J. exp. Biol. 145, 395–402 (1989)
Printed in Great Britain © The Company of Biologists Limited 1989

SODIUM- AND CALCIUM-DEPENDENT MECHANISMS IN THE ACTION POTENTIAL OF THE SECRETORY EPITHELIUM OF A CLAM MANTLE

BY PAULO S. BEIRÃO

Departamento de Bioquimica e Imunologia, Instituto de Ciências Biológicas, UFMG, Caixa Postal 2486, 30.161 Belo Horizonte, MG, Brazil

AND JOSÉ HAMILTON M. NASCIMENTO

Departamento de Biologia Animal, Universidade de Viçosa, 36.570 Viçosa, MG, Brazil

Accepted 31 March 1989

Summary

The secretory epithelium of the mantle of the clam *Anomalocardia brasiliana* is excitable. The ionic dependence of its action potentials was investigated. Two distinct phases could be recognized by their ionic dependences. The early spike phase, that appeared in all action potentials, was dependent on the Na^+ concentration of the solution in the interstitial space and was insensitive to tetrodotoxin (TTX) at concentrations as high as $36\,\mu mol\,l^{-1}$. It was inhibited by local anesthetics, and its repolarization was inhibited by veratrine. The data show this electrogenesis is caused by TTX-insensitive sodium channels located at the basolateral membrane of this epithelium. Cardiac-like action potentials were recorded in several specimens: the rapid Na^+-dependent spike was followed by a slower repolarization phase that formed a plateau and increased the action potential duration. The plateau amplitude was markedly increased when the external Ca^{2+} concentration was increased to $60\,mmol\,l^{-1}$ and it was inhibited by the addition of inorganic calcium channel blockers such as Mn^{2+} and Cd^{2+}. These observations suggest that inward Ca^{2+} currents cause the sustained depolarization during the plateau.

Introduction

The generation of action potentials has been shown in epithelia of several animal species from different and widely separated phyla (Anderson, 1980). In spite of this, much biophysical work is still required for the elucidation of the ionic mechanisms and for comparison with better-known excitable tissues.

One of the most interesting examples of epithelial excitability occurs in the clam mantle, whose outer (shell-facing) epithelium, which is responsible for the shell secretion, is excitable. This epithelium shows two types of action potentials,

Key words: clam mantle, epithelial excitability, TTX-insensitive sodium channel, calcium channel, secretory epithelium.

depending on its physiological state. During periods in which the animal is engaged in shell secretion, electrical stimulation elicits action potentials with a spike phase followed by a prominent plateau, resembling some cardiac action potentials. During the phases of the animal's life in which it is resorbing the shell, the action potentials are spike-like, with no plateau (Beirão & Sorenson, 1986).

The present paper describes the ionic dependence of these action potentials. A Na^+-dependent TTX-insensitive electrogenesis, with otherwise similar properties to the electrogenesis mediated by sodium channels, is responsible for the generation of the spike. Evidence for the occurrence of Ca^{2+} currents during the plateau is given. A preliminary account of the data has appeared elsewhere (Nascimento & Beirão, 1983).

Materials and methods

Animals and morphology

Specimens of the clam *Anomalocardia brasiliana* were obtained from Rio de Janeiro or from São Vicente, Brazil. They were kept in an aquarium with aerated sea water containing plankton and algae as food sources. An animal was selected for use only if it actively pumped water when undisturbed and if it closed its valves quickly when handled. The largest possible area of the mantle (whole mantle, usually more than 1 cm^2) was carefully removed and immediately assembled in the recording chamber. In some experiments, a narrow (2–3 mm) strip of the mantle was used, allowing the solution in the interstitial space to be easily exchanged with the bathing solution.

The impalements were restricted to the central part of the mantle, which consists of two single-layered epithelia separated by an interstitial space containing hemolymph. The outer (secretory) epithelium is formed by columnar cells, whose basolateral membranes are convoluted and communicate directly with the interstitial space (Beedham, 1958; Neff, 1972).

