Excitatory Neurotoxic Properties of Pontamine Sky Blue Make It a Useful Tool for Examining the Functions of Focal Brain Parts

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Abstract: Pontamine sky blue (PSB) is used in brain studies to mark the position of microelectrode and micropipette tips. However, few studies have been made on the effects of PSB on neurons; therefore we examined these effects. When puffed on isolated sensory ganglion cells of rats, PSB increased membrane conductance, depolarized membrane potential, and reduced the amplitude of action potentials. When dripped on frog sympathetic ganglion, much like hexamethonium, PSB decreased the amplitude of compound action potentials of the postganglionic strand. A bath application of PSB to sartorius muscle fibers that had been treated with tetrodotoxin depolarized the membrane potential and increased the frequency and amplitude of miniature end-plate potentials. All these effects were reversible. When injected into the rat's pontine part corresponding to the location of the canine pontine defecation reflex center, PSB produced repetitive colorectal contractions and irreversibly abolished them in response to analcanal stimulation. The excitatory and blocking effects of PSB and its staining ability make it a useful tool for examining the functions of focal brain parts. [The Japanese Journal of Physiology 54: 61–70, 2004]

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antiemetic action [11]. An injection of the antagonist into the medulla area corresponding to the central pattern generator for vomiting [13–15] produced no detectable effects. However, an injection of the same volume of PSB solution into the same site to estimate the diffusion area of the antagonist abolished vomiting responses to the stimulation of abdominal vagal afferents. As a result, we assumed that PSB produces neuronal dysfunction. If so, it may be useful for determining the function of focal brain parts. Therefore we conducted this study to elucidate the effects of PSB on excitable cells and to examine the usefulness of PSB in determining the function of focal brain parts.

MATERIALS AND METHODS

To define the neurotoxic properties of PSB, we examined the following effects in an ordinary illuminated room: (1) effects on transmission in the 9th sympathetic ganglion in the bullfrog, Rana catesbeiana; (2) effects on the conduction of compound action potentials along the tibial and peroneal nerves in the bullfrog; (3) effects on membrane potentials and miniature end-plate potentials of sartorius muscle fibers in the leopard frog, Rana nigromaculata; (4) effects on membrane potentials of isolated ganglion cells in rats; and (5) effects of microinjections of the dye into the pontine part of rats that corresponds to the location of the pontine defecation reflex center in dogs [16–18].

Experiments reported in this study were carried out in accordance with the “Principles of laboratory animal care” (NIH publication No. 86-23) and “The Guiding Principles for the Care and Use of Animals” approved by the Council of the Physiological Society of Japan.

Experiments in the 9th sympathetic ganglion. The 9th ganglion with the white and gray rami and the sympathetic trunk that bridged the 8th and 9th ganglia was isolated from the pithed bullfrog. It was placed on six parallel platinum wire electrodes, and the middle two were used as the ground electrodes. The ganglion was placed on a small sheet of tissue paper that bridged the gap of the two ground electrodes and was covered with another sheet of tissue. The pre- and postganglionic nerve strands were laid on the pairs of stimulating and recording electrodes, respectively. The sheets were soaked with Ringer’s solution, and each of the nerve strands was covered with one drop of mineral oil that was then stabilized by a small piece of lapping film placed on the strand. The preganglionic strand was continuously stimulated at 0.2 Hz with pulses of 0.2 ms and 10 V. The responses of the postganglionic strand were averaged 50 times in accordance with the experimental timetable. Hexamethonium bromide (1 mg ml⁻¹ in Ringer’s solution) and PSB (1–3% in Ringer’s solution) were applied to the ganglion as drops and washed out with drops of Ringer’s solution.

Experiments on the tibial and peroneal nerves. The nerve strands (20–25 mm long) were dissected from the tibial and peroneal nerves and stored in Ringer’s solution at room temperature. They were placed on the electrode assembly as described above. The proximal parts of the nerve strands were stimulated, and compound action potentials were recorded from the distal part and averaged as described above. In moststrands, the perineurium was removed from a region 5–8 mm long at the middle part, and the fiber bundle was sleeved to enhance penetration by PSB.

