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Structural Characteristics of the Saxitoxin Receptor on Nerve

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Summary. The effects of uranyl ion (UO_2^{2+}; at low concentrations binds specifically to phosphate groups) and the cationic dye methylene blue (MB^+; binds strongly to carboxyl groups) on saxitoxin (STX) potency in crayfish axon has been studied by means of intracellular microelectrodes. At pH 6.00 ± 0.05 and 13.5 mM Ca^{2+}, addition of 10.0 μM UO_2^{2+} + 5.0 nM STX had only slightly, if any, less effect on the spike's maximum rate of rise [0.79 ± 0.04 (viz., mean ± SEM) of control value] than did addition of 5.0 nM STX alone (0.72 ± 0.05). Under the same conditions of pH and Ca^{2+} concentration, 1.0 mM MB^+ had approximately the same effect: 1.0 mM MB^+ + 5.0 nM STX, 0.76 ± 0.03; 5.0 nM STX alone, 0.70 ± 0.04. However, at pH 7.00 ± 0.05 and lower Ca^{2+} concentrations, 1.0 mM MB^+ significantly reduced STX potency. Using 6.0 mM Ca^{2+}: 1.0 mM MB^+ + 5.0 nM STX, 0.92 ± 0.01; 5.0 nM STX alone, 0.68 ± 0.08. Using 3.0 mM Ca^{2+}, the corresponding values were 0.94 ± 0.03 and 0.67 ± 0.04. It is concluded that: (1) In accord with previous suggestions, the ionized acidic group known to exist in the Na channel (and to which a guanidinium group of STX appears to bind) is very likely a carboxyl group and not a phosphate group. (2) The accessible part of the Na channel mouth serving as the saxitoxin receptor probably does not include phospholipid in its structure proper.

Over the last decade, considerable evidence has been gathered to indicate that the potent marine toxins saxitoxin (STX) and tetrodotoxin (TTX) block sodium channels in nerve by binding at the external openings of these channels (*see* Evans, 1972, and Narahashi, 1974, for reviews). At the same time, it has become increasingly clear that many similarities exist in the way the two toxins interact with their common receptor on the excitable membrane (Kao & Nishiyama, 1965; Narahashi, Haas & Therrien, 1967; Hille, 1975*b*). Most studied among these interactions is an apparent electrostatic attraction between a guanidinium group found in both toxin molecules and an ionized, metal-cation-binding site within

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the Na channel (e.g., Henderson, Ritchie & Strichartz, 1974; Ulbricht & Wagner, 1975). Henderson *et al.* (1974) have combined their findings with those of other workers to argue that this ionized site is probably identical to the one described by Hille (1971) as part of a postulated selectivity filter within the Na channel, and evidently near its external opening (Woodhull, 1973). Concerning this site, Hille stated "The pK_a [i.e., 5.2] indicates that the group is probably an oxygen acid such as a carboxylic acid or a phosphate", although the carboxyl group was favored in his model. One reason for uncertainty when attempting molecular group identification from pK_a measurements becomes clear upon examination of the wide pK_a range possible for a given type of group, and hence the overlap of pK_a's of different molecular groups, depending upon inductive (i.e., electron-withdrawing and electron-releasing) effects (e.g., Garvin & Karnovsky, 1956; Edsall & Wyman, 1958; White, Handler & Smith, 1964). The precise nature of these effects are, in turn, determined by the nature and number of other chemical groups and molecules in the immediate vicinity of the ionizable group in question. The situation can be further complicated by the presence of surface charges (*cf.* Davies & Rideal, 1963) near the Na channels of nerve (*see* D'Arrigo, 1973, 1974, for references and discussion). An interesting approach toward more certain identification of the ionized acidic group in sodium channels was later taken by Shrager and Profera (1973). They reported a substantial (85%) reduction in the binding of TTX to crab nerve fibers after rather selective, covalent modification (with a water-soluble carbodiimide at 100 mM) of accessible, membrane carboxyl groups. However, Keana and Stampfli (1974) have raised the possibility that the carbodiimide could have caused a nonspecific breakdown of the crab nerve (analogous to their results with frog nerve using a structurally similar carbodiimide at 10 mM) "with concomitant effects on tetrodotoxin binding having little to do with the 'intact' Na^+ channel. It is pertinent to note that the crab nerves did not conduct an action potential after treatment for 40 min with carbodiimide." (More recently, Baker and Rubinson (1975) have carried out a similar covalent modification of crab nerves and found reaction conditions under which the nerves continue to conduct impulses. Although the carbodiimide reaction sequence did cause a greatly reduced sensitivity to both TTX and STX, the authors point out that the specific reaction conditions used were insufficient to determine whether the modified membrane-bound site was a carboxyl or phosphate group. Moreover, the fact that nerve conduction persisted in the presence of the covalent label argues against an