Recording

The mantle was pinned down with its secretory epithelium facing upwards. Supramaximal isolated current pulses were delivered through a suction electrode placed on top of the epithelium.

To study the effect of Na^+ concentration on the spike overshoot, the mantle was stretched and assembled between the two compartments of a chamber, shutting off a 0·008 cm^2 passageway that communicated them. Isolated current pulses were passed across this small area of the mantle, stimulating it almost simultaneously (see Beirão & Sorenson, 1986).

In either chamber, resting and action potentials were recorded with conventional intracellular (8–30 MΩ) glass microelectrodes connected to a capacity-compensated electrometer (BAK, Instruments for Life Sciences, Rockville, Maryland). Recordings were displayed and photographed on a storage oscilloscope (Tektronix, model 5111, Beaverton, Oregon). Resting potentials were

Table 1. *Composition of the saline solutions*

Solution	Na^+	Choline	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	Hepes
Normal	410	–	10	10	50	490	25	5
0-Na^+(Ch)	–	410	10	10	50	490	25	5
0-Ca^{2+}	410	–	10	–	60	490	25	5
High-Ca^{2+}	410	–	10	60	–	490	25	5

recorded on a digital voltmeter (ECB, São Paulo, SP). Mantles with cardiac-like and spike-like action potentials were used but, for simplicity, only experiments with the latter were chosen to show the effects on the spike. The data shown are typical of three or more experiments.

Solutions

Table 1 shows the composition of the solutions used. The normal solution resembled the extrapallial fluid (Crenshaw, 1972). Intermediate Na^+ concentrations were obtained by the combination of normal solution with 0-Na^+ solution. All reagents were analytical grade. Tetrodotoxin, veratrine and lidocaine were from Sigma Chemical Co. All experiments were carried out at room temperature (21–26°C).

Results

Sodium-dependence of the spike

The spike phase of the action potential of the whole mantle was very insensitive to ionic substitutions in the bathing solution. In the first experiment (Fig. 1), a narrow strip of the mantle was used. Na^+ removal caused a reversible abolition of the action potential. Partial (50%) removal of Na^+ caused a decrease of the spike amplitude by 15 mV. This approach had the limitation that whenever Na^+ was decreased to 100 mmol l^{-1} or less, the Na^+ current became too small to cause the

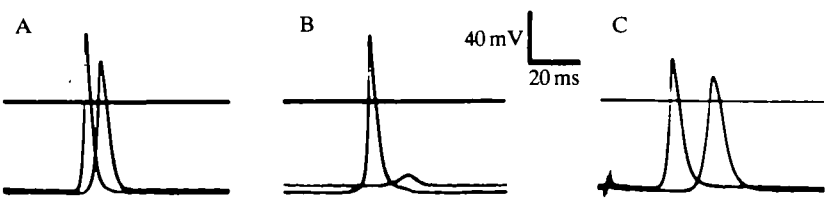

Fig. 1. Effect of sodium removal on action potentials recorded from a strip of mantle. (A) Superimposed records of a control action potential (RP = −65·5 mV) and a smaller action potential obtained after 12 min in 0-Na^+(Ch) solution (RP = −66 mV). (B) No action potential was seen after 23 min in 0-Na^+ solution (RP = −62·5 mV) and it was fully restored 9 min after normal solution was reintroduced (RP = −66·5 mV). (C) Superimposed records of a control action potential (RP = −63·5 mV) and an action potential recorded 17 min after partial substitution of choline for Na^+ that decreased Na^+ concentration to 205 mmol l^{-1} (RP = −65·5 mV).