Experiments in the sartorius muscle. The muscle was removed from either leg of the pithed leopard frog and stored in Ringer’s solution containing tetrodotoxin (1 µg ml⁻¹) for 1–3 h at room temperature. The muscle was placed on and pinned to a paraffin plate fixed to the bottom of a 5 ml acrylic resin chamber perfused with Ringer’s solution containing tetrodotoxin. PSB (2%) was dissolved in Ringer’s solution containing tetrodotoxin and applied by perfusion. Membrane potentials were recorded with a glass micropipette filled with 3 M KCl and amplified with DC amplifiers. The output of the amplifiers was further amplified with an AC amplifier to record miniature end-plate potentials.

Experiments in isolated petrosal and geniculate ganglion cells. Sprague-Dawley rats were anesthetized with sodium pentobarbital (over 50 mg kg⁻¹, I.P.). The petrosal and nodose ganglia were removed and placed in HEPES buffer containing (in mM) 124 NaCl, 5 KCl, 5 MgCl₂, 10 sodium succinate, 15 dextrose, 15 HEPES, and 2 CaCl₂, and gassed with O₂. The pH was adjusted to 7.4 with 1 M NaOH. The ganglia were transferred to 5 ml of HEPES buffer containing 1 mg ml⁻¹ trypsin (type III) and 1 mg ml⁻¹ collagenase (type IVA). The tissue was incubated for 30 min at 37°C. The ganglia were then triturated with a series of fire-polished Pasteur pipettes that were progressively smaller in diameter to produce a suspension of dissociated neurons that was then placed on a poly-L-lysine–coated coverslip in a 35 mm diameter plastic petri dish. The cell suspension was superfused with oxygenated HEPES buffer. The enzymes were obtained from Sigma and prepared daily. Recordings were obtained from 30 min to 3 h after plating. Patch electrodes, pulled in two stages from 1.5 mm OD borosilicate filament glass, were filled with a solution containing (in mM) 130 potassium gluconate, 10
HEPES, 10 EGTA, 1 MgCl₂, 1 CaCl₂, and 2 ATP. The pipette solution was adjusted to a pH of 7.2 with KOH and had an osmolarity of 272–292 mOsm. Electrode resistance was from 5 to 8 MΩ. The petri dish containing the neurons was mounted on the stage of an inverted microscope. The electrodes were manipulated under visual control, and conventional patch-clamp recordings were performed in whole cell mode. Current-clamp recordings were made with an Axoclamp 2A amplifier (Axon Instruments). The bridge balance was carefully monitored throughout the experiments and adjusted when necessary. The criteria for a successful recording included a minimum of 10 min of recording time with a stable resting membrane potential more negative than −40 mV, an action potential amplitude larger than 50 mV, and a neuron input resistance larger than 300 MΩ. The cells were then clamped at the selected holding potential, and a series of hyperpolarizing or depolarizing command potentials, or both, was delivered. A three-barrel pipette filled with a different concentration of PSB was positioned ~40 μm from the neuronal cell body. The dye was ejected from the pipette with a Picospritzer at low pressure. We fitted the concentration-response curves by using the Hill equation, and experiments were conducted at room temperature.

Microinjection of PSB in the pons to identify the location of the pontine defecation reflex center. Adult male Sprague-Dawley rats, each weighing 350–450 g, were obtained from Japan Clea (Hyogo, Japan). They were housed in a climate-controlled, 12 h light/12 h dark room and allowed free access to water and food. The Animal Research Committee of Kawasaki Medical School approved the experimental protocols.

