BRIEF COMMUNICATION

CURRENT-DEPENDENT BLOCK OF NERVE MEMBRANE SODIUM CHANNELS BY PARAGRACINE

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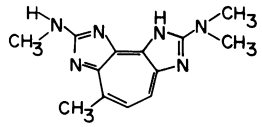
ABSTRACT Paragracine, isolated from the coelenterate species *Parazoanthus gracilis*, selectively blocks sodium channels of squid axon membranes in a frequency-dependent manner. The blocking action depends on the direction and magnitude of the sodium current rather than on the absolute value of the membrane potential. Paragracine blocks the channels only from the axoplasmic side and does so only when the current is in the outward direction. This block may be reversed by generating inward sodium currents. In axons in which sodium inactivation has been removed by pronase, the frequency-dependent block persists, and a slow time-dependent block is observed. A slow interaction with its binding site in the channel may account for the frequency-dependent block.

Progress in the research of neurotoxins and other neuro-active substances derived from marine sources has greatly elucidated the mechanisms of membrane excitation in nerves and muscles. Tetrodotoxin (TTX), saxitoxin (STX), and neurotoxins from sea anemones are the best examples of marine neurotoxins which have been extensively utilized for this purpose. Other neurotoxins such as palytoxin (1), maculotoxin (2), and the red tide toxin(s) from the dinoflagellate *Gymnodinium breve* (3) hold promise as useful probes for the functional characteristics of ionic channels. We report here our recent study on the action of the marine natural product paragracine that has an interesting chemical structure and exerts a unique action on sodium channels in squid giant axons. Paragracine is isolated from the coelenterate species *Parazoanthus gracilis* (4). This compound and other related pigments isolated from the Mediterranean species *P. axinellae* and *Epizoanthus arenaceus* are classified as zoanthoxanthins (5, 6). The basic structure of paragracine, 6-methyl-1,3,7,9-tetraazacyclopent[*e*]azulene, contains a 7-carbon troponoid ring with two guanidine groups attached to it, carrying two positive charges at pH 7 (see formula below). The pharmacological actions of



Paragracine

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paragracine on various autonomic effector organs have recently been published (7). Our results of experiments on squid axons indicate that it selectively blocks sodium channels in a current-dependent manner.

Voltage clamp experiments on giant axons of the squid *Loligo pealei* were performed at the Marine Biological Laboratory, Woods Hole, Mass. Isolated axons were internally perfused and voltage clamped by the axial wire technique (8). About 40% of series resistance was compensated for, and leakage and capacity currents were subtracted from the membrane current records using an analog circuit. Normal external solution contained 445 mM NaCl, 50 mM $CaCl_2$, 10 mM KCl, and 5 mM Hepes buffer. Low-sodium external solution contained 111 mM NaCl. Tetramethylammonium chloride was used as a replacement for the NaCl. In K-free external solution, the KCl was replaced by CsCl. The pH was adjusted to 8.0. Normal internal solution had the following composition: 320 mM K-glutamate, 50 mM NaF, 333 mM sucrose, and 30 mM K-phosphate buffer. The K-free internal solution contained 250 mM Cs-glutamate, 20 mM NaF, 400 mM sucrose, and 30 mM Na-phosphate buffer. The pH was adjusted to 7.3. The temperature of external solutions was maintained between 7° and 9° C. Paragracine was a gift from Dr. Yasuo Komoda of Tokyo Medical and Dental University.

When papagracine was applied internally to the axons at a concentration of 0.20–0.25 mM, the inward sodium current (I_{Na}) remained unchanged while the outward I_{Na} decreased progressively when pulsed repetitively at a certain frequency. Fig. 1 illustrates the results of an experiment with internal application of 0.23 mM paragracine in K-free media. Repetitive depolarizing pulses to either 0 or +100 mV were applied after a period of rest at a frequency of 1 Hz. Inward I_{Na} elicited by the 0 mV pulses remained constant during the pulsing as shown by the selected records at 1, 5, 10, and 30 s. In contrast, pulsing to +100 mV caused a progressive reduction in the amplitude of outward I_{Na} with each succeeding pulse. The amplitude of the outward I_{Na} reached a steady-state level at 50% of the control after 30 pulses. Once the block was established, it would persist even after 30 min if the membrane potential was held at −80 mV. However, the block could be relieved rapidly by repetitive pulses to 0 mV to generate inward I_{Na} (Fig. 1). Note that there was no detectable hook in the tail current during the establishment of block. As soon as the perfusion established the drug concentration, these effects appeared. They could be completely reversed by washing.