intra-channel location for the membrane-bound site modified by their procedure.)

Another major structural question in regard to the STX-TTX receptor concerns whether it is composed only of protein (*cf.* Benzer & Raftery, 1972; Henderson & Wang, 1972) or includes both protein and lipid in its structure proper (*cf.* Villegas, Barnola & Camejo, 1970, 1975; Villegas & Barnola, 1972). Besides providing a better molecular understanding of the receptor, experimental data relating to this question would have a direct bearing on any all-protein model of the sodium channel (e.g., Smythies, Benington, Bradley, Bridgers & Morin, 1974).

In view of all the foregoing considerations, the objective of the present study was to acquire group-specific (and reversible), chemical data on the STX-TTX receptor using fully excitable axons. The experimental results indicate that: (1) In accord with previous suggestions, the ionized acidic group in the Na channel is very likely a carboxyl group and not a phosphate group. (2) The accessible part of the Na channel mouth serving as the saxitoxin receptor probably does not include phospholipid in its structure proper.

Materials and Methods

Preparation

Axons from the abdominal nerve cord of the crayfish, *Procambarus clarkii*, were used in all experiments. A portion of the cord's connective-tissue sheath was removed between the third and fourth abdominal ganglia. The dissection and mounting of the preparation in the perfusion chamber has been described in detail previously (D'Arrigo, 1973).

Electrical Recording

The electrical recording techniques are discussed by D'Arrigo (1973). Briefly, surface fibers in the desheathed section of the cord were impaled with glass microelectrodes filled with 5 M potassium acetate. These electrodes had resistances that were between 15 and 30 MΩ and tip potentials which did not exceed −5 mV. A bridge circuit was used (in conjunction with a constant current source) to apply current pulses intracellularly through the recording electrode. Current pulses were adjusted (amplitude ranged between 1×10^{-8} and 5×10^{-8} A, while duration was held constant at 1.8 msec) so that spike initiation occurred after the termination of the stimulus. The rate of rise of the action potential was obtained by differentiating the output signal of the bridge with a circuit having a time constant of 10 μsec.

Solutions

The bathing saline used during the dissection and initial recording was a modification of Van Harreveld's (1936) solution. It contained (mM) Na, 205; K, 5.4; Ca, 13.5; Mg,