Anesthesia and decerebration. The rats were anesthetized with an injection of α-chloralose (60 mg kg⁻¹) into the tail vein. When the animals became flaccid about 10 min after the injection, pentobarbital sodium (16 mg kg⁻¹) was injected into the tail vein to obtain surgical anesthesia. A tracheal cannula was inserted to maintain the respiratory tract, and the head was fixed on a head-holder. The dorsolateral surface of the skull was exposed through an incision along the midline, and the temporal muscles were stripped off to expose the parietal and temporal bones. Both bones and the underlying dura mater were carefully removed to expose the cerebral hemispheres. A slender knife (1.5-mm width), specially made from a razor blade, was inserted through the hemisphere at the level of the lambdoid suture, and the midbrain was severed at the rostral part.

Recording intraluminal pressure. Intraluminal pressure was recorded from the large intestine by a balloon-pressure transducer method. An electrode assembly was used to stimulate the anal-canal mucosa. To deliver stimulating current over the mucosa, the assembly consisted of three pairs of platinum-wire electrodes (0.3 mm in diameter and 8 mm long) arranged on the outer surface of an end of a vinyl tube (5 mm in diameter). Balloons were made from the head of a condom and placed on the end of a polyethylene tube. The contractility of the proximal and distal parts of the descending colon and the rectum was recorded with three 15-mm–long balloons, arranged at 5 mm intervals, and the tubes were bundled to make a balloon array. The three tubes of the array were passed through the vinyl tube of the electrode assembly and fixed to it. The array was then inserted through the anal canal to the rectum and descending colon until the electrode assembly fit into the canal. The descending colon was ligated at the most oral part. To record intraluminal pressure, each balloon was connected to a pressure transducer and a 1 ml syringe via a T-cock. The syringe was used to fill the balloon with air, 0.2 ml in the proximal part of the descending colon and 0.1 ml in the distal part of the colon and rectum. Arterial pressure was also recorded from the femoral artery through a thin polyethylene cannula filled with heparinized Tyrode’s solution. The anal mucosa was usually stimulated with pulses of 10 V, 20 Hz, and 0.5 ms duration.

Microinjection of PSB. The rats were fixed on a stereotaxic head holder for the microinjections of PSB. A glass micropipette with a tip diameter of about 10 μm was connected to a 50 μl Hamilton syringe and filled with PSB (2%) freshly dissolved in saline and filtered through a 0.2 μm millipore filter. The syringe was attached to a microinjector (Nihon Kohden, XF-320J) on the stereotaxic head holder. The micropipette was inserted in the pons through the cerebellum, and 0.3–1.0 μl of PSB was injected at 0.07 μl min⁻¹. After the experiments, the rat was reanesthetized with pentobarbital sodium (50 mg kg⁻¹) and perfused through the aorta with 100 ml of Tyrode’s solution, followed by 100 ml of fixative (0.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer) administered with a peristaltic pump. The brain stem was removed, fixed overnight in the same fixative, and sectioned at 50 μm in the transverse plane with a vibratome. Methyl green pyronin solution (Sigma Diagnostics, Inc.) was used as a counterstain.
RESULTS

Experiments in the 9th sympathetic ganglion

Figure 1 shows a record of compound action potentials recorded from a postganglionic strand of the 9th sympathetic ganglion of the bullfrog and averaged 50 times. Fast and late peaks were constantly elicited by each pulse in the 0.2 Hz stimulation of the preganglionic strand (Fig. 1A). The amplitudes of the two peaks decreased during the 30 min period of hexamethonium (C6) application, and the responses averaged during the last 250 s of C6 application were about a third of those at the outset, as shown in Figs. 1B and 2. Therefore both the fast and late peaks appear to originate from postganglionic neurons and to correspond to the action potentials of myelinated B and unmyelinated C postganglionic fibers, respectively [19]. The amplitudes of the peaks were almost fully recovered in the last 250 s of the 30 min washout period. Both peaks reversibly decreased in amplitude during the 30 min application of PSB solution (Figs. 1D–G and 2). The decreases in amplitudes were both significant ($p<0.05$, in the paired $t$-test, $n=6$) and dose-dependent, as shown in Fig. 2.