The current-voltage (I-V) relationship for sodium current shown in Fig. 2 indicates a

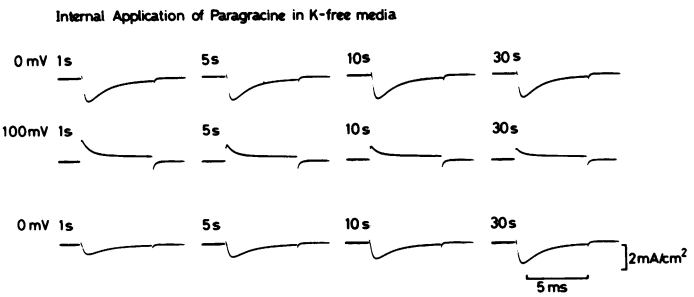

FIGURE 1 Effects of internal application of 0.23 mM paragracine on sodium currents. Repetitive depolarizing pulses to either 0 or +100 mV were applied at a frequency of 1 Hz. Selected records of sodium currents at 1, 5, 10, and 30 s from the beginning of the stimulations are shown after the application of paragracine.

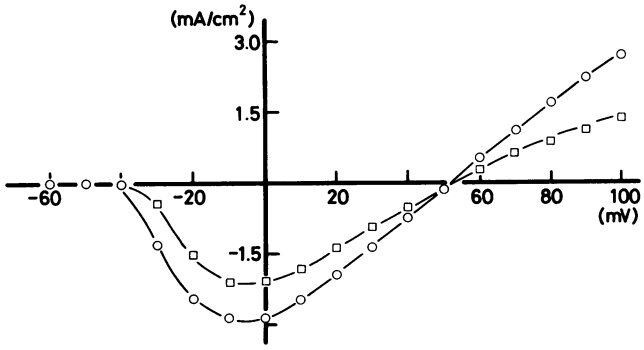

FIGURE 2. Current-voltage relationship of peak sodium currents before and during internal application of 0.23 mM paragracine. Measurements were taken at the 30th pulse given at 1 Hz.

curvature in the linear portion of the I-V curve after the block established by the repetitive pulsing. Between −60 and +50 mV, where I_{Na} is inward, the I-V curve is slightly reduced in amplitude. Beyond the reversal potential, there is a downward deviation of the I-V curve, reflecting a progressive decrease in the conductance with larger depolarization. The degree of block by paragracine correlated well with the outward current from +60 through +100 mV, they being linearly related with a correlation coefficient of 0.95. In contrast to the results with internal application, paragracine did not affect the sodium currents in either direction when applied externally.

Potassium currents were not affected by paragracine applied either internally or externally. In Fig. 3 are shown superimposed records of membrane currents during internal application of 0.23 mM paragracine. Current tracing *a* was obtained on the 30th pulse of repetitive depolarizations to +100 mV at a frequency of 1 Hz. There was a reduction in the peak outward sodium current but no change in potassium current as compared to the control (not shown in this figure). Recovery from the block in sodium currents was obtained by applying 50 repetitive pulses to 0 mV (tracings *b*). Current tracing *c* elicited by a test pulse to +100 mV shows that the potassium current did not change while the sodium current was restored to the control level.

Since the kinetics of inhibition of sodium current by paragracine may run parallel to that of the sodium inactivation, it is difficult to directly observe the time course of paragracine

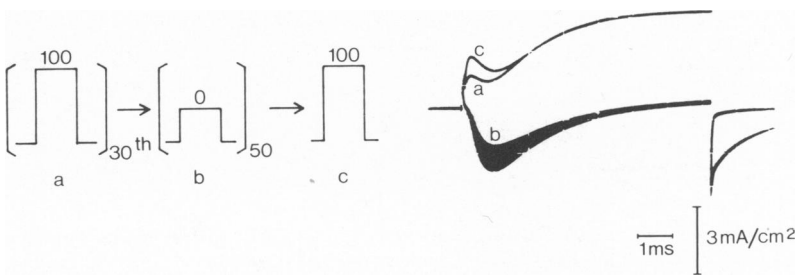

FIGURE 3 Absence of effects on potassium currents during paragracine block of sodium currents. *a*, *b*, and *c* are records of membrane currents elicited by the pulses as shown in the pulse protocol.

blocking action in axons with intact sodium inactivation mechanism. In addition, recent evidence indicates that for some blocking agents such a 9-aminoacridine (9), QX-314 (10, 11), and strychnine (12), the frequency-dependent blocking action is mediated by drug interaction with sodium inactivation. Thus, removal of the sodium inactivation by agents such as pronase, *N*-bromoacetamide, or deoxycholate eliminates the frequency-dependent component of blocking action for these agents (13). Therefore, this procedure serves not only to reveal the time course of paragracine block but also to assess the involvement of sodium inactivation in the frequency-dependent component of paragracine block.