2.6; Cl, 242; HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), 3.0, and the pH was 7.00 ± 0.05. Test solutions employed when performing the measurements of the spike's maximum rate of rise were prepared by adding varying amounts of $CaCl_2 \cdot 2H_2O$ to a solution which contained (mM) Na, 205; K, 5.4; Cl, 210.4; MES (2-(N-morpholino)-ethanesulfonic acid) or HEPES, 5.0; saxitoxin (purified compound obtained from Dr. E.J. Schantz, Food Research Institute, University of Wisconsin), 5.0 nM. Other test solutions contained, in addition, uranyl ion (UO_2^{2+}), 10.0 μM, or the cationic dye methylene blue ($C_{16}H_{18}N_3S^+$; hereafter abbreviated MB^+), 1.0 mM. At each calcium concentration considered (i.e., 3.0 mM, 6.0 mM, or 13.5 mM), the solution used as the standard differed from the corresponding test solutions only by not containing any STX, UO_2^{2+}, or MB^+. $UO_2Cl_2 \cdot 3H_2O$ was obtained from Ventron Corp. (Alfa Products) and $C_{16}H_{18}N_3S\ Cl \cdot 3H_2O$ from Aldrich Chemical Co. Once dissolved, neither salt contacted glass at any time until after it had passed out of the perfusion chamber; aliquots of the various test solutions were taken, before and after UO_2^{2+} or MB^+ addition, for pH measurement and then discarded. The pHs of the MES-buffered (pK_a 6.15) solutions were 6.00 ± 0.05 and the HEPES-buffered (pK_a 7.55) solutions were 7.00 ± 0.05. In the concentrations used, all of the cations tested were completely soluble at the pHs of their respective solutions (Sillén & Martell, 1971). However, it was noted in test solutions containing MB^+ that the dye slowly (i.e., over a period of days) formed a precipitate with some of the calcium present. Consequently, all solutions containing MB^+ (and also those containing UO_2^{2+}) were made immediately before the start of each experiment. The lower calcium concentration used in some of the test solutions made them slightly hypotonic with respect to the physiological saline. However, ionic strength and osmolarity remained virtually constant among the standard and test solutions to be compared. Moreover, in control experiments where the osmolarity of the bathing solution was lowered to 386 mosmols (from the normal 440 mosmols, approximately) by removal of 15% of the NaCl content of the solution, no significant change in the shape or amplitude of the action potential was noted as compared with that occurring in a standard solution of normal osmolarity containing sucrose and an identical salt concentration (*cf.* D'Arrigo, 1973). All experiments were performed at temperatures between 22.8 and 24.2 °C. The flow rate through the 4-ml perfusion chamber was kept between 6 and 8 ml/min.

Results

When crayfish axons were bathed in the standard calcium salines (containing either 3.0 mM, 6.0 mM, or 13.5 mM Ca^{2+}) at pH 6.00 or 7.00 ± 0.05, spike amplitudes were between 104 and 139 mV starting from a resting potential of −85 to −101 mV. The overshoot potentials of the spikes were between 19 and 44 mV. In control experiments involving application periods of up to 10 min, neither 10.0 μM UO_2^{2+} at pH 6.00 nor 1.0 mM MB^+ (*cf.* Burmistrov, Lyudkovskaya & Shuranova, 1969; Poppers, Mastri, Lebeaux & Covino, 1970) at pH 6.00 or 7.00 ± 0.05 had any significant effect on the maximum rate of rise or the amplitude of the spike or on the resting potential of the axons.

The maximum rate of rise of the action potential in the various standard calcium salines usually ranged between 700 and 950 V/sec. Fig. 1 summarizes the decreases in the spike's maximum rate of rise observed,

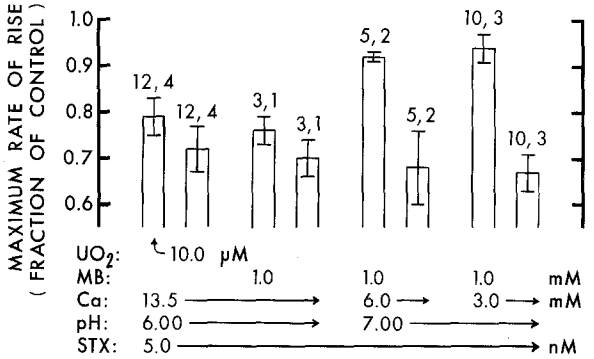

Fig. 1. The effects of uranyl ion (UO_2^{2+}) and methylene blue (MB^+) on saxitoxin (STX) potency in crayfish axons. The ordinate plots the decreases in the spike's maximum rate of rise observed when a standard saline was first replaced by a solution containing (in addition) either test cation +5.0 nM STX and thereafter replaced by a solution with 5.0 nM STX added alone. The data are grouped along the abscissa according to the test cation used and the Ca^{2+} concentrations and pHs (±0.05) at which the measurements were made. The number of separate measurements and the number of axons used are listed in succession next to each experimental value

at the different Ca^{2+} concentrations and pHs used, when a standard saline was first replaced by a solution containing (in addition) a test cation +5.0 nM STX and thereafter replaced by a solution with 5.0 nM STX added alone. Four to twelve test solutions, having the same Ca^{2+} concentration and pH, were usually used with the same fiber and the preparation was returned to the standard saline after each test run (i.e., a two-solution sequence). Maximum-rate-of-rise measurements in any given fiber were terminated if the spike amplitude, monitored during control periods, had fallen by 10%.