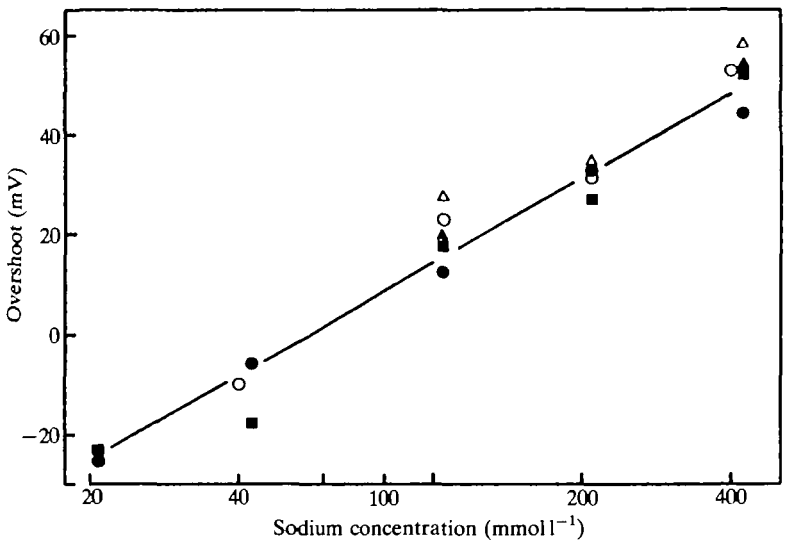

Fig. 2. Effect of the external sodium concentration on the overshoot of the action potential. Plot of the peak of the action potential as a function of the logarithm of the external Na^+ concentration. The five different symbols refer to different preparations.

spike to propagate and no action potential was seen. In order to use low Na^+ concentrations, non-propagated ('membrane') action potentials were generated in mantles assembled between two compartments. The result of partial Na^+ removal in both compartments is shown in Fig. 2, in which the peak value of the spike is plotted against the logarithm of the Na^+ concentration. The straight line obtained by linear regression has a slope of 54·5 mV for a 10-fold increase in Na^+ concentration, which closely approaches the theoretical value for a Na^+ electrode (59 mV).

Effect of sodium channel modifiers

To test if the increase in Na^+ permeability was affected by sodium channel blockers, TTX was added to the medium bathing a strip of the mantle (Fig. 3A). No effect was observed at concentrations up to 36 μmol l^{-1}, even after 67 min of exposure to TTX. The results from one experiment (Fig. 3B) showed that it was unlikely that an unusually long delay in exchanging the solution in the interstitial space could have prevented TTX reaching the basolateral membrane. The strip of mantle was preincubated in 0-Na^+ solution, resulting in the disappearance of action potentials, and then in normal solution containing 6 μmol l^{-1} TTX. After 10 min, normal action potentials were restored. They maintained the same amplitude for up to 40 min, showing that the solution in the interstitial space had been exchanged and that TTX had no effect. TTX from the same stock solution inhibited action potentials of frog skeletal muscle at a concentration of 30 nmol l^{-1}.