Axons bathed in K-free media were first treated with pronase applied internally to remove the sodium inactivation (14). Control sodium inward and outward currents of an axon in which sodium inactivation has been removed are shown in Fig. 4 (tracings *a* and *b*). After the application of paragracine, repetitive pulsing to +100 mV again induced the block of outward I_{Na} (tracings *c* and *d*), which was reversed by repetitive pulsing to 0 mV to generate inward I_{Na} (tracings *e* and *f*). The persistence of frequency-dependent block in the pronase-treated axons suggests that the presence of sodium inactivation is not necessary for the blocking action to

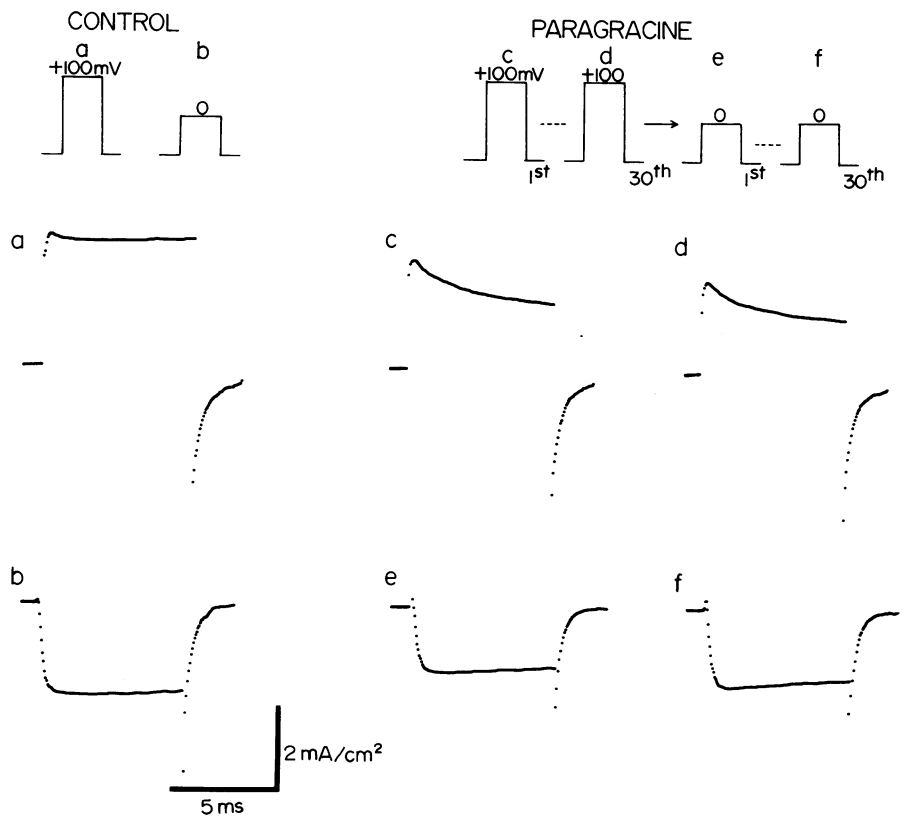

FIGURE 4 Frequency-dependent blocking action by paragracine in a pronase-treated axon. *a* and *b* are records of control sodium currents. *c-f* are those during internal application of 0.23 mM paragracine with repetitive pulsing to +100 mV (*c* and *d*) and subsequently to 0 mV (*e* and *f*) given at 1 Hz and preceded by a period of rest. Records (*c* and *e*) and (*d* and *f*) are associated respectively with the first and the last pulses of a train of 30 pulses.

occur. In superimposed records of the outward currents at +100 mV (not shown), we found that the rising phase of sodium currents during paragracine treatment was superimposable with that of control sodium currents. Only after sodium current had reached a maximum could a time-dependent block be seen (tracings *c* and *d*). This result is consistent with the notion that paragracine can enter and block the channels only when the normal voltage-dependent activation gates are open.

The blocking action at higher depolarizations and unblocking at lower depolarizations may be a function of the absolute value of the membrane potential E_m (voltage-dependent block). Alternatively, it may depend on the value of the driving force for sodium current ($E_M - E_{Na}$), which determines the amplitude and direction of sodium current (current-dependent block). To decide whether the block by paragracine is voltage- or current-dependent, we examined the effect of changing the driving force on the block at a fixed membrane potential. By shifting the reversal potential E_{Na}, a change in the direction or amplitude of current flow at a fixed potential can be obtained. If the block is voltage-dependent, the degree of block should not be affected by such a maneuver since the membrane potential is maintained at a fixed level. If the block is current-dependent, it is expected to be affected depending on the change in the direction or magnitude of the current flow.