The experimental results given in Fig. 1 show that in crayfish axon:

(1) At pH 6.00±0.05 and 13.5 mM Ca^{2+}, addition of 10.0 μM UO_2^{2+} +5.0 nM STX had only slightly, if any, less effect on the spike's maximum rate of rise (0.79±0.04 (viz., mean±SEM) of control value) than did addition of 5.0 nM STX alone (0.72± 0.05).

(2) Under the same conditions of pH and Ca^{2+} concentration, 1.0 mM MB^+ had approximately the same effect: 1.0 mM MB^+ +5.0 nM STX, 0.76±0.03; 5.0 nM STX alone, 0.70±0.04.

However, at pH 7.00±0.05 and lower Ca^{2+} concentrations, 1.0 mM MB^+ significantly reduced STX potency.

(3) Using 6.0 mM Ca^{2+}: 1.0 mM MB^+ +5.0 nM STX, 0.92±0.01; 5.0 nM STX alone, 0.68±0.08.

(4) Using 3.0 mM Ca^{2+}, the corresponding values were 0.94±0.03 and 0.67±0.04.

Discussion

In experiments concerning the action of TTX on single Ranvier nodes of *Xenopus laevis* and *Rana esculenta*, Schwarz, Ulbricht and Wagner (1973) have examined the relation between the maximum rate of rise of the axonal spike and the inward sodium current. For experimental values between 0.25 and 0.75 of the control value, the spike's maximum rate of rise was observed to be approximately proportional to inward sodium current (*see* Fig. 1 in Schwarz *et al.*, 1973). In the present experiments the mean maximum-rate-of-rise values for the 5.0 nM STX solutions, not containing a test cation, all fell between 0.67 and 0.72 (*see Results*); any significant effect of a given test cation on STX binding to Na channels (and hence on inward sodium current) can therefore be expected to produce a significant change in the measured maximum rate of rise.

From the observed measurements of the spike's maximum rate of rise (*see* (1) in *Results*), 10.0 μM UO_2^{2+} (at pH 6.00 and 13.5 mM Ca^{2+}) appears to have little, if any, effect on the binding of STX to Na channels. The effect is of borderline significance and, if real, could well be explained in terms of a change in surface potential from surface binding of UO_2^{2+} (*cf.* D'Arrigo, 1975) near (but not adjacent to) Na channels. Such an effect of UO_2^{2+} on STX binding would only be indirect (i.e., through alteration of the surface concentration of the divalent STX cation) and not involve competitive binding at the toxin receptor itself (*see* below).

As pointed out in a previous paper, there is much evidence (*see* D'Arrigo, 1975 for references) to show that UO_2^{2+}, in a bulk concentration range of 1.0 to 10.0 μM, binds extensively and specifically to phosphate groups at various surface charge densities. Evidence for such binding at pH 6.00 ± 0.05 and a much higher Ca^{2+} concentration (50 mM) than that used in this study has already been reported in the earlier paper. (At similar negative surface charge densities, the relative apparent binding affinity of UO_2^{2+} for carboxyl or sulfate groups is without exception at least one or two orders of magnitude weaker than that for phosphate groups, while UO_2^{2+} binds negligibly (if at all) to sulfhydryl groups (*see* D'Arrigo, 1975).) More appropriate for the present study, however, is supplementary evidence to indicate that UO_2^{2+} has a similar binding affinity for individual phosphate groups (Sillén & Martell, 1971). (Note, for example, that uranyl mono-H-phosphate is insoluble in water, whereas, 7.7 gm of uranyl acetate will dissolve in 100 ml of cold water (Weast, 1973). Another striking example of the extreme affinity of UO_2^{2+} for isolated phosphate groups is observed in the commercial extraction

of uranyl nitrate from various organic solvents; a highly successful extractant for uranyl nitrate, that does not require a "salting-out" agent for useful ratios, is tributyl phosphate (Cotton & Wilkinson, 1972).)