Experiments on the tibial and peroneal nerves

A compound action potential recorded from a nerve strand dissected from the petrosal nerve of a bullfrog is shown in Fig. 3a. The conduction velocities of the 1st and 2nd peaks were about 25 m s$^{-1}$ and 3.5 m s$^{-1}$, corresponding to the action potentials of A and B fibers, respectively. In early experiments, we applied PSB to the middle part of the nerve strand. Compound action potentials were averaged during the last 250 s of the 30 min application of PSB. No obvious changes were recognized in the 1st peak, but the latency of the 2nd peak was slightly elongated. We wondered whether or not this slight effect was positive. Therefore we unsheathed the nerve-strand under a binocular microscope and found that nerve fibers in the perineurium were scarcely stained by the dye, suggesting that it cannot penetrate the blood-nerve barrier composed of the perineurium [20]. Next, we applied the dye to the unsheathed part of the nerve strand, and a typical result is shown in Fig. 3b. In the unsheathed preparations, the amplitude of the 1st peak decreased to $9.2\pm1.6$ µV, from $92.9\pm16.8$ µV (mean±SE, $n=5$), and the latent period from the onset of stimula-

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Fig. 1. Effects of hexamethonium (C6) and pontamine sky blue (PSB) on transmission in the 9th sympathetic ganglion of bullfrogs. Compound action potentials in response to single-pulse stimulation (10 V, 0.2 Hz, 0.2 ms duration) of the preganglionic strand were recorded from the postganglionic strand and averaged 50 times during the last 250 s of the 30 min period for each procedure. Amplitudes of the 1st and 2nd peaks were measured to statistically evaluate drug effects, as shown in 1 and 2 in A. The amplitudes of both peaks decreased during the drip application of C6 (B, 1 mg ml$^{-1}$) and PSB (D, 1% and E, 2%) and recovered at least partially during the 30 min washout period with the dripping of Ringer’s solution.

Fig. 2. Graphical representation of the effects of PSB and C6 on transmission in the frog sympathetic ganglion. Each column represents the mean amplitude and standard error of the 1st (open) and 2nd (hatched) peaks measured in 6 preparations. Asterisks indicate statistically significant differences ($p<0.05$) as evaluated by Student’s $t$-test (paired).
tion to the top of the 2nd peak was prolonged to 11.5±0.4 ms, from 7.2±0.7 ms, during the 30 min application period. Both changes partially recovered to 24.6±8.5 mV in amplitude and to 9.0±0.9 ms in latency during the 30 min washout period (Fig. 3c). These changes were significant (p<0.007–0.03)

Experiments in the sartorius muscle
Since nerve stimulation did not produce contractions in the nerve-sartorius muscle preparations stored in Ringer’s solution containing tetrodotoxin (1 μg ml⁻¹) for 1–3 h at room temperature, the Na⁺-channel may be blocked in these preparations. We recorded membrane potentials and miniature end-plate potentials from a muscle fiber of the preparation and observed the effects of PSB. Figure 4 shows a typical recording in the experiments. As shown, a depolarization of the membrane potential developed soon after the bath application of PSB (2%) and nearly reached an equilibrium value at about 1 min after application. The mean resting membrane potential (−87.4±3.5 mV, n=8) was depolarized to −50.6±6.3 mV at 1–2 min after the application of the dye. In three preparations, the depolarized membrane potential partially recovered after PSB was replaced by tetrodotoxin-Ringer’s solution. Miniature end-plate potentials progressively increased in frequency and amplitude after the application of PSB (Fig. 4). Similar increases were observed in the three other preparations.