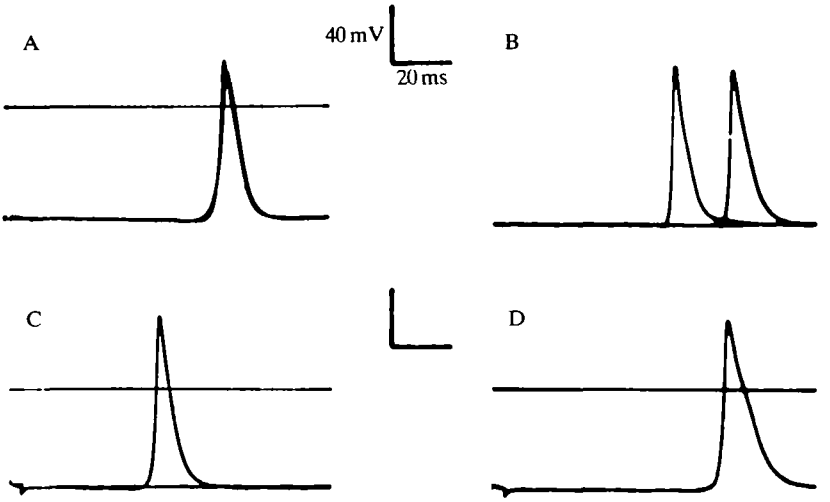

Fig. 3. Effect of sodium channel blockers. (A) Superimposed records of a control action potential obtained at about 0·1 mm from the edge of a strip of mantle (RP = −70 mV) and an action potential (with a slightly higher peak) obtained at the same position 67 min after addition of 36 μmol l^{-1} TTX (RP = −70 mV). (B) Three superimposed records showing: no action potential elicited in a strip of mantle soaked for 30 min in 0-Na^+(Ch) solution (RP = −50 mV); partial recovery of the action potential 5 min after this solution had been replaced with normal solution containing 6 μmol l^{-1} TTX (RP = −50 mV); a fully recovered action potential recorded 25 min after addition of Na^+ and TTX (RP = −50 mV). (C) Control action potential (RP = −61 mV) superimposed on a record showing no action potential 9·5 min after the addition of 5 mmol l^{-1} lidocaine (RP = −62 mV). (D) Action potential obtained 29 min after the removal of lidocaine and replacement with normal solution (RP = −63·5 mV).

Local anesthetics block sodium channels by a mechanism different from that of TTX (Hille, 1977). Fig. 3C,D shows the reversible inhibition produced by 5 mmol l^{-1} lidocaine. Procaine (1·4 mmol l^{-1}) and tetracaine (1 mmol l^{-1}) also blocked the spike electrogenesis. Veratrine, which specifically acts on sodium channels and inhibits their inactivation, caused long-lasting afterpotentials at low concentrations (data not shown).

Ionic dependence of the plateau

Our results confirm the previous report by Beirão & Sorenson (1986), that the removal of Ca^{2+} markedly decreased the plateau. Fig. 4 shows that the increase in Ca^{2+} concentration caused by substitution of Ca^{2+} for Mg^{2+} increased the height and duration of the plateau.

The Ca^{2+}-dependence of the plateau suggests that calcium channels are activated during its electrogenesis. This possibility was further investigated using inorganic calcium channel blockers. Mn^{2+} reversibly inhibited the plateau with a short delay, often less than 5 min (Fig. 5). The addition of Mn^{2+} did not transform

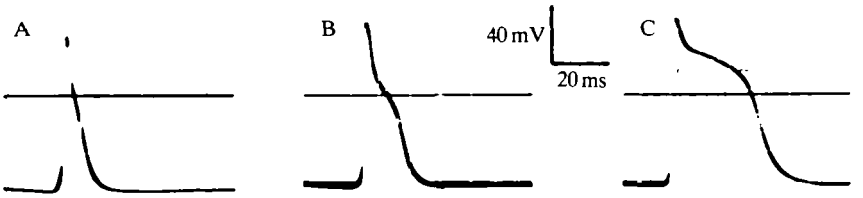

Fig. 4. Effect of the external calcium concentration. (A) Action potential recorded in a mantle soaked for 40 min in 0-Ca^{2+} solution (RP = −64·5 mV). (B) Action potential 15 min after substitution of normal solution for the 0-Ca^{2+} solution (RP = −67 mV). (C) Action potential recorded 20 min after substitution of high-Ca^{2+} solution for the normal solution (RP = −68 mV).