Consequently, the observed absence of a significant effect of UO_2^{2+} on STX potency in crayfish axon indicates the following: The accessible part of the Na channel (including the selectivity filter) serving as the STX receptor probably does not possess any phosphate groups and, therefore, probably does not include phospholipid in its structure. (This does not exclude the possibility that this portion of the Na channel does actually include lipids whose phosphate groups cannot be bound by UO_2^{2+} due to steric hindrance. Such a situation is rather improbable, however, in view of the likely bilayer arrangement of phospholipids in axonal membrane (*see* D'Arrigo, 1972, for references) and the thermodynamic favorability of having the polar groups of membrane phospholipids exposed to the aqueous phase.) Should it be established that the STX-TTX receptor of nerve is composed exclusively of protein, presence of surrounding membrane lipids might still be essential to maintain the native or "active" conformation of the receptor (*cf.* Benzer & Raftery, 1972, 1973).

In line with the conclusion drawn from the UO_2^{2+} data are suggestions that the ionized acidic group in the Na channel, to which a guanidinium group of STX and TTX apparently binds (Henderson *et al.*, 1974), is a carboxylic acid (Hille, 1971; Shrager & Profera, 1973). However, direct support for this hypothesis using excitable axons would obviously be more desirable than the indirect UO_2^{2+} data. The immediate questions then were (1) whether a physiologically acceptable cation which was known to bind strongly to carboxyl groups could be found, and (2) does this cation measurably and reversibly compete with STX for binding at the acidic site in the Na channel? Simply raising the concentration of UO_2^{2+} considerably beyond 10.0 μM (so that this cation might now also bind to membrane carboxyl groups) was not a satisfactory answer to the first problem. The limiting solubility of UO_2^{2+} at pH 6.0, before it begins precipitating as $UO_2(OH)_2$, is 10.0 μM (Sillén & Martell, 1971).

A much better candidate was the vital, cationic dye methylene blue (MB^+). It has been used extensively to determine the carboxyl content of oxidized cellulose (Davidson, 1948*a, b*, 1950; Davidson & Nevell, 1948) and oxidized starches (Cheung, Carroll & Weill, 1960). Published data on the relative apparent binding affinities of MB^+ and Na^+ for carboxyl groups of oxidized cellulose (Davidson, 1948*b*) indicated MB^+ binding to such sites on the crayfish axon, at the maximum practical

dye concentration of 1.0 mM, ought to be favored in the presence of a normal concentration of Na^+. H^+ (Davidson, 1948*a*; *cf.* Shrager, 1974) and Ca^{2+} were also expected to compete with MB^+ for occupancy at membrane carboxyl groups, Ca^{2+} being the more worrisome competitor at pH 6. While Ca^{2+} has a relative apparent binding affinity one to two orders of magnitude less than that of MB^+ for the carboxyl groups of oxycellulose (Davidson, 1948*b*), the normal Ca^{2+} concentration (13.5 mM) for crayfish axon was 13.5 times greater than the maximum practical dye concentration. The concern seemed appropriate. At pH 6.00 ± 0.05 and 13.5 mM Ca^{2+} (*see* (2) in *Results*), addition of 1.0 mM $MB^+ + 5.0$ nM STX had only slightly less effect on the spike's maximum rate of rise than did 5.0 nM STX alone. This difference was barely significant.

To determine whether the lack of a sizable MB^+ effect on STX potency was in fact due to interference from competing cations, two changes were made in the test solutions. The pHs were raised to 7.00 ± 0.05 and the Ca^{2+} concentrations lowered. Using 6.0 mM Ca^{2+} (*see* (3) in *Results*), 1.0 mM MB^+ now significantly reduced STX potency; the mean values obtained for the relative decreases in the spike's maximum rate of rise indicated that roughly 50–75% (*cf.* Fig. 1 of Schwarz *et al.*, 1973) fewer channels were blocked in the solution containing 1.0 mM $MB^+ + 5.0$ nM STX as compared with the solution having 5.0 nM STX alone added. At 3.0 mM Ca^{2+} (*see* (4) in *Results*), the data suggested that even fewer channels were blocked in the presence of 1.0 mM MB^+.