Experiments in isolated petrosal and geniculate ganglion cells
Recordings were made in 35 petrosal and 16 geniculate ganglion neurons. Depolarizing and hyperpolarizing currents were injected to investigate the action potential and passive membrane properties, respectively. None of the neurons was spontaneously active. The application of PSB (0.1–3%) induced a decrease in the input resistance and depolarized all the neurons (Fig. 5A). A higher concentration of the dye induced action potentials. The response was dose-dependent and returned to control levels within 30 s after the ap-
Application was terminated. To investigate the effects of the dye on action potentials, 0.5% PSB was continuously applied to a petrosal ganglion neuron. An example is shown in Fig. 6. This neuron responded to the injection of a depolarizing current of 100 pA with a sharp spike, and the duration measured at the half-amplitude of neurons (half-duration: HD) was 6 ms before dye application (Fig. 6A). However, multiple spikes were induced by the same current injection, and HD was 8 ms at 30 s after dye application (Fig. 6B). HD was increased when the application was continued further and was 19 ms at 11 min (Fig. 6C). As previously described in regard to other ganglion neurons [21, 22], the action potentials had a deflection, a "hump," in the repolarization phase of the spike (Fig. 6C). Furthermore, to determine the neurotoxic effect of the dye, PSB was applied in 50 sequential puffs at 10 s intervals (Fig. 7). A high concentration of the dye elicited action potentials without depolarizing current injection. The membrane potential gradually depolarized, and the amplitude of the action potentials decreased. This neuron was finally unable to produce action potentials after the 35th application. Similar neurotoxic effects of 3% PSB were confirmed in 2 other neurons. After sequential puffs were discontinued, the membrane potential partially recovered.

A result of the experiments using PSB as a tool to determine the function of a brain part

To define the area of the pontine defecation reflex center in rats, we injected PSB into the pontine part corresponding to the location of the reflex center in dogs [16–18]. A typical result is shown in Fig. 8. As a control, colorectal propulsive contractions were first produced by stimulation of the anal canal (A). Next, a micropipette filled with PSB (2%) was inserted into the right pons through the cerebellum at a point 1 mm caudal to the lambda point and 2 mm lateral to the midline until the pipette tip reached a depth of 7 mm from the dorsal surface of the cerebellum. Small colorectal propulsive contractions were produced just after the insertion. This result suggests that an insertion of the pipette itself stimulates the pontine defecation.
tion reflex center. Soon after this response, the injection of PSB was begun. The dye produced a series of repetitive propulsive contractions (B). Although this series subsided within 10 min after the onset of injection, the injection was continued (C) until 1.4262 ml of PSB had been injected at 14 min after the onset. A stimulation of the anal canal mucosa produced small propulsive contractions at 20 min after the end of the injection (D). PSB was then injected into the left pons at 30 min after the end of the right-side injection. The left-side injection produced repetitive propulsive contractions that were comparable to the colorectal responses to the right-side injection (E). The injection was stopped at 8 min after the onset, just after the propulsive contractions subsided. A stimulation of the anal canal applied 15 min after the end of the left-side injection produced no colorectal contractions (G). The stained area on the right side was also outlined with a blue line. The outlines of the left side were flipped horizontally and superimposed on the photograph of the right side (B). Bar, Bar- rington's nucleus; LC, locus coeruleus; SubCA, subcoeruleus nucleus α.

Fig. 9. Pontine areas were stained with PSB. The results obtained in this rat are shown in Fig. 8. Photographs show the stained area on the left (A) and right (B) sides at the pontine level corresponding to the rostral end of the motor trigeminal nucleus (Mo5). The outlines of the stained area, the Mo5 and the mesencephalic trigeminal nucleus (Me5) on the left side, were flipped horizontally and superimposed on the photograph of the right side (B). Bar, Barrington's nucleus; LC, locus coeruleus; SubCA, subcoeruleus nucleus α.