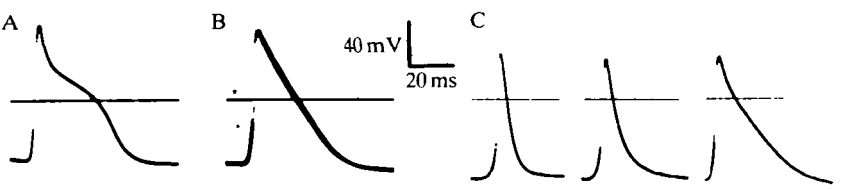

Fig. 5. Effect of inorganic calcium channel blockers. (A) Control cardiac-like action potential (RP = −49 mV). (B) Two superimposed action potentials recorded 12 and 15 min after the addition of 5·6 mmol l^{-1} Mn^{2+} (RP = −52 mV). (C) Sequence of three records showing: control spike-like action potential (RP = −65 mV), 13 min after addition of 11 mmol l^{-1} Mn^{2+} (RP = −67 mV) and 27 min after addition of Mn^{2+} (RP = −67 mV).

a cardiac-like action potential into a spike-like action potential, because it slowed the rate of repolarization. Fig. 5C shows that the addition of Mn^{2+} to a preparation with a spike-like action potential reduced its repolarization rate. The same effects were seen with Cd^{2+}.

Discussion

Beirão & Sorenson (1986) described the occurrence of spike-like and cardiac-like action potentials in the clam mantle, and proposed that the latter were made up of two distinct components: an earlier, faster one that originates the spike phase, and a later, slower one that forms its plateau. The data presented in this paper show that these components have distinct ionic dependences.

Sodium-dependence of the spike electrogenesis

In conditions where the diffusion of Na^+ into or out of the interstitial space was improved, the spike amplitude was markedly dependent on the external Na^+ concentration. During its peak, the membrane behaved as a Na^+ electrode, as shown in Fig. 2, in which the straight line was drawn according to the equation:

$$V_{peak} = V + 54{\cdot}5 \log[Na^+] ,$$

where $V = -95$ mV when $[Na^+]$ is given in mmol l^{-1}. This behavior indicates a fast and transient increase in the Na^+ conductance. Furthermore, local anesthetics blocked the generation of the spike and veratrine produced long-lasting depolarizing afterpotentials.

A remarkable observation was the absence of any effect of TTX at concentrations three orders of magnitude higher than the concentrations effective in nerve and skeletal muscle. This lack of effect cannot be attributed to a barrier for its diffusion to the site of the sodium channels, as shown by Fig. 3. Besides, there is no structure in the mantle that could provide a physical barrier specifically blocking TTX diffusion (Neff, 1972). We conclude that there are TTX-insensitive sodium channels in the basolateral membrane that generate the spike.

The existence of sodium channels with lower affinity for TTX has been reported in denervated mammalian muscle (Redfern & Thesleff, 1971), in cultured rat myoblasts and myotubes (Frelin *et al.* 1984), cultured rat heart cells (Catterall & Coppersmith, 1981) and in embryonic heart cells (Renaud *et al.* 1981). In these preparations, the concentration for half-maximal inhibition ($K_{0.5}$) for TTX is between 0·1 and 1 μmol l^{-1}. Our results show that TTX had no effect on the spike electrogenesis, even at a concentration as high as 36 μmol l^{-1}, showing an unusually high insensitivity of this preparation to TTX.

This property of the sodium channels of the clam mantle is probably related to its adaptation. It was shown that clams can accumulate saxitoxin (STX) at very high concentrations when dinoflagellates are present in the sea water. STX and TTX bind to the same receptor and have the same inhibitory mechanism (Colquhoun *et al.* 1972). Twarog *et al.* (1972) have shown that many shellfish species show STX- and TTX-resistant nerve excitability. Spalding (1980) showed that blocking a carboxyl group of the sodium channel with trimethyloxonium ion makes it insensitive to 90 μmol l^{-1} TTX without interfering with its sensitivity to local anesthetics and with its ionic selectivity.

Calcium-dependence of the plateau

Beirão & Sorenson (1986) showed that the substitution of Mg^{2+} for Ca^{2+} markedly decreased or blocked the plateau of cardiac-like action potentials. Fig. 4 shows that the increase in Ca^{2+} concentration increases the amplitude and duration of the plateau.