The MB^+ data (including competition of MB^+ with other cations), combined with the UO_2^{2+} data, indicate that an ionized carboxyl group is very likely part of the STX receptor in crayfish axon. The only ionized site on the STX-TTX receptor, for which there is evidence, is located within the postulated selectivity filter of the Na^+ channel (Hille, 1975*a*, *b*). Although MB^+ is also known to bind to hydroxyl groups and an $-OH$ group may well be part of the STX-TTX receptor (*cf.* Camougis, Takman & Tasse, 1967), at a position within the Na channel mouth and external to the selectivity filter (Hille, 1975*b*), the dye's affinity for such groups is much lower than that for carboxyl groups. In addition, MB^+ binding to hydroxyl groups is not inhibited by the presence of other cations as is the case with carboxyl groups (Davidson, 1948*a*). Moreover, the single acidic site in the Na channel is known to bind Ca^{2+} at the normal calcium concentration for nerve (Woodhull, 1973; *cf.* Baker, Hodgkin & Ridgway, 1971) so that the observed interaction of Ca^{2+} (and H^+), MB^+, and STX in crayfish axon is almost certainly

occurring at this same specific site. Since methylated cations are believed to be able to reach (but not proceed past) the acidic site from the external solution (Hille, 1975*a*, *b*), one can understand how the charged group ($=N^+(CH_3)_2$, (Davidson, 1948*a*)) of MB^+ is able to bind to this channel site. (For the same geometric reason, uranyl ion should have been able to bind (Evans, 1963) to the anionic site if it was a phosphate group.) This molecular interpretation may at first seem to result in a paradox: How can (1) Ca^{2+}, MB^+, and STX compete for occupancy at the acidic site (very likely a carboxyl group) in the selectivity filter, and yet (2) MB^+ be more effective than Ca^{2+} in interfering with STX binding to Na channels? The answer resides in the fact that MB^+ has a considerably higher binding affinity for the channel site than does Ca^{2+} (*see Results*); in other words, the dissociation of a MB^+ molecule from the channel site occurs less frequently than in the case of a Ca^{2+} ion. As a result, a MB^+ molecule is more likely to interfere with site occupancy by an STX molecule which, with its extremely high binding affinity for the mouth of the Na channel, spends the greatest amount of time at the site. (Conversely, the much lower binding affinities (and hence much shorter "average occupancy times") of Ca^{2+} and MB^+ as compared with that of STX for the channel explain why Ca^{2+} (Woodhull, 1973) and MB^+ (*see Results*) are much *less* effective than is STX in preventing Na^+ ion passage through the Na channel.)

There is still another aspect of the present data that needs to be considered; namely, the lack of a significant effect on the potency of 5.0 nM STX (without UO_2^{2+} or MB^+ present) when the external Ca^{2+} concentration was altered (*see Results*). (Also, note the similar finding of Takata, Moore, Kao & Fuhrman (1966) with lobster axon using TTX.) Several factors may have contributed to this result. As explained above, the relatively low binding affinity of Ca^{2+} for the acidic site in the Na^+ channel is expected (from kinetic considerations) to result in little effect on STX binding. The discernable effect, reported by Henderson *et al.* (1974), of Ca^{2+} on STX and TTX binding to garfish nerve may be due to a somewhat higher binding affinity of Ca^{2+} for the channel site in that preparation. (This difference in binding affinity would be for the same reasons, outlined in the introduction, that the pK_a of a given group can vary in somewhat different chemical environments.) However, a large part of the Ca^{2+} effect observed by Henderson *et al.* was due to a change in axonal surface potential. It may therefore be the case that, in crayfish axon as compared with garfish axon, (1) the distance is greater (*cf.* Henderson *et al.*, 1974) between the Na channel

mouth and the negative surface charges near the m-gate voltage sensor (D'Arrigo, 1973, 1974), and/or (2) there are fewer, if any, negative surface charges adjacent to the Na channel openings (*cf.* Bengenisich, 1975). Finally, there is also the possibility that there is in fact a small Ca^{2+} effect on STX potency in crayfish axon, but the standard errors of the measurements obscured the effect.

In summary, it is concluded that: (1) in accord with previous suggestions, the ionized acidic group in the Na channel is very likely a carboxyl group and not a phosphate group; (2) the accessible part of the Na channel mouth serving as the saxitoxin receptor probably does not include phospholipid in its structure proper.

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