DISCUSSION

The PSB depolarized ganglion cells and produced ac-
tion potentials, increased the frequency of miniature end-plate potentials, and blocked transmission in the sympathetic ganglion and the conduction of action potentials along myelinated axons. These results suggest that this dye produces a sustained depolarization of excitable membranes and continuously facilitates transmitter release; secondarily, it causes the inactivation of voltage-dependent Na\(^+\) and Ca\(^{2+}\) channels and finally blocks the generation of action potentials and synaptic transmission. We found only one previous report of this stimulating effect of PSB. Durmuller et al. [24] demonstrated that when injected into the cerebral ventricle in rats, PSB (2%) causes electrical and behavioral seizures that resemble those induced by the glutamate analogue kainate. They also reported that rats that suffered from such seizures exhibited neuronal loss in the hippocampus 3 days later. Therefore it is assumed that the increase in intracellular Ca\(^{2+}\) during hyperactivity caused by PSB induces delayed neuronal death [25].

When this dye is microinjected into the brain, it first produces stimulating effects and then blocks the function of the stained brain part, as seen in the example shown in Fig. 8. In this case, the microinjection of the dye into the dorsal part of the pontine tegmentum produced serial propulsive contractions of the descending colon and rectum, then blocked propulsive contractions in the reflex response to anal stimulation after bilateral microinjections. In a previous study, we showed that the microinjection of less than 5 µl of aqueous solution produced no mechanical effect [11]. Therefore our present results with the microinjection of less than 1 µl of PSB are not thought to be produced by mechanical effects. Thus these results indicate that the essential area for these stimulating and blocking effects exists in the dorsal part of the subcoeruleus nucleus [23]. This pontine area in rats corresponds to the location of the pontine defecation reflex center in dogs, as demonstrated by Okada and his colleagues based on the results of serial pontine transections and electrical lesion experiments [16, 17] and on the recordings of neuronal activity [18]. These results suggest that the pontine defecation reflex center exists in a similar part of the pons in dogs and rats. Therefore this dye seems to be a useful tool for clarifying the functions of the small parts of the brain.

The functions of focal brain parts have been investigated by various methods, e.g., electrical and chemical stimulation, reversible blocking of neuronal function by focal cooling and the microinjection of local anesthetics, and irreversible electrical and chemical destruction of neuronal tissue. The microinjection of various agonists or antagonists can produce effects only through corresponding receptors, though these electrical methods and PSB commonly produce effects on neurosoma and axon. All these previous methods are thought to be deficient because of the difficulty of delimiting the influenced area. This difficulty is resolved by PSB, which stains the influenced neurons. Moreover, PSB produces an excitatory effect and subsequently blocks neuronal functions, similar to excitatory neurotoxins, e.g., kainic acid and ibotenic acid. This excitatory neurotoxic characteristic of PSB is convenient for investigators and can help them to observe the function and dysfunction of a focal brain part after a single microinjection of PSB. Because it has been reported that PSB does not permeate through vascular continuous endothelium, it is thought not to penetrate the blood-brain barrier [5]. Therefore PSB injected into the brain could not be washed out. This characteristic makes its blocking effect irreversible and provides a useful tool for chronic experiments.

Possible mechanisms that may underlie these effects of PSB are briefly discussed below.

**Possibility of destruction of cell membrane.** PSB increased membrane conductance in isolated ganglion cells and depolarized the membrane potential of ganglion cells and frog muscle fibers treated with tetrodotoxin. This depolarization was rapidly elicited after an application of the dye. However, when dye application was continued, action potentials gradually decreased and the membrane potential finally reached a steady level of about −40 mV in the ganglion cells and −50 mV in frog muscle fibers. These steady levels were maintained throughout the observation period of up to 30 min. The depolarizing effects were reversible, as were other effects observed in this study; i.e., blocking of synaptic transmission and conduction along myelinated axons. The properties of these effects of the dye are clearly different from those in membrane destruction induced by nonspecific detergents. Liu et al. [26] reported that a nonspecific membrane detergent, saponin (0.005%), abruptly and irreversibly depolarized isolated guinea pig ventricular myocytes, and the I–V curve obtained by a whole-cell patch-clamp method at 0.5 s after the application of saponin revealed a large nonselective current reversal near 0 mV.

