Induced Pacemaker Activity on Toad Skin

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ABSTRACT The electrical transients produced on the isolated abdominal skin obtained from *Bufo arenarum Hensel*, under the influence of inward current pulses of constant intensity have been studied. When both faces of the skin are bathed with Ringer's solution, short pulses of inward current give rise to transient variations of the potential difference between both faces of the skin with "all-or-nothing" characteristics (action potentials, AP). When the outer face is bathed with a modified Ringer solution with low sodium content (2.4 mM), the transients are longer and they are only evident when the pulse is several hundred milliseconds long. With even longer pulses (several seconds) a repetitive activity can be elicited, with the electrical characteristics of a "pacemaker" activity. In all these "excitability" phenomena Na^+ may be replaced by Li^+ in the outer solution. The logarithm of the duration of AP's is inversely related to the logarithm of the increase in concentration of Na^+ or Li^+ in the solution bathing the external face of the skin. The duration of AP's is increased when the Ca^{++} concentration in the outer solution is raised. This effect is more evident with low sodium concentration on the outside. The evolution of the slope conductance during repetitive activity has been determined. The site and mechanisms of the "excitable" behavior of the skin and the induced repetitive activity are discussed. Under the experimental conditions employed the behavior of the skin is compared with that of normally excitable plasma membranes.

INTRODUCTION

During the last decade a number of studies have been carried out to elucidate the mechanism of active sodium transport in isolated surviving frog skin. The information obtained gives insight on ion transporting properties of other epithelial membranes. Koefoed-Johnsen and Ussing (1) demonstrated that the electrical potential through the skin is dependent upon both the concentrations of sodium bathing the outer surface and of potassium bathing the inner surface. They proposed a model based on their results to explain the electrical behavior of the skin. This model assumes the existence of two major diffusion barriers: (*a*) an outward facing barrier impermeable to all cations except Na^+ and Li^+, and (*b*) an inward facing barrier permeable to K^+ and with an extremely low permeability to free Na ions. In the absence of a permeant anion, the outer barrier would behave as a Na^+ electrode and the inner barrier as a K^+ electrode. Although there is no general agreement upon the actual localization of these two barriers within the epithelial layer, their functional existence is supported by several lines of evidence. It has already been pointed out (2) that the properties of the inner barrier resemble those of resting plasma membranes of normally excitable cells, while the properties of the outer barrier resemble those of plasma membranes in the excited state. This similarity goes so far as to permit, under special conditions, the production of transient responses of the skin potential with the characteristics of an action potential (AP)(3, 4).

The present paper explores further this similarity. Its main purposes are: (*a*) To describe the behavior of toad skin and the influence of current pulses, especially on low sodium concentration on the outside, (*b*) to establish the conditions in which repetitive firing could be obtained, (*c*) to correlate the all-or-nothing responses with the ionic variation of the outer bathing solution, and (*d*) to discuss the site and mechanism of the response and compare them with plasma membrane characteristics of normally excitable tissues.

TABLE I

Ringer's*	Na^+	Cl^-	K^+	Ca^{++}	Li^+	HCO_3^-	SO_4^{--}	MOSM/liter
	mM/liter	*mM/liter*	*mM/liter*	*mM/liter*	*mM/liter*	*m/Mliter*	*mM/liter*	
Control	115	116.6	2	1	—	2.4	—	237
Sulfate	113.4	—	1.9	0.89	—	2.4	57.34	176
Na^+ Cl^- variable	2.4–115	4–116.6	2	1	—	2.4	—	12.8–237
Li^+ Cl^- variable	—	5.59–117	2.4	1	3.59–115	2.4	—	14.98–238
Ca^{++} variable	3.59	5.19–67.19	2	1–32	—	2.4	—	14.18–139
K^+ variable	3.59	5.19–21.59	2–16	1	—	2.4	—	14.18–45
$Na_2^+SO_4^{--}$ variable	2.4–113.4	—	1.9	0.89	—	2.4	1.84–57.4	9.43–176

* After equilibration with air pH = 8.1.

METHODS

All the experiments here reported were performed on abdominal skin of *Bufo arenarum Hensel* during spring, summer, and early autumn at room temperature (20–23°C). After dissection the skin was mounted between two cylindrical Lucite chambers of the Ussing-Zerahn (5) type providing 8.55 cm^2 area. Control Ringer's or modified Ringer's solutions were employed to fill the outer and the inner chambers. The composition of the solutions is shown in Table I. Unless otherwise stated control Ringer's was used on the inside. Air was bubbled through both chambers except when voltage and current were determined. The potential across the skin was measured through saturated KCl-agar bridges connected with two calomel half-cells to a double beam oscilloscope (Tektronix 520) (Tektronix, Inc., Beaverton, Ore.) with an input impedance of 10 MΩ. Current intensity was measured with the other beam of the oscilloscope as a potential difference through a 100 Ω resistance in the circuit. Two kinds of pulses were employed: (*a*) *long* current pulses (>1 sec) were delivered with a manually operated switch from a 200 v DC stabilized power supply; and (*b*) *short* current pulses (<1 sec) were supplied with a stimulator (modified Tektronix 160 Series). The current circuit was coupled to the solution in the chambers through two Ag-AgCl electrodes. A resistance larger than 100 Ω was employed to stabilize pulse current intensity in spite of variations of skin resistance and polarization resistance of the electrodes when low sodium concentration solutions $[Na]_o$, filled the outer chamber. When solutions of high sodium concentration were employed on the outside, the total resistance of the skin was so low that the value of the intercalated resistance had to be diminished; in these cases the constancy of the pulse intensity was controlled on the oscilloscope tracing. All experiments in which the intensity varied during the voltage response were discarded. Relative slope conductance was measured as a potential difference (width of the tracing) passing a small continuous 50 msec square wave pulse simultaneously with the main pulse. The small pulse was delivered with another stimulator (Tektronix 160 Series). Voltages were expressed as potential difference between the outside and the inside of the skin; that is in the opposite sense to the normal skin polarization. The expression "action potential" is used throughout this paper to characterize the all-or-nothing behavior of the

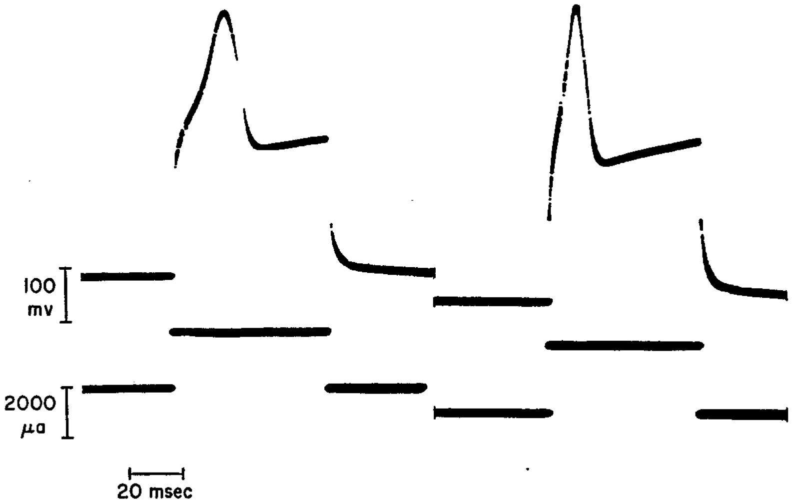

FIGURE 1. Effect of a 50 msec pulse of inward current upon the isolated abdominal skin of *Bufo arenarum*. In both records the inner and outer solutions were control Ringer's. (*a*) The pulse intensity employed carried the skin potential to its threshold and an action potential was elicited. (*b*) Same skin stimulated with a suprathreshold pulse. Note that the latent period is shorter and the ascending limb is steeper. There is no plateau and the AP*t* is shorter when the pulse intensity is increased.

skin, that is with the same meaning already given by Finkelstein (3, 4) in frog skin. Its actual signification is discussed below. Standard statistical methods were employed for the evaluation of data (6).

RESULTS

Characteristics of the Transient Response

When the skin was mounted with control Ringer's on both sides and 50 msec inward pulses of increasing intensity were passed through the current circuit, a parallel skin depolarization passing through zero to positive values on the outside was observed. Eventually a polarization was reached at which a nonlinear response was obtained. When the current pulse had enough intensity to polarize the outside of the skin to about +227 mv, a transient response developed (Fig. 1). This transient response was similar to the AP's described by Finkelstein in frog skin (3, 4). For each preparation there was a typical value of polarization at which the all-or-nothing response could be evoked (threshold). After an AP, the skin developed a refractory state during which it was not possible to obtain a new AP until some time (5–10 sec) had elapsed. The time and voltage characteristics of AP's in five different skins measured at threshold are listed in Table II.

TABLE II

	External solutions	
	2.4 mM Na$^+$	115 mM Na$^+$
	Mean value ± standard error of the mean	
Skin polarization, *mv*	67±5.83	46.4±15.4
Short circuit c., μa *cm*$^{-2}$	17.25±3.9	41.64±11.08
Skin resistance, *ohms cm*$^{-2}$	4551±881	1029±250
Threshold, *mv*	162±22.5	227±10.2
Threshold, *μa cm*$^{-2}$	60.70±17.1	190.48±26
AP duration, *msec*	178±42.5	37.4±3.33
Total height	273±12.4	240±13.8

These results indicate that the AP's obtained with the abdominal skin of *Bufo arenarum* are similar in several respects to those obtained with *Rana pipiens* and *Rana temporaria*, and that toad skin is also a suitable experimental model for this kind of experiment.

Effect of Low Sodium Concentration Solution in the Outer Chamber

Some evidence (3, 4) seems to point out that the site of the AP mechanism is close to or associated with the outer barrier and involves sodium (4). For that reason, the effect of this ion was explored in a series of experiments.

In preliminary experiments it was observed that with zero Na, the skin presented an inverse polarization and it was not possible to obtain an AP. If a solution containing 2.4 mM of sodium is employed on the outside the skin polarizes normally (i.e. with the positive on the inside). With this setup, 50 msec pulses of inward current were passed through the skin and the results were recorded. Except for the lack of production of action potentials the effect of these pulses at low Na^+ outer concentration was similar to that already described in the experiments in which control Ringer's was used.

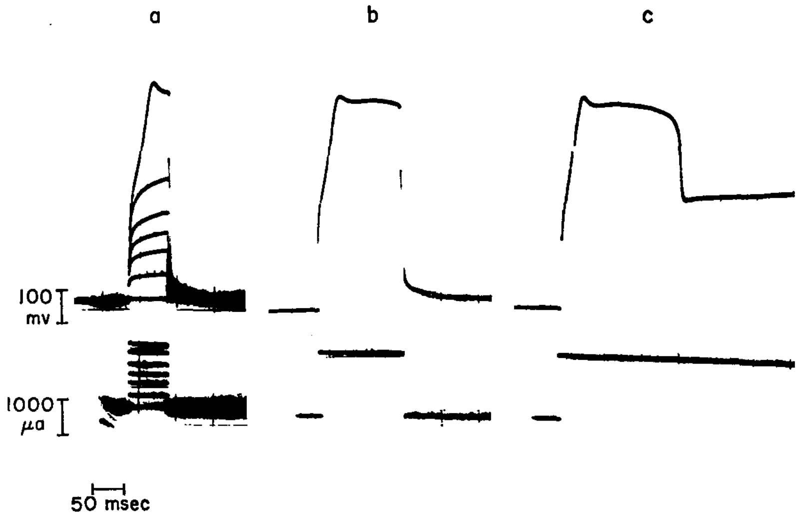

FIGURE 2. Effect of increasing intensity of an inward pulse passing through the skin. In the three experiments the inner chamber was filled with control Ringer's and the outer chamber with a solution containing 2.4 mM sodium. The skin was stimulated in (*a*) with a 50 msec pulse, in (*b*) with a 100 msec pulse, in (*c*) with a 500 msec pulse. Increasing pulse intensity produced a corresponding voltage pulse that showed nonlinearity especially with the higher intensities. In (*a*) the stronger current pulse produced a transient that was not completed when the pulse was over. In (*b*) the transient of the voltage pulse is interrupted. In (*c*) the transient is completed and shows the same characteristics as those obtained with control Ringer's in the outside chamber.

However, two major differences were observed: (*a*) the ratio $_{\Delta}V/_{\Delta}I$ was higher at low Na^+ as a consequence of the increment on the skin resistance when solutions of low sodium concentration bathe the outer surface of the skin, and (*b*) for pulses of relatively high intensities the voltage transient was not completed when the pulse was over. Therefore, the effect of 500 msec pulses was explored (Fig. 2). Fig. 3 shows a typical response in which a 500 msec pulse with 180 $\mu a\ cm^{-2}$ intensity developed a long-lasting AP with a rounded spike and a plateau. The electrical characteristics of the AP's are summarized in Table II. The AP's had a definite threshold, a rather constant shape, and were followed by a refractory period. In other words, they presented all the characteristics already seen in the experiments performed with control Ringer's on the outside. Two main differences were nevertheless observed: the action potential duration (AP*t*) was longer, and the refractory state was shorter in low Na.

In several experiments, it was observed that after an AP had been elicited by a 500 msec current pulse the potential difference through the skin showed a steady depolarization that ended when the current pulse was over. To further investigate this point longer pulses were employed. For inward *long* current pulses of relatively low intensity, the voltage developed remained constant as long as the pulse lasted, but when the pulse intensity produced a polarization of about 100–150 mv, the voltage through the skin did not remain at a steady level but developed a slow hyperpolarization and a repetitive activity ensued (Fig. 4).

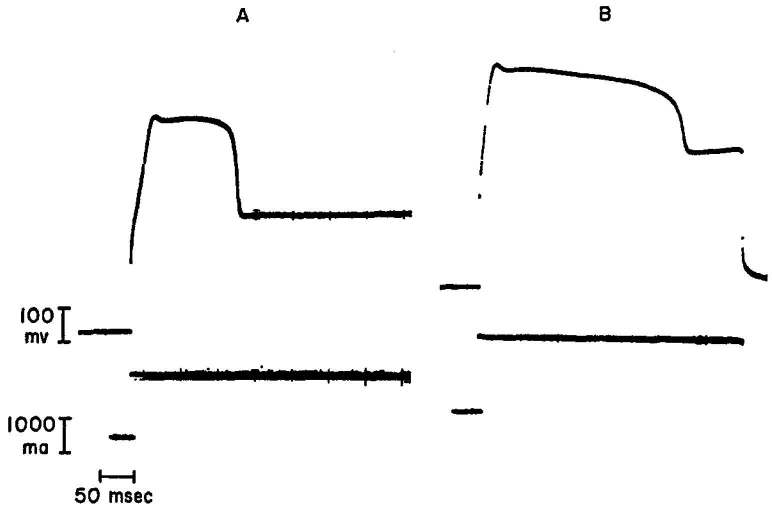

FIGURE 3. Records of two typical action potentials developed by toad skin under the influence of inward pulse when 2.4 mM Na^+ (record A) or 2.4 mM Li^+ (record B) filled the outer chamber. Note that both AP's are very much alike and that with low ionic concentrations the AP duration is often greater with Li^+ than with Na^+ solutions.

The AP of this repetitive firing had approximately the same characteristics as the single AP obtained with 500 msec pulse. From these experiments it could be seen that with low sodium concentration bathing the outside of the skin, it was possible to obtain not only AP's but also a repetitive activity that resembled pacemaker activities of normally excitable tissues.

EFFECT OF THE PULSE INTENSITY (*A*) Single AP's. When the pulse intensity was increased beyond threshold levels, the latency of the response measured from the ascending limb of the voltage pulse to the beginning of the AP diminished and the ascending phase was steeper. With still higher pulse intensities, the ascending phase of the AP continued directly the front of the voltage pulse. The duration of the AP's was also increased (Fig. 5).

(*B*) Repetitive firing. During repetitive firing the increase in pulse intensity produced a transient increase of discharge frequency. Sometimes the first AP at the new current level was longer (Fig. 4).

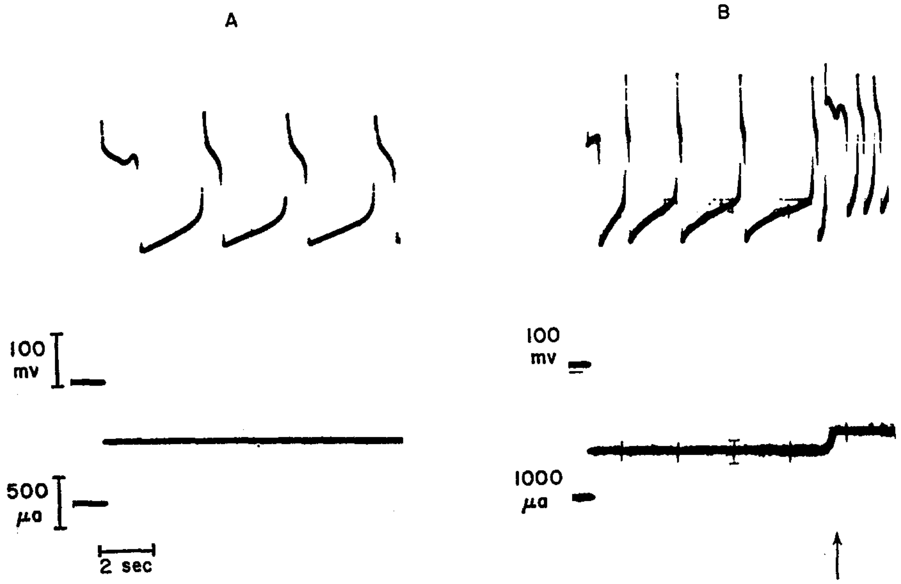

Figure 4. Effect of a long-lasting inward pulse upon toad skin. The inner chamber was filled with control Ringer's and the outer chamber with 2.4 mm sodium. (*a*) Repetitive activity developed with a long-lasting pulse. After each AP, a steadily increasing hyperpolarization triggers the mechanism for the next response. (*b*) The rate of firing increased when the current pulse was raised (arrow).

With current intensities up to 80–100 μa cm^{-2}, the repetitive firing could be held several minutes with no apparent damage to the electrical characteristics of the skin. As soon as the pulse was over a quick repolarization to normal values of open circuit conditions was obtained. With greater intensities repetitive firing was also observed but the skin was soon irreversibly damaged.

Effect of Variation of the $[Na^+]_o$

As was reported above one of the major differences between the AP's in low $[Na^+]_o$ as compared to those with control Ringer's in the outer chamber, was the duration of the response. For that reason it was planned to investigate the effect of the $[Na^+]_o$ on the AP duration (AP*t*). It was not intended to measure the duration of the refractory period with this low [Na+] solution because of its spontaneous variation and its strong dependence on pulse intensity. However, the interval between two AP's during repetitive firing clearly demonstrates that the refractory period is considerably diminished.

In these experiments the outer sodium concentration was varied from 2.4 to 115 mM. The duration of the AP was measured in single AP's developed with a 500 msec pulse as well as in AP's corresponding to repetitive firing produced by *long* pulses. All measurements were performed at threshold current intensity levels. The primary effect of the increasing sodium chloride concentration on the outside was a reduction on the skin resistance and accordingly the intensity of the pulse had to be increased to reach a definite degree of polarization.

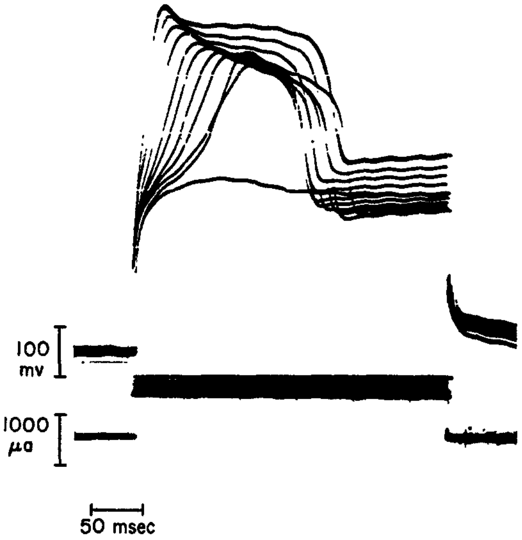

FIGURE 5. Effect of increased pulse intensity upon single skin AP. The inner chamber was filled with control Ringer's and the outer chamber with 2.4 mM sodium. The pulses were increased every 20 sec. The smaller intensity pulse produced a graded response, all others a corresponding AP. Note that with increasing pulse intensity the latent period is shorter, the ascending limb is steeper, and the duration is longer mainly as a consequence of a longer duration of the plateau phase.

(*A*) Single AP's. The effect of the increasing $[Na^+]_o$ was a decrease in the AP*t*. By plotting log AP*t* vs. log $[Na^+]_o$, a significant negative correlation was obtained ($r = -0.95, p < 0.001, b = -0.622, n = 35$) (Fig. 6).

(*B*) Repetitive firing. When the $[Na^+]_o$ was raised, the AP*t* decreased during repetitive firing. Plotting log AP*t* vs. log $[Na^+]_o$ a significant negative correlation was obtained ($r = -0.784, p < 0.001, b = -0.74, n = 30$). The repetitive firing could be obtained up to 57.5 mM $[Na^+]_o$. With greater concentrations the intensity of the pulse needed was so high that it very soon irreversibly damaged the skin.

EFFECT OF OSMOLARITY To discard the possible effect of increasing osmolarity upon AP*t* several experiments were performed in which isotonicity of the outer solution was attained with sucrose. Essentially the same effects as those already described were observed. When plotting log AP*t* vs. log $[Na^+]_o$, a negative significant correlation was obtained in single AP's as well as in AP's corresponding to repetitive activity (single activity: $r = -0.92$,