The addition of inorganic calcium channel blockers, such as Mn^{2+}, inhibited the plateau in a few minutes. It is noteworthy that the repolarization rate in the presence of these cations was slow, producing a triangular action potential. This also occurred when Mn^{2+} was added to a preparation with spike-like action potentials, showing that it decreases the repolarization rate.

We suggest, therefore, that the presence of calcium channels, mostly in the apical membrane, accounted for the short time it took for the effects of calcium channel blockers and Ca^{2+} substitutions to take place, even when the whole mantle was used. The reason for the absence of the plateau in some mantles is under investigation.

The authors are indebted to Dr H. Masuda for his generous supply of clams and to Dr A. L. Sorenson for helpful discussions. This work was supported in part by grants 40.2912/82 and 30.0303/78-BF from the Conselho Nacional de Desenvolvimento Cientifico e Tecnológico-CNPq and by Convênio FINEP-Bioquimica. JHMN was a fellow from CAPES.

References

ANDERSON, P. A. V. (1980). Epithelial conduction: its properties and functions. In *Progress in Neurobiology*, vol. 15 (ed. G. A. Kerkut & J. W. Phillis), pp. 161–203. Oxford, New York: Pergamon Press.

BEEDHAM, G. E. (1958). Observations on the mantle of the lamellibranchia. *Q. Jl microsc. Sci.* **99**, 181–197.

BEIRÃO, P. S. & SORENSON, A. L. (1986). Two types of action potentials in a secretory epithelium of a clam mantle. *J. exp. Biol.* **121**, 179–195.

CATTERALL, W. A. & COPPERSMITH, J. (1981). Pharmacological properties of sodium channels in cultured rat heart cells. *Molec. Pharmac.* **20**, 533–542.

COLQUHOUN, D., HENDERSON, R. & RITCHIE, J. M. (1972). The binding of labelled tetrodotoxin to non-myelinated nerve fibres. *J. Physiol., Lond.* **227**, 95–126.

CRENSHAW, M. A. (1972). The inorganic composition of molluscan extrapallial fluid. *Biol. Bull. mar. biol. Lab., Woods Hole* **143**, 506–512.

FRELIN, C., VIJVERBERG, H. P. M., ROMEY, G., VIGNE, P. & LAZDUNSKI, M. (1984). Different functional states of tetrodotoxin sensitive and tetrodotoxin resistant Na^+ channels occur during the in vitro development of rat skeletal muscle. *Pflügers Arch. ges. Physiol.* **402**, 121–128.

HILLE, B. (1977). Local anesthetics: Hydrophilic and hydrophobic pathways for the drug–receptor reaction. *J. gen. Physiol.* **69**, 497–515.

NASCIMENTO, J. H. M. & BEIRÃO, P. S. (1983). Electrogenesis of the plateau of the action potential of a secretory epithelium. *Braz. J. med. biol. Res.* **16**, 429.

NEFF, J. M. (1972). Ultrastructure of the outer epithelium of the mantle in the clam *Mercenaria mercenaria* in relation to calcification of the shell. *Tissue & Cell* **4**, 591–600.

REDFERN, P. & THESLEFF, S. (1971). Action potential generation in denervated rat skeletal muscle. The action of tetrodotoxin. *Acta physiol. scand.* **82**, 70–78.

RENAUD, J. F., ROMEY, G., LOMBET, A. & LAZDUNSKI, M. (1981). Differentiation of the fast sodium channel in embryonic heart cells: interaction of the channel with neurotoxin. *Proc. natn. Acad. Sci. U.S.A.* **78**, 5348–5352.

SPALDING, B. (1980). Properties of toxin-resistant sodium channels produced by chemical modification in frog skeletal muscle. *J. Physiol., Lond.* **305**, 485–500.

TWAROG, B. M., HIDAKA, T. & YAMAGUCHI, Y. (1972). Resistance to tetrodotoxin and saxitoxin in nerves of bivalve molluscs. A possible correlation with paralytic shellfish poisoning. *Toxicon* **10**, 273–278.