**Possible effects on Ca\(^{2+}\) channels.** PSB produced a reduction of membrane resistance and depolarized resting membrane potentials in isolated ganglion cells. Both results suggest that the dye increases the conductance of Na\(^+\) and/or Ca\(^{2+}\). The dye also produced a hump in the repolarizing phase of action potentials. Since it has been reported that the hump phase is formed by the influx of extracellular Ca\(^{2+}\)
[27], the dye may enhance Ca$^{2+}$ conductance. The dye also decreased resting membrane potential in frog sartorius muscle fibers that had been treated with tetrodotoxin. Because Na$^+$ channels in the muscle fibers are blocked by tetrodotoxin, this depolarization seems to demonstrate that PSB increases Ca$^{2+}$ conductance in the muscle fibers. The dye increased the frequency of miniature end-plate potentials in the same preparation. It is well established that the frequency of miniature end-plate potentials, i.e., quantal release of acetylcholine, increases with an increase in the Ca$^{2+}$ concentration in the nerve terminal [28]. Therefore PSB should have caused an increase in the intracellular Ca$^{2+}$ concentration in the nerve terminal. The increased Ca$^{2+}$ concentration is not very likely caused by depolarization induced by an increase in Na$^+$ permeability, since Na$^+$ channels were blocked by tetrodotoxin in the nerve terminal. Consequently, the dye is thought to increase Ca$^{2+}$ permeability in the nerve terminal. Therefore we suppose that an increase of Ca$^{2+}$ channel activity participates in the excitatory effects of PSB. However, this supposition is not to exclude the possibilities that other cation channels, e.g., tetrodotoxin-insensitive Na$^+$ channels and the ligand-activated cation channels, are also involved in the effects of PSB.

**Possible effects on Na$^+$ channel.** PSB decreased the amplitude of the compound action potentials of frog nerve strands. This effect is presumed to be caused by depolarization, since this dye may increase Ca$^{2+}$ conductance and depolarized ganglion cells. To the best of our knowledge, there have been no reports on the existence of Ca$^{2+}$ channels on the membrane of Ranvier’s node in myelinated fibers, but Ca$^{2+}$ channels have been reported to exist in vagal unmyelinated fibers in rats [29] and in squid giant nerve fibers [30]. Therefore Ca$^{2+}$ channels may not exist in the Ranvier’s node membrane, as indirectly suggested by Waxman and Ritchie [31] and Lehning et al. [32]. If this is correct, the present result suggests the possibility that PSB also increases the Na$^+$ permeability of nerve membranes.

**Possible mechanisms underlying the changes in cation permeability.** Chang et al. [33] investigated the entrapment of 4 polysulfonated compounds, i.e., suramin, Evans blue, trypan blue, and PSB, into phosphatidylcholine multilamellar liposomes and suggested that it results from their binding onto the surface of the phospholipid bilayer or intercalation into the liposomal bilayer. Therefore PSB seems to be incorporated into the cell membrane. Lundbaek et al. [34] postulated that membrane stiffness affects the properties of incorporated ion channels and demonstrated that cholesterol, which increases phospholipid bilayer stiffness, shifts the inactivation of N-type Ca$^{2+}$ channels toward positive potentials. Synthetic detergents (Triton X-100 and β-octyl glucoside), however, reduce bilayer stiffness and reversibly shift the inactivation to negative potentials. Moreover, Mauricio and Ferreira [35] demonstrated that a bile salt, deoxycholate, activates both anionic and cationic channels in cultured cell lines from the frog kidney (A6) and from human colonic adenocarcinoma (Caco2). PSB has been presumed to have an almost planar conformation [12], which is well known to be the conformation of bile salts.

These findings suggest that the incorporation of PSB into the cell membrane affects Ca$^{2+}$ and Na$^+$ channels and consequently causes an increase in membrane conductance and a decrease in membrane potential.

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