Axons were first bathed in the normal external solution containing 445 mM Na (the internal perfusate contained 50 mM Na). Depolarizing pulses to +30 mV were applied at 1

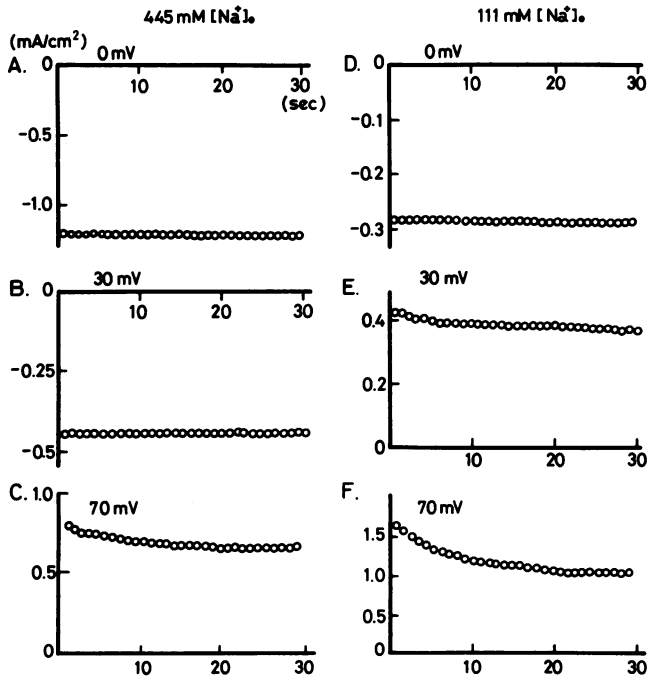

FIGURE 5 Changes in the amplitude of peak sodium currents elicited by repetitive pulses to 0, +30, and +70 mV during the internal application of 0.23 mM paragracine. Panels *A*, *B*, and *C* were obtained with axon in bathing solutions containing 445 mM $[Na]_o$ and 50 mM $[Na]_i$. Panels *D*, *E*, and *F*, 111 mM $[Na]_o$ and 50 mM $[Na]_i$.

Hz to elicit inward I_{Na}, and no block by paragracine (0.23 mM) was observed (Fig. 5*B*). When the external sodium concentration was reduced from 445 to 111 mM, the direction of I_{Na} was changed from inward to outward at this potential. Under this condition, the frequency-dependent block occurred (Fig. 5*E*). This figure also shows the effect of changing the amplitude of current flow on the blocking action. As the amplitude of outward I_{Na} at +70 mV was increased by 108% by a such a maneuver, the steady-state level of the frequency-dependent block was increased by 105% (Fig. 5*C* and *F*). Based on these results, it seems reasonable to conclude that the block by paragracine depends primarily on the direction and magnitude of the sodium current.

The present study has demonstrated that paragracine selectively blocks sodium channels in a frequency-dependent manner. It blocks the channels only from the axoplasmic side and does so only when they are open. The blocking action seems to be a function of both the direction and magnitude of the sodium current rather than the absolute value of the membrane potential. Paragracine enters and blocks the open channels when the current is in the outward direction. The blocked channels can then be cleared of the blocking molecule by the influx of Na^+ ions through the channel. Upon repolarization the blocked channels can close which prevents the drug from leaving the channel. Experiments with pronase-treated axons show that the normal sodium inactivation mechanism does not appear to mediate the frequency-dependent blocking action.

The paragracine molecules, when applied from outside, are not able to block sodium currents in either direction. This lack of action from outside implies either its inability to reach the receptor located on the axoplasmic side of the channel or the absence of similar binding site on the outer surface. Despite the presence of two guanidine groups on the structure, it does not seem to be able to bind to the receptor for TTX or STX.

Among several drugs reported to block sodium channels with frequency dependence, strychnine is the only agent that has been shown to exhibit some aspects of blocking action characteristic of current dependence (12, 15, 16). Like paragracine, the block of sodium current occurs only when the current flow is in the outward direction. However, strychnine exhibits other blocking effects which are not observed with paragracine. Strychnine induces a pronounced hook in the sodium tail current. More significantly, pronase treatment completely abolishes the frequency-dependent component of strychnine blocking action, suggesting the involvement of sodium inactivation in modulating its block. In the pronase-treated axons, strychnine exerts a faster time-dependent block than paragracine. We can conclude from these differences that the frequency-dependent blocking action may arise from either an interaction with the sodium inactivation gate or a slow interaction with the channel receptor site or both. In the case of paragracine, the frequency-dependent blocking action is primarily due to the slow interaction with its binding site in the open channels.

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