J. Membrane Biol. 22, 255–263 (1975)
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Axonal Surface Charges: Evidence for Phosphate Structure

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Received 9 December 1974; revised 10 March 1975

Summary. The effect of different extracellular alkaline-earth cations (Ca^{2+}, Mg^{2+}, Sr^{2+}, Ba^{2+}) upon the threshold membrane potential for spike initiation in crayfish axon has been studied by means of intracellular microelectrodes. This was done at the following extracellular concentrations of the divalent uranyl ion (UO_2^{2+}): 1.0×10^{-6} M, 3.0×10^{-6} M, and 9.0×10^{-6} M. At each concentration employed, extensive neutralization of axonal surface charges by UO_2^{2+} was evidenced by the fact that equal concentrations (50 mM) of alkaline-earth cations did not have the same effect on the threshold potential. The selectivity sequences observed at the different uranyl-ion concentrations were: 1.0×10^{-6} M UO_2^{2+}, $Ca^{2+} > Mg^{2+} > Sr^{2+} > Ba^{2+}$; 3.0×10^{-6} M UO_2^{2+}, $Ca^{2+} > Mg^{2+} > Ba^{2+} \geqq Sr^{2+}$; 9.0×10^{-6} M UO_2^{2+}, $Ca^{2+} \approx Ba^{2+} > Sr^{2+} > Mg^{2+}$. These selectivity sequences are in accord with the equilibrium selectivity theory for alkaline-earth cations. At each of the concentrations used, uranyl ion did not have any detectable effect on the actual shape of the action potential itself. It is concluded that many (if not most) of the surface acidic groups in the region of the sodium gates represent phosphate groups of membrane phospholipids, but that the *m* gates themselves are probably proteinaceous in structure.

Previous work (D'Arrigo, 1973) with crayfish axon has demonstrated the existence of dense clusters of surface negative charges around the sodium gating structures. In a subsequent study (D'Arrigo, 1974), it was found that a large percentage of these surface acidic groups are protonated within a rather narrow range of external pH, i.e. from pH 5.35 to 4.65 ± 0.05 of the bulk aqueous phase. Since the surface charge density on the crayfish axon at an external pH of 7.0 has already been determined as close to that of cholesterol-free phosphatidyl serine and phosphatidyl glycerol bilayers at about the same pH (D'Arrigo, 1973; *cf.* Vogel, 1974), one might hypothesize that most of the acidic sites being protonated in this pH range represent phosphate groups of membrane phospholipids. The present

study using crayfish axons was undertaken to test this hypothesis. An abstract giving some of the results has appeared (D'Arrigo, 1975).

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, 1974). 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 −4 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 4×10^{-8} and 9×10^{-8} A, while duration was held constant at 1.8 msec) so that spike initiation occurred after the termination of the stimulus. Threshold potential was defined as the most negative membrane potential at which a spike could repeatedly be elicited. [By way of comparison between threshold-potential and voltage-clamp measurements, it is interesting to note that Vogel (1974) has recently performed a voltage-clamp analysis of polyvalent-cation action on the nodal membrane of *Xenopus laevis*. He describes a situation that largely resembles the nearly pure screening situation found with crayfish axon, where threshold-potential measurements were employed (D'Arrigo, 1973). Furthermore, the value he calculates for the negative surface charge density in the vicinity of the sodium "activation" gates is quite consistent with the value that was obtained for crayfish axon (*see* the Discussion).]

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; Tris (hydroxymethyl)aminomethane, 3.0, and the pH was 7.00 ± 0.05. The test solutions employed when performing the measurements of threshold membrane potential were prepared by adding 50 millimoles/liter of the cloride salts of different alkaline-earth cations (Ca^{2+}, Mg^{2+}, Sr^{2+}, Ba^{2+}) to a solution which contained (mM) Na, 205; K, 5.4; Cl, 210.4; MES (2-(N-morpholino)ethanesulfonic acid), 10.0; uranyl ion (UO_2^{2+}), variable concentration. At each UO_2^{2+} concentration considered (i.e., 1×10^{-6} M, 3×10^{-6} M, or 9×10^{-6} M), the solution chosen as the standard contained 50 mM Ca^{2+}. $UO_2Cl_2 \cdot 3H_2O$ was obtained from Ventron Corp. (Alfa Products). Once dissolved, this salt did not contact 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+} addition, for pH measurement and then discarded. The pH of the MES-buffered (pK_a 6.15) solutions were 6.00 ± 0.05. In the concentrations used, all of the divalent cations tested were completely soluble at pH 6.0 (Parsons, 1959). [In particular, uranyl ion only precipitates as $UO_2(OH)_2$ when present at concentrations in excess of 10^{-5} M at pH 6 (Sillén & Martell, 1971).] The 50 mM alkaline-earth cation concentration of the various test solutions made them moderately hypertonic with respect to the physiological

saline, i.e. approximately 530 milliosmols versus 440 milliosmols, respectively. However, ionic strength and osmolarity remained virtually constant among the test solutions themselves. Moreover, it has already been determined in control experiments (D'Arrigo, 1973) that when the osmolarity of the bathing solution is raised to 630 milliosmols by the addition of sucrose or lowered to 386 milliosmols by removal of 15% of the NaCl content of the solution, no measurable change in threshold membrane potential occurs. The elevated alkaline-earth cation concentration of the test solutions was used to help protect the axon from the elevated hydrogen-ion concentration (*see* Hafemann, 1969) and the possibility of irreversible effects from the uranyl-ion concentrations employed (*cf.* Hagiwara & Takahashi, 1967). All experiments were performed at temperatures between 20.3 and 22.1° C. The flow rate through the 4-ml perfusion chamber was kept between 5 and 7 ml/min.

Results

When crayfish axons were bathed in the 50 mM Ca^{2+} standard solution at several concentrations of uranyl ion (1×10^{-6} M, 3×10^{-6} M, 9×10^{-6} M), spike amplitudes were between 108 and 137 mV starting from a resting potential of -80 to -99 mV. The overshoot potentials of the spikes were between 26 and 44 mV. For application periods under 6 min, most of the divalent-cation test solutions did not have any measurable effect on the amplitude of the spike or on the resting potential of the axons. Shorter application periods in some cases, under 3 min for the Ba^{2+} test solutions containing 1×10^{-6} or 3×10^{-6} M UO_2^{2+} and under 4 min for the Mg^{2+} test solution containing 3×10^{-6} UO_2^{2+}, were necessary for this condition to be met. The Ba^{2+} test solutions were often noted to prolong the falling phase of the spike so that spike duration, which was normally under 1 msec, increased to a maximum of 2.0 msec. The prolongation of the spike was largely reversed upon returning to the standard Ca^{2+} solution.

The threshold membrane potential for spike initiation under standard conditions was more positive than the resting potential of the axons by 34–68 mV at 1×10^{-6} UO_2^{2+}, by 27–66 mV at 3×10^{-6} M UO_2^{2+}, and by 35–68 mV at 9×10^{-6} UO_2^{2+}. Fig. 1 summarizes the shifts in the threshold membrane potential observed, at the different uranyl-ion concentrations used, when the 50 mM Ca^{2+} standard solution was replaced by test solutions containing an equal concentration of the other alkaline-earth cations. (The pH of all the standard and test solutions was 6.00 ± 0.05.) Four to ten test solutions, having the same UO_2^{2+} concentration, were usually used with the same fiber and the preparation was returned to the standard solution after the application of each test solution. Threshold potential measurements in any given fiber were terminated if the amplitude of the spike had fallen by 10%.

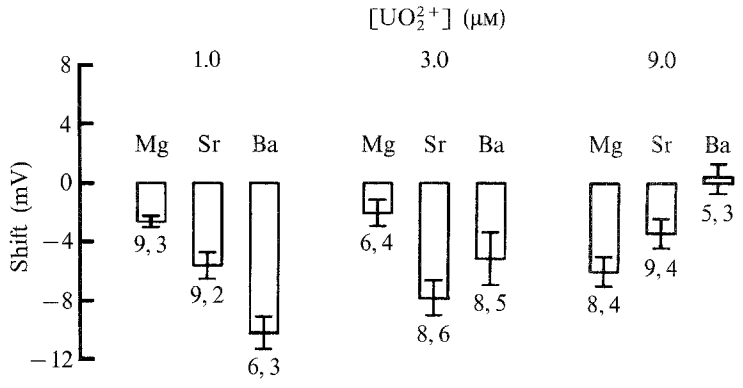

Fig. 1. The effect of extracellular alkaline-earth cations on the threshold membrane potential for spike initiation in crayfish axons. The ordinate plots the shift (in mV; mean values ±SE) of threshold potential observed, at different uranyl-ion (UO_2^{2+}) concentrations, when the 50 mM calcium standard solution was replaced by a test solution containing an equal concentration of some other alkaline earth. The data are grouped along the abscissa according to the UO_2^{2+} concentration (1.0×10^{-6} M, 3.0×10^{-6} M, or 9.0×10^{-6} M) at which the measurements were made. The pH of all the standard and test solutions was 6.00±0.05. The number of separate measurements and the number of axons used are listed in succession next to each experimental value

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

(1) With 1×10^{-6} M UO_2^{2+} present, replacement of 50 mM Ca^{2+} with an equal concentration of the other alkaline-earth cations produced the following mV shifts (mean±SE) of threshold potential: Mg^{2+}, −2.6±0.4; Sr^{2+}, −5.6±0.9; Ba^{2+}, −10.2±1.1.

(2) With 3×10^{-6} M UO_2^{2+} present, the corresponding mV shifts (mean±SE) of threshold potential were as follows: Mg^{2+}, −2.0±0.9; Sr^{2+}, −7.8±1.2; Ba^{2+}, −5.1±1.8.

(3) With 9×10^{-6} M UO_2^{2+} present, the corresponding mV shifts (mean±SE) of threshold potential were as follows: Mg^{2+}, −6.0±1.0; Sr^{2+}, −3.4±1.0; Ba^{2+}, +0.4±0.9.

(4) At each of the concentrations used, uranyl ion did not have any detectable effect on the actual *shape* of the action potential itself. (However, uranyl ion did consistently cause a concentration-dependent shift of the threshold potential in the positive direction (*cf.* Hagiwara & Takahashi, 1967; Sokoll & Thesleff, 1968). This effect was evidenced by the fact that threshold potentials measured in each 50 mM Ca^{2+} standard solution containing uranyl ion were without exception more positive than those measured from the same axon bathed in saline, at the same pH, containing 50 mM Ca^{2+} as the only divalent ions present.)

Discussion

Previous experiments with crayfish axons (D'Arrigo, 1974) conducted at an external pH of 6.00 ± 0.05, in the *absence* of uranyl ion, have shown that the four alkaline-earth cations have essentially the same effect upon the threshold membrane potential. This finding is indicative of a non-selective ("non-binding") or nearly pure screening situation. However, the densely-packed, negative surface charges giving rise to this screening situation (as concerns alkaline-earth cations) might still bind other types of divalent ions very effectively. An outstanding candidate is uranyl ion (UO_2^{2+}). UO_2^{2+} has been clearly shown (*see* Fig. 3*a* in McLaughlin, Szabo & Eisenman, 1971) to bind very effectively at extremely low concentrations (10^{-6} to 10^{-5} M in the bulk aqueous phase) to negative surface charges on phosphatidyl serine bilayers, whereas much higher concentrations (10^{-3} to 10^{-1} M in the bulk phase) of the alkaline earths bind minimally and predominantly screen these charges. In a similar manner, one might expect UO_2^{2+} to bind extensively at very low concentrations to the numerous negative charges known to exist (*see* D'Arrigo, 1974) on the crayfish axon surface at pH 6.0. Such extensive uranyl-ion binding would cause the negative surface charge density on the axon to be decreased (i.e. "neutralized") considerably, which in turn would now cause any alkaline-earth cations present to no longer screen but rather to also bind [although with a much weaker affinity (*cf.* Rothstein, 1962)] to the remaining negative surface charges (*cf.* D'Arrigo, 1974).

In the present experiments where UO_2^{2+} was added to the test solutions (having a pH of 6.00 ± 0.05), stereospecific binding of alkaline-earth cations to the negatively-charged groups on the crayfish axon exterior was indeed evidenced by the fact that equal concentrations of the alkaline earths did not have the same effect on the threshold potential of the spike. At a given concentration, the strength of binding of a given alkaline earth will be reflected by the magnitude of the threshold membrane potential (*cf.* Huxley, in Frankenhaeuser & Hodgkin, 1957; Hille, 1968; Gilbert & Ehrenstein, 1970). When UO_2^{2+} was present at a bulk concentration of 1.0×10^{-6} M, the shifts in average threshold potential (*see* (1) in the Results) produced when the standard 50 mM Ca^{2+} was replaced by an equal concentration of the other alkaline earths indicate the following order of binding or selectivity sequence: $Ca^{2+} > Mg^{2+} > Sr^{2+} > Ba^{2+}$ (Eisenman divalent sequence VI). At UO_2^{2+} concentrations of 3×10^{-6} M and 9×10^{-6} M (*see* (2) and (3) in the Results), the selectivity sequences are $Ca^{2+} > Mg^{2+} > Ba^{2+} \geqq Sr^{2+}$ (Eisenman sequence V) and $Ca^{2+} \approx Ba^{2+} > Sr^{2+} > Mg^{2+}$ (Eisenman sequence III or II), respectively.

The selectivity sequences derived at the different UO_2^{2+} concentrations are in accord with the predictions of the basic theory of alkaline-earth equilibrium selectivity, as developed by Sherry (1969), Truesdell (1962), Truesdell & Christ (1967), and by Eisenman (1965). As discussed previously (D'Arrigo, 1974), it is well established both theoretically and experimentally in artificial systems that when alkaline-earth cations genuinely bind to a negatively charged surface, the selectivity pattern shifts with decreasing charge density and/or field strength in the direction from a strong-field sequence (VII) to a weak-field sequence (I).

At this point it is useful to reconsider the extremely low bulk concentration range, i.e. 10^{-6} to 10^{-5} M, within which UO_2^{2+} so effectively neutralized membrane surface charges both in the present experiments on crayfish axon and the phospholipid-bilayer study alluded to earlier. There is extensive evidence (Bungenberg de Jong, 1949; Rothstein & Meier, 1951; Rothstein, 1962; Bangham, De Gier & Greville, 1967; Reader, Parisi & DeRobertis, 1973) to show that the uranyl ion possesses an "apparent" binding affinity this strong *only* for closely-spaced phosphate groups. At similar negative charge densities, the relative "apparent" binding affinity of uranyl ion for carboxyl or sulfate groups is without exception at least one or two orders of magnitude weaker (Bungenberg de Jong, 1949; Rothstein & Meier, 1951) than that for phosphate groups, while UO_2^{2+} binds negligibly (if at all) to sulfhydryl groups (Rothstein, 1962; Sandow & Isaacson, 1966). The overall conclusion therefore is that many, if not most, of the surface acidic groups on crayfish axon (in the region of the sodium "activation" gates) represent phosphate groups of membrane phospholipids.

Considering the above conclusion combined with (1) the aforementioned concordance between the surface charge density calculated for crayfish nerve at pH 7.0 and certain phospholipid bilayers at about the same pH, and (2) the fact that Gouy-Chapman theory—which was successfully applied to polyvalent cation action on crayfish axon at pH 7.0 (D'Arrigo, 1973)—assumes that all the surface charges lie in one plane and are uniformly distributed (*cf.* Davies & Rideal, 1963; Haydon, 1964; Adamson, 1967), I believe one can also reasonably conclude the following: The external surface of the axonal membrane in the region surrounding a sodium gate, at least in the crayfish, essentially represents a portion of a phospholipid bilayer (or possibly monolayer). [Recent work by Vogel (1974) suggests that this conclusion is in fact applicable to other axonal preparations as well. From the effects of different polyvalent cations on the sodium conductance-voltage curve of the nodal membrane of *Xenopus*

laevis, he concludes that "the observed shifts reflect a change in membrane surface potential due to electrostatic screening by the cations of the external solution. On this basis, a negative fixed charge density of approximately 1/70 Å^2 is calculated for the vicinity of the Na channel". His value of $1e^-/70$ Å^2 is somewhat smaller than the value of $1e^-/43$ Å^2 calculated for the crayfish (D'Arrigo, 1973), but this is to be expected since the secondary effects of stereospecific cation binding at the nodal membrane noted by Vogel were more extensive than those noted with the crayfish. In addition, the mean area occupied by one electronic charge in either case corresponds very closely to values obtained for the cross-sectional areas of different types of phospholipids (*see* Small, 1967; Hayashi, Muramatsu & Hara, 1972).] However, the observation (*see* (4) in the Results) that UO_2^{2+} (bulk) concentrations as high as 9×10^{-6} M had no detectable effect on the actual *shape* of the action potential argues against the notion that the sodium "activation" gates (i.e. the *m* gates) are themselves also represented by phospholipid polar groups. Although past work by numerous investigators (*see* D'Arrigo, 1972) suggested such a possibility, more recent and direct investigations of the actual sodium gating currents (Armstrong & Bezanilla, 1974; Bezanilla & Armstrong, 1974; Keynes & Rojas, 1974) have provided other indications that the *m* gates are more likely proteinaceous (Meves, 1974; *cf.* Begenisich & Lynch, 1974) in structure. These recent findings and the present results agree well with the often-mentioned structural scheme, increasingly supported by experimental evidence, of a protein-lined sodium channel (e.g. Albuquerque, Sasa, Avner & Daly, 1971; Henderson & Wang, 1972; Levinson & Ellory, 1973) within which the *m* gate is located (Strichartz, 1973; Keynes & Rojas, 1974; Smythies, Benington, Bradley, Bridgers & Morin, 1974).

The author wishes to thank Messrs. J. Fisher and B. Ovard for expert technical assistance. This work was supported by USPHS Grants HL-16348 and NS-07938 from the National Institutes of Health and Grant BMS 74-20391 from the National Science Foundation.

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