Calcium and Sodium Contributions to Regenerative Responses in the Embryonic Excitable Cell Membrane

Abstract. *Ionic dependence of regenerative responses of the embryonic cell membrane has been studied successively at each stage of development from the unfertilized egg to the differentiated striated muscle in the tadpole larva of the tunicate. The unfertilized egg cell itself showed a type of regenerative response dependent on both sodium and calcium ions, while the spike potentials exclusively dependent on calcium ions were elicited in the differentiated muscle cell.*

The increase in permeability to specific ions in excitable membranes has been established by analysis of the voltage clamp type (*1*) and also by studies of the effects of various agents, such as tetrodotoxin and tetraethylammonium and transition metal ions (*2*). The general developmental features of electrical excitability were described for the embryonic muscle cell membrane of certain tunicates (*3*). Therefore, it is of interest to know its selective permeabilities to cations such as sodium, calcium, and potassium on the basis of their differential establishment during embryogenesis.

The general experimental techniques have already been described (*3*). The embryos used were mostly those of *Halocynthia aurantium* Pallas. The external solution bathing an embryo was exchanged by continuous flow through a peristaltic pump. The ionic composition of standard artificial seawater (ASW) was also the same as before (*3*). Sodium-free ASW was obtained by isosmotically replacing NaCl with tris-(hydroxymethyl)aminomethane hydrochloride (tris). To obtain Na-containing ASW or Na-free ASW with ten times the Ca concentration in normal seawater (10 Ca ASW or 10 Ca, Na-free ASW), Ca was substituted for corresponding amounts of Na or tris. Experiments were done at a temperature below 2.5°C, except where noted.

The development of the embryo has been separated (*3*) into four successive stages, A, B, C, and D, according to the responses of the cell membrane to depolarizing current pulses: stage A, from the period just before fertilization to the initiation of invagination (128-cell stage); stage B, from the period of an early gastrula to the stage of a young tadpole larva with a tail that is 50 percent grown; stage C, to the period of hatching; stage D, to the period just before metamorphosis of a hatched tadpole. The penetrated cell was a presumptive muscle cell, identified by referring to Conklin's map (*4*), or a differentiated muscle cell. All blastomeres were electrically coupled to each other and equivalent potentials in an embryo were practically assured at stage A and the gastrula stage (*3*). A Ca-deficient solution has been reported to dissociate intercellular coupling (*5*). In an experiment with an embryo at the gastrula stage, the Ca concentration was reduced to 1 m*M* (1/10 Ca), but no sign of intercellular dissociation was found as far as electrotonic potentials were concerned.

At an early stage in A (a mature but unfertilized egg, a fertilized egg before first cleavage, or a two-cell embryo) the resting potentials were as low as 0 to −20 mv and no regenerative response was observed with a depolarizing current (*3*). The hyperpolarization at less than −20 mv was also passive electrotonic. It was difficult to obtain a steady level of the potential in the range between −20 and −60 mv under constant current application (Fig. 1A). With the hyperpolarizing current the effective resistance was extremely high (*3*), and in some cases the slope resistance in that potential range might be slightly negative.

On the contrary, in the potential range below −60 mv the slope resistance was less than 100 megohms in the current-voltage (*I-V*) relation. The hyperpolarizing responses to a current above 10^{-10} amp showed the saturation of the potential change below −60 mv, as shown in Fig. 1A. These results indicate that the steady-state *I-V* relations of the egg cell membrane have a marked nonlinear character, including the inward rectifying region. From this nonlinearity, one expects that the cessation of hyperpolarization beyond −60 mv may not show a simple exponential return of the potential, but a rapid return to −60 mv and thereafter a slower return. In the actual response illustrated in Fig 1A (standard ASW) the membrane hyperpolarization more negative than −70 mv brought a characteristic off response with an inflection at −45 mv, in addition to the nonexponential return expected from the nonlinearity. The off response

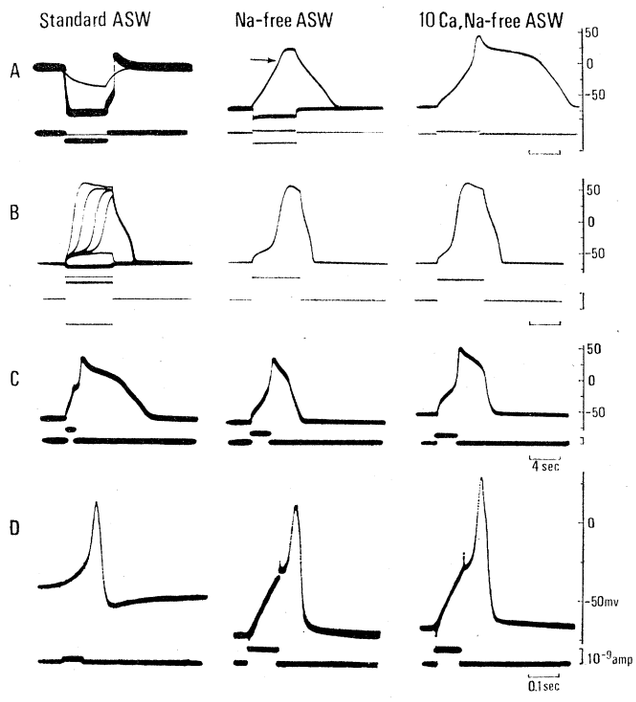

Fig. 1. Membrane responses to current pulses at four stages, A, B, C, and D. Each series of records was obtained in the same blastomere or muscle cell, while the external solution was changed successively from standard ASW to Na-free ASW and then to 10 Ca, Na-free ASW. (A) The two-cell stage; (B) the middle of the gastrula stage (4.5°C); (C) the stage of the young tadpole with a tail 50 percent grown; and (D) the stage of the hatched tadpole. All records were taken with single electrodes filled with 3*M* KCl and connected to the modified bridge circuit. The figure on the potential scale represents the membrane potential in each series (in Fig. 2 as well). Further description, including the explanation of the arrow, is in the text.

even showed an overshoot 10 to 20 mv above the original resting potential following the steep depolarization from the inflection. These characteristics suggest that the off response is produced as a result of an increase of the permeability of the membrane to a certain cation or cations.

The removal of Na ions from ASW shifted the resting membrane potential from 0 to −20 mv to about −70 mv. Hence, Na ions are considered to play an important role in determining the low resting potential in standard ASW at this stage. Since the resting potential is −70 mv in Na-free ASW, a response comparable to the off responses observed in standard ASW might be expected upon the application of an outward current pulse, that is, an inflection at −45 mv and a steep rise from it might be observed in the rising phase of depolarization. As shown by Fig. 1A, however, no sharp inflection was found at −45 mv. Instead, a slight inflection was found in the rising phase at 0 mv (Fig. 1A, horizontal arrow). When the Ca concentration was increased ten times (10 Ca, Na-free ASW) (Fig. 1A), a steep potential rise from this inflection became more apparent, the peak being more than 40 mv positive to the inflection. In the high-Ca condition, the inflection was sometimes a few millivolts more positive than in the standard-Ca condition. The potential change outlasted the current pulse by about 10 seconds and showed a plateau at about +20 mv. By adjusting the intensity of the applied current, it is possible to elicit a regenerative action potential in 10 Ca, Na-free ASW (Fig. 2A). Therefore, the slight inflection at 0 mv in the Na-free condition probably indicates the presence of potential-dependent increase of membrane permeability to Ca ions, while the disappearance of the sharp inflection at −45 mv indicates that the off response found in standard ASW may include the permeability increase to Na ions. The cause of the inflection, however, cannot be attributed solely to the Na contribution for the following reasons. First, close inspection revealed the slight increase of the potential slope at about −50 mv, even in Na-free ASW. The residual inflection was suspected to be due to incompleteness of Na removal, but it is likely to be derived from possible negative resistance in the *I-V* relation, as discussed in connection with stage B. Second, the responses in the presence and absence of Na were compared for different values of the resting potentials because of the inevitable shift caused by Na removal. Thus, it was desirable to compare the effects of Na ions at the same resting potential.

When 10 Ca, Na-free ASW was replaced by 10 Ca ASW, in spite of the addition of Na the resting potential remained at −70 mv, probably because of the stabilizing effect of Ca (Fig. 2A). In the presence of Na, two distinct inflections were seen in the rising phase of the response to outward current pulses. The first inflection was found at −40 mv, which was the same as or slightly more positive than the critical level found for the off response in standard ASW (Fig. 2A, horizontal arrow 1). The second inflection (Fig. 2A, horizontal arrow 2) was at +5 mv, which was the same as the potential seen in 10 Ca, Na-free ASW. The first inflection almost disappeared on returning the preparation to the Na-free medium (Fig. 2A). The potential change lasted much longer after the termination of the outward current pulse in the presence of Na. This result again suggests that the first inflection at about −45 mv is produced by the increase of the membrane permeability to Na ions.

With the advancement of stages within A, Na and Ca contributions to the off response in standard ASW became less clear, and a region of negative resistance developed in the steady-state *I-V* relation.

At stage B a resting potential of about −70 mv was observed in standard ASW (*3*). The penetrated cell showed only electrotonic responses to inward current pulses and to relatively small outward pulses (Fig. 1B). With an increase of the outward current above the critical value, however, a steep potential rise occurred abruptly with an inflection at about −45 mv in the rising phase of the response. The potential tended to saturate at about +50 mv without a second inflection. With larger current intensities, the potential change attained an initial peak and then smoothly declined to the steady potential level. Decay to the resting level after the pulse required about 4 seconds, including the plateau phase. The inflection at about −45 mv might be expected due to Na ions, as appeared to be the case at the early stage in A. However, that is not the case at stage B, for the inflection was never eliminated in Na-free ASW (Fig. 1B). Moreover, it did not disappear even in the Na- and Ca-free condition. Therefore, the inflection observed at stage B is not due to an increase in permeability to Na or Ca. Analysis of steady-state *I-V* relations of the cell membrane has shown that there is inward-going rectification of the K conductance at all stages in this embryo and that it is most prominent at stage B. Further, it

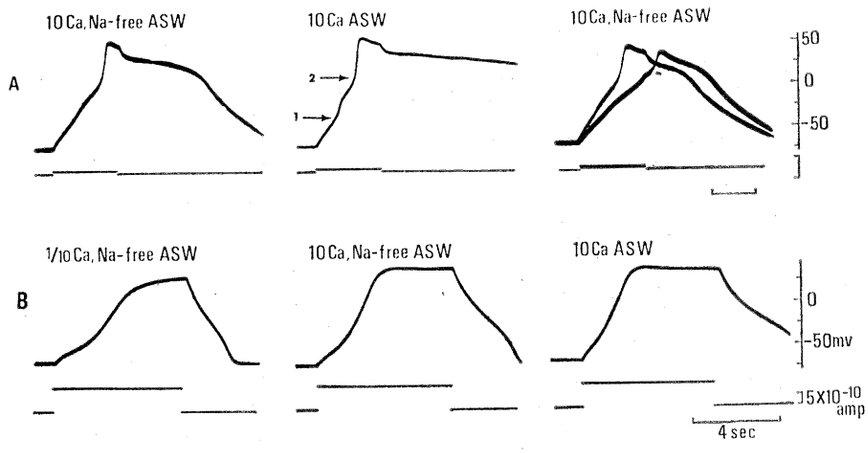

Fig. 2. (A) Records showing the two components of the regenerative response in a mature but unfertilized egg (6.5°C). The solution was changed from 10 Ca, Na-free ASW to 10 Ca ASW and then returned to the Na-free condition. Note the two inflections indicated by two arrows in 10 Ca ASW (see text). A regenerative response is shown in 10 Ca, Na-free ASW (right side). (B) Comparison of the rising phase in three depolarizing responses under three successive conditions. The records were obtained in a gastrula. Note the steeper potential rise in accordance with the high Ca concentration or the presence of Na ions. The figure on the potential scale represents the membrane potential in each series.

has been shown that the abrupt potential transition at −45 mv in stage B occurs as a result of the negative resistance region in the steady-state *I-V* curve, which is possibly caused by the abrupt decrease in K conductance, as discussed in connection with the eel electroplaque membrane (*6*). Both of the presumed Na and Ca inflections should be overwhelmed by the abrupt potential shift resulting from a decrease in K conductance even if they may exist.

The possible participation of Na was indicated by the fact that the duration of the plateau outlasting a current pulse became shorter in Na-free ASW (Fig. 1B). A somewhat clearer demonstration of the existence of Na and Ca components is illustrated in Fig. 2B. These records were obtained from the same embryo in different media with current pulses of the same intensities. While there was an inflection at −45 mv in 1/10 Ca, Na-free ASW, there was a steeper rise of the potential above −20 mv in 10 Ca, Na-free ASW. There was only a slight increase of the slope of the rising phase from −45 to −20 mv in 10 Ca ASW. It will be necessary to examine further the Na contribution at stage B.

At a later stage in B, the response to an outward current showed a steep potential rise with an inflection at approximately −10 mv and a sharp peak (not shown here). The response is a transitional one to the very long action potential at early stage C.

At stage C, the resting potential was in the range −70 to −55 mv, and an action potential with an overshoot of about +30 mv was elicited in all-or-none fashion with a critical membrane potential of approximately −15 mv in the muscle cells of young tadpole larvae (Fig. 1C). The action potential consisted of an initial steep rise followed by a plateau and then a slowly falling phase. The total duration of the action potential tended to shorten from the longest value of 10 seconds to about 100 msec with the advancement of stages within C. In Na-free ASW the resting potential usually remained unchanged or slightly increased. The action potential was still elicited, and the initial steep rise and the overshoot were not altered, but the slowly falling phase was almost eliminated. The latter indicates that, even at stage C, Na ions probably participate in the later phase of the action potential. When the external Ca concentration was raised ten times, the overshoot was increased by 20 mv.

A spike potential with an overshoot of 0 to +10 mv was obtained at a critical membrane potential of −30 mv in muscle cells at stage D (Fig. 1D). The spike duration was short, less than 50 msec at half amplitude. The resting potential was in the range −50 to −40 mv in standard ASW, and the spike was always followed by a large positive afterpotential. In Na-free ASW, although the resting potential increased to −60 to −70 mv, the afterpotential became a negative one, and the threshold and the overshoot of the spike were the same as those in standard ASW. As shown in Fig. 1D, 10 Ca, Na-free ASW increased the overshoot by 15 mv. The spike potentials were abolished by 20 m*M* Co ions in standard ASW, while tetrodotoxin (5×10^{-6} g/ml) had no effect on them. Therefore, it can be said that the fully differentiated muscle cell shows an almost pure "Ca spike."

In conclusion, the egg cell membrane even before the two-cell stage is capable of producing a regenerative potential change due to an increase in permeability to Ca and probably to Na ions. This is true even of mature but unfertilized eggs. A "Ca spike" is observed in the fully differentiated muscle, while the Na component in the action potential disappears in young tadpoles some time before hatching. It is possible that in some other tissues, such as nerve tissues, the Na component may remain and the Ca component disappear (*7*).

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7. It has been reported that plateau responses are elicited besides spike responses in the cultured skeletal muscle cells of the chick embryo (M. Kano, Y. Shimada, K. Ishikawa, *J. Cell. Physiol.*, in press).
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Behavioral Thermoregulation by Fishes: A New Experimental Approach

Abstract. *Electronic equipment allows fishes, by their spatial movements, to regulate the temperature in experimental tanks. Swimming into warmer water causes the temperature of the entire tank to increase; conversely, swimming into cooler water causes the temperature to decrease. The technique may be adapted for studying simultaneous behavioral regulation of temperature and nonthermal factors.*

Fishes, as mobile organisms living in heterothermal environments, can exercise substantial behavioral control over the temperatures they experience. They achieve this by swimming into, or remaining in, areas with certain temperatures and leaving, or avoiding, parts of the habitat with other temperatures.

Understanding how different fishes behave in heterothermal environments is crucial in predicting the ecological impact of heated effluents. Discharges from the rapidly expanding steam–electric power industry not only heat the receiving waters but do so unevenly, causing strong spatial variation in tempeature. Where fishes live in such waters largely determines what metabolic and ecological effects actually accrue.

Behavioral thermoregulation by fishes has been investigated primarily through two experimental techniques. The more