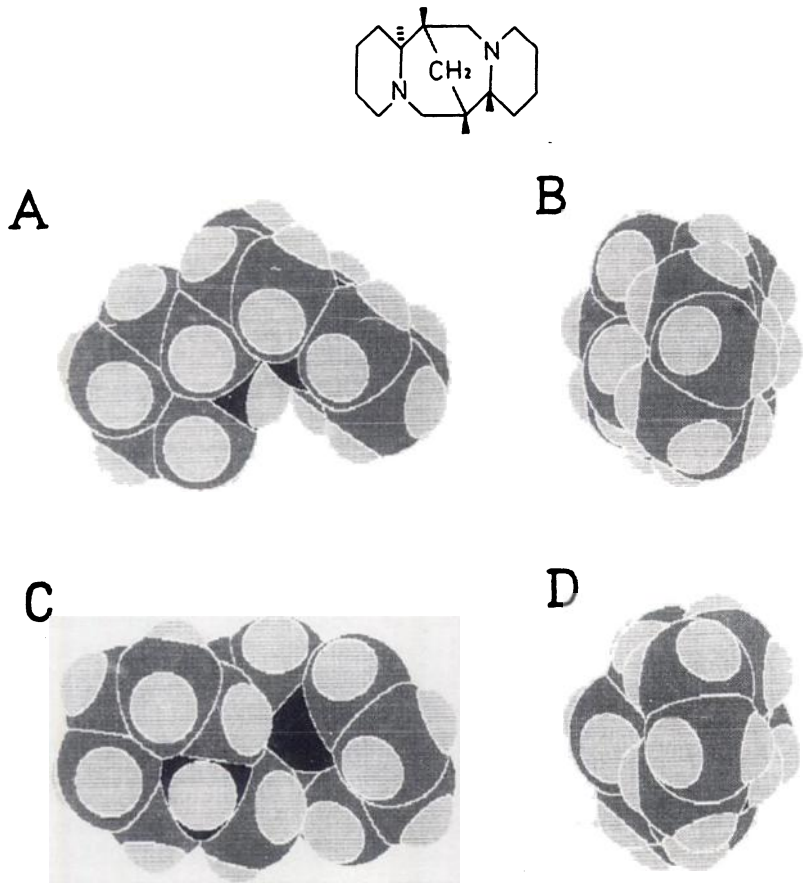

Fig. 1. Space-filling displays of two minimum-energy conformers of (+)-sparteine. Two views of the *cis*-conformer (A and B) and of the *trans*-conformer (C and D) are illustrated. Carbon, nitrogen, and hydrogen atoms are *gray*, *black*, and *sand*, respectively. *Inset*, structure of (+)-sparteine.

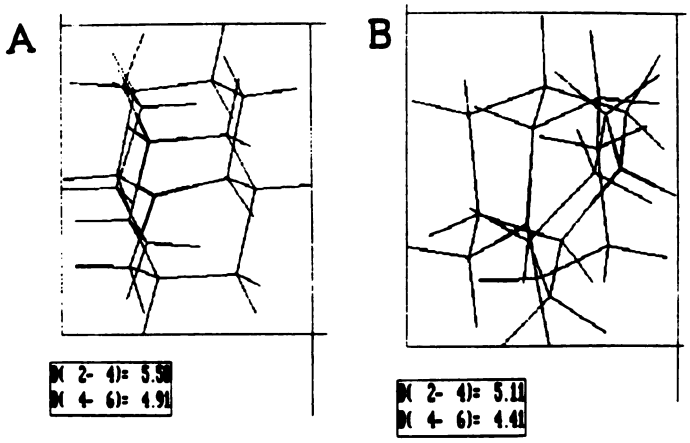

Fig. 2. Minimal profile wire-frame computer displays of two minimum-energy conformers of (+)-sparteine. A, *Cis*-conformer; B, *trans*-conformer. A rectangle described over each conformer has been constructed using a special computer technique; three dummy molecules, two linear and one angular, have been introduced. The outlined distances between the atoms in the dummy molecules represent the dimensions of the rectangle (indicated at the *bottom* in Å). Note the larger scale in B than in A.

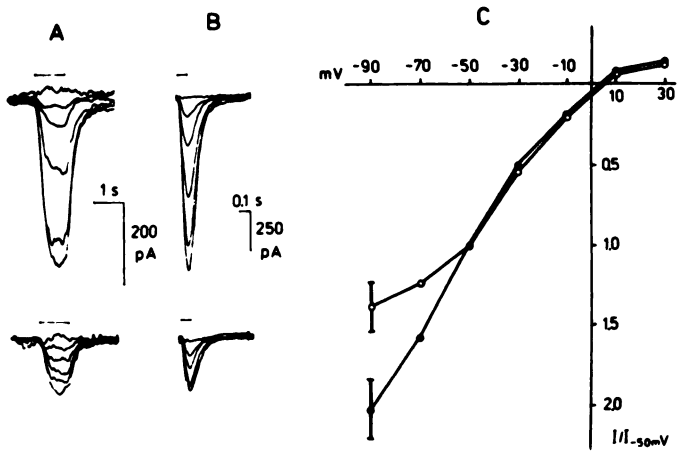

Fig. 3. Voltage dependence of the effect produced by (+)-sparteine on the ACh-induced membrane current. A and B, Membrane currents evoked by 1-sec (A) and 0.1-sec (B) ionophoretic applications of ACh in two different neurons, at membrane potentials of −90, −70, −50, −30, −10, and 10 mV, in the absence (*upper records*) and in the presence (*lower records*) of 2 μM (+)-sparteine. *Horizontal bars*, time of ACh application. C, Mean values of the ACh-induced membrane currents obtained from seven neurons (three neurons with 1-sec and four neurons with 0.1-sec ACh applications) in normal saline solution (●) and in the presence of 2 μM (+)-sparteine (○) and normalized relative to those obtained at −50 mV, plotted against membrane potentials. For −90 mV, mean ± standard error is shown.

(see Ref. 1). The values of $[\lambda(-90) - 1]$ and $[\lambda(-50) - 1]$ calculated for the same neurons ($n = 9$) with 2 μM (+)-sparteine were 2.0 ± 0.3 and 1.2 ± 0.3, and their ratio was 2.3 ± 0.5 (mean ± standard error from the paired data). On the other

hand, [λ(−30) − 1] was 1.1 ± 0.3, i.e., was almost similar to [λ(−50) − 1]. Therefore, the blocking effect of (+)-sparteine on the ACh-induced current was voltage independent at membrane potentials more positive than about −50 mV and was voltage dependent at more negative membrane potentials.

We also investigated the effect of 2 μM (+)-sparteine on the ACh dose-response relationship. Fig. 4 shows that (λ − 1) clearly decreases with increased ionophoretic currents, both at −50 mV and at −90 mV. Although the absolute values of the ACh doses applied could not be measured accurately with this technique, their relative values could be compared by assuming that the ACh dose depends directly on the ionophoretic current.

In one more group of neurons, (λ − 1) for 2 μM (+)-sparteine was calculated in each neuron for two ionophoretic currents, weak and strong. The "weak" current caused a response of a minimal amplitude that could be accurately measured above noise level, whereas the "strong" current was 3 times stronger (although it caused a response not higher than 1 nA). For weak and strong currents, [λ(−50) − 1] was 2.18 ± 0.32 and 1.29 ± 0.27 (n = 9) and [λ(−90) − 1] was 2.44 ± 0.33 and 1.62 ± 0.28 (n = 7), respectively (the data at −50 mV and at −90 mV were obtained from different neurons). The differences in (λ − 1) between weak and strong ionophoretic currents, as well as the differences between [λ(−50) − 1] and [λ(−90) − 1] for each of two ionophoretic currents, were significant.

The decrease in (+)-sparteine-induced blockade caused by increased ACh dose suggests a competitive blocking mechanism. At the same time, the fact that the blockade is increased by hyperpolarization implies that there is an open-channel-blocking component in (+)-sparteine-induced blockade (for more detailed discussion, see below).

Effect of (+)-sparteine on the ACh-induced current fluctuations. In five neurons, the power density spectra of the ACh-induced current fluctuations were obtained at −90 mV, both in normal saline solution and in the presence of 2 μM (+)-sparteine. The difference spectrum can be approximated by a Lorentzian function

$$S(f) = \frac{S(0)}{1 + (f/f')^2}$$

where $S(f)$ and $S(0)$ are power densities at the frequencies f and 0, respectively, and f' is a cutoff frequency that corresponds to $S = S(0)/2$ (see Ref. 17). In all five neurons studied, the spectrum was satisfactorily fitted by one Lorentzian function, with only very small improvement if a sum of two Lorentzian functions was used for the fit. Therefore, the approximation by a sum of two Lorentzian functions was not necessary, although both possibilities were tested in each neuron. An example is shown in Fig. 5. The mean open time of the AChR channel, estimated as the inverse of the cutoff frequency f', in normal saline solution varied from 10.9 to 20.4 msec, with a mean of 14.0 ± 1.7 msec, whereas in the presence of (+)-sparteine the corresponding values were 10.4 to 21.6 msec and 13.7 ± 2.0 msec (n = 5). The difference between the two means was not significant.

For comparison, the effect of hexamethonium, a well known open-channel-blocker for ganglionic AChRs, was studied in two neurons. Their mean channel open times, being equal to 23.3 msec and 11.8 msec in normal saline solution, dropped to 7.5 msec and 2.9 msec in the presence of 20 μM and 30 μM hexamethonium, respectively. This result was consistent with the effects of hexamethonium observed in rat submandibular ganglion (1) and in rabbit superior cervical ganglion (2, 4).

It can be concluded that (+)-sparteine (2 μM), in contrast to hexamethonium, does not decrease mean channel open time, as estimated from the power spectrum for the ACh-induced current fluctuations.

Effect of (+)-sparteine on EPSC. The EPSC decay curve could be fitted by a single exponential, if examined in the region from 90% to 10% of the EPSC maximum amplitude. The effect of (+)-sparteine on the EPSC was studied in five neurons. At −50 mV, (+)-sparteine at concentrations of 5 μM and 10 μM reduced the EPSC amplitude by 36 ± 6% and 40 ± 8%, respectively (n = 5). The effect of 10 μM (+)-sparteine is shown in Fig. 6A. The EPSC amplitude was reduced in a voltage-

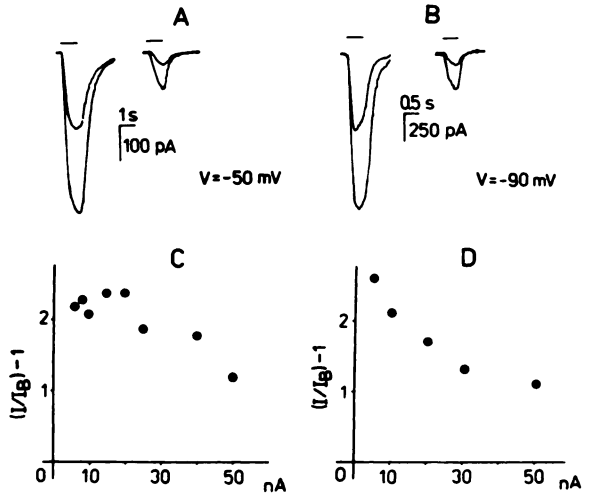

Fig. 4. Effect of ACh dose on (+)-sparteine-induced blockade. A and B, ACh-induced responses to near-threshold ionophoretic current (*right records*) and to 5 times stronger ionophoretic currents (*left records*), obtained in the absence and in the presence of 2 μM (+)-sparteine, at −50 mV (A) and at −90 mV (B). *Horizontal bars*, time of ACh application. C and D, Blocking effect of 2 μM (+)-sparteine plotted against different ionophoretic currents. I and I_B, responses to ACh in the absence and in the presence of the blocker, respectively. The results in A and C and in B and D were obtained from two different neurons.

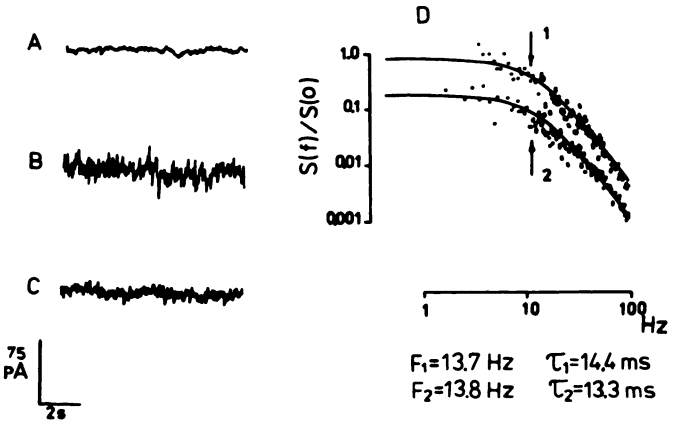

Fig. 5. Effect of (+)-sparteine on the power density spectrum of the ACh-induced fluctuations recorded at −90 mV. The records were obtained before (A) or during ionophoretic application of ACh in the absence (B) and in the presence (C) of 2 μM (+)-sparteine. Difference spectra 1 and 2 (D) were obtained from records similar to those shown in B and C, respectively. The *ordinate* values are normalized to $S(0)$ obtained in the absence of (+)-sparteine. The cutoff frequencies (*arrows*) and corresponding mean channel open times are indicated at the *bottom*.

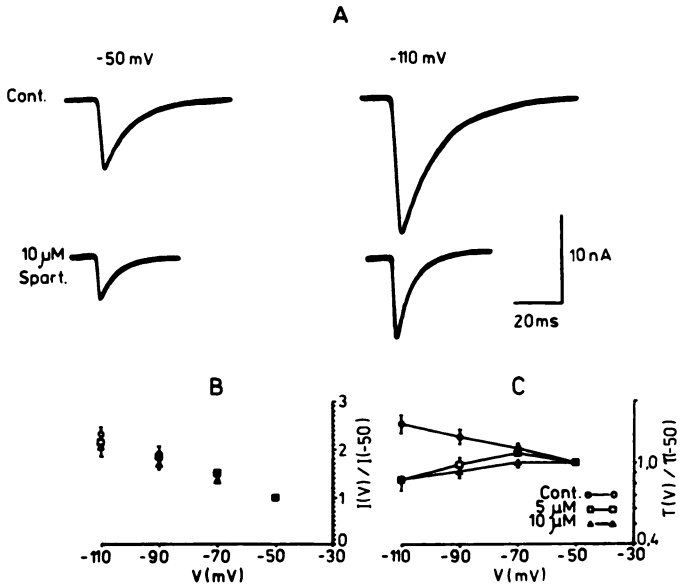

Fig. 6. Effects of (+)-sparteine on EPSC observed at different membrane potential levels. A, EPSCs recorded in normal saline solution (*upper records*) and in the presence of 10 μM (+)-sparteine (*lower records*), at −50 mV (*left*) and at −110 mV (*right*). B and C, Plots of the EPSC peak amplitude (B) and decay time constant (C), measured in normal saline solution (○) and in the presence of 5 μM (□) and 10 μM (+)-sparteine (△), against membrane potential (mean ± standard error; $n = 5$). The *ordinate* values are normalized to those obtained at −50 mV.

independent fashion; the slope of the current-voltage relationship curve for EPSC was unchanged by antagonist (Fig. 6B).

With (+)-sparteine concentrations lower than 5 μM, the blocking effect was too small to be measured sufficiently precisely, in contrast to the aforementioned effect of (+)-sparteine on ACh-induced current. The reason for this difference was not clear. One possibility was that it was due to a difference in pharmacology between the synaptic and nonsynaptic AChRs. Another possibility was that the concentration of ACh in the synaptic cleft was higher than that which could be attained with the ionophoretic ACh application.

(+)-Sparteine also markedly reduced the time constant of the EPSC decay (τ_{syn}). In the absence of antagonist at −50 mV, τ_{syn} was 12.3 ± 1.7 msec ($n = 6$). This τ_{syn} value, if taken as a unit, was decreased by 5 μM and 10 μM (+)-sparteine to 0.75 ± 0.54 and 0.67 ± 0.03, respectively (this difference was significant). In contrast to the reduction in the EPSC amplitude, the reduction in τ_{syn} was strongly voltage dependent; $\tau_{syn}(-70)$ was decreased by 5 μM and 10 μM (+)-sparteine from 1.17 ± 0.06 [normalized to $\tau_{syn}(-50)$] to 0.81 ± 0.02 and to 0.67 ± 0.02, respectively ($n = 5$; this difference was significant). At −90 mV the corresponding values were 1.34 ± 0.11, 0.72 ± 0.02, and 0.60 ± 0.04. The voltage dependence of the (+)-sparteine-induced decrease in τ_{syn} is illustrated in Fig. 6C.

The rate constant (k^*_{+B}) of a blocker binding to the open channel can be calculated from the equation

$$k^*_{+B} = [(\tau')^{-1} - \tau^{-1}]\ X_B^{-1}$$

where τ' and τ are the time constants of the EPSC decay measured in the presence and in the absence of the blocker, respectively, and X_B is the concentration of the blocker B. Mean values of $k^*_{+B}(-50)$ and $k^*_{+B}(-90)$ found from this equation were (3.4 ± 0.2) × 10^6 M^{-1} sec^{-1} and (6.6 ± 1.1) × 10^6 M^{-1} sec^{-1}. The mean H value, a shift in the membrane potential corresponding to an e-fold change in k^*_{+B}, was −59 mV.

Discussion

The results obtained in this work suggest that (+)-sparteine has a competitive blocking action and an additional open-channel-blocking action upon nicotinic AChRs in sympathetic ganglion neurons. The evidence for the competitive blocking mechanism is provided by the following results: 1) the blockade of the ACh-induced current by (+)-sparteine decreases with increasing ACh dose, 2) the blockade is voltage independent at membrane potentials more positive than about −50 mV, and 3) the EPSC amplitude is decreased by (+)-sparteine in a voltage-independent fashion.

The open-channel-blocking mechanism for (+)-sparteine is suggested by a blocker-induced decrease in τ_{syn} at membrane potentials more negative than about −50 mV and by a voltage-dependent depression of the ACh-induced currents at −70 mV and more negative membrane potentials. However, the two interpretations given above are by no means the only possible ones, and some other mechanisms (e.g., voltage-dependent "closed channel" block) (4) can also be considered.

The puzzling result is that 2 μM (+)-sparteine does not decrease the mean open time of AChR channels, as estimated from noise analysis at −90 mV, i.e., at the membrane potential at which 5 μM (+)-sparteine markedly decreases the τ_{syn} and at which all blocking effects of (+)-sparteine (except the depression of the EPSC amplitude) are clearly voltage dependent. It should be noted, however, that a decrease in mean channel open time predicted for the action of 2 μM (+)-sparteine at −90 mV, from the $k^*_{+B}(-90)$ value given above, is only about 20%. Such a small change in mean channel open time can barely be detected by noise analysis (see Ref. 18).

The question arises why $[\lambda(-90) - 1]$ for 2 μM (+)-sparteine is modified by increasing agonist dose in a competitive fashion, notwithstanding the voltage sensitivity of the (+)-sparteine blocking effects [including $(\lambda - 1)$] at −90 mV. This inconsistency is probably due to a small contribution of the open-channel-blocking component to total (+)-sparteine-induced blockade, relative to that of a competitive component. This possibility is supported by the fact that at least one half of $[\lambda(-90) - 1]$ is voltage independent, as follows from the ratio of 2.3 between $[\lambda(-90) - 1]$ and $[\lambda(-50) - 1]$ (see Results).

It should be noted that the $k^*_{+B}(-50)$ value for (+)-sparteine (3.4 × 10^6 M^{-1} sec^{-1}) is much lower than those for pempidine and hexamethonium, pure open-channel blockers in ganglionic AChRs (16.7 × 10^6 M^{-1} sec^{-1} and 7.3 × 10^6 M^{-1} sec^{-1}, respectively) (4).

The voltage sensitivity of (+)-sparteine binding to the open channel is very close to that found for some other ganglionic blocking compounds, e.g., tetramethonium. Assuming that the net charge of (+)-sparteine at pH 7.2 is 1 (see Ref. 19), the fraction of the effective field sensed by (+)-sparteine binding would be about 40%, as calculated for $H = -59$ mV (see Ref. 4).

Recent findings suggest that the profile of the ganglionic AChR channel at the level where the open-channel blockers interact with the channel wall is 6.1 × 8.3 Å (5). The minimal sizes for the high- and low-populated (+)-sparteine conformers (7.3 × 7.9 Å and 6.8 × 7.5 Å, respectively) are larger than this profile. Yet, the sizes of both (+)-sparteine conformers are

intermediate between that of trimethaphan, a pure competitive ganglionic antagonist, and that of pempidine, a pure open-channel-blocker for ganglionic AChRs (8.0 × 10.8 Å and 6.0 × 6.8 Å, respectively).[1] One can, thus, speculate that the (+)-sparteine molecule, because it is smaller than trimethaphan, is able to approach the binding site in the channel at strong hyperpolarization of the membrane, in contrast to what is observed with trimethaphan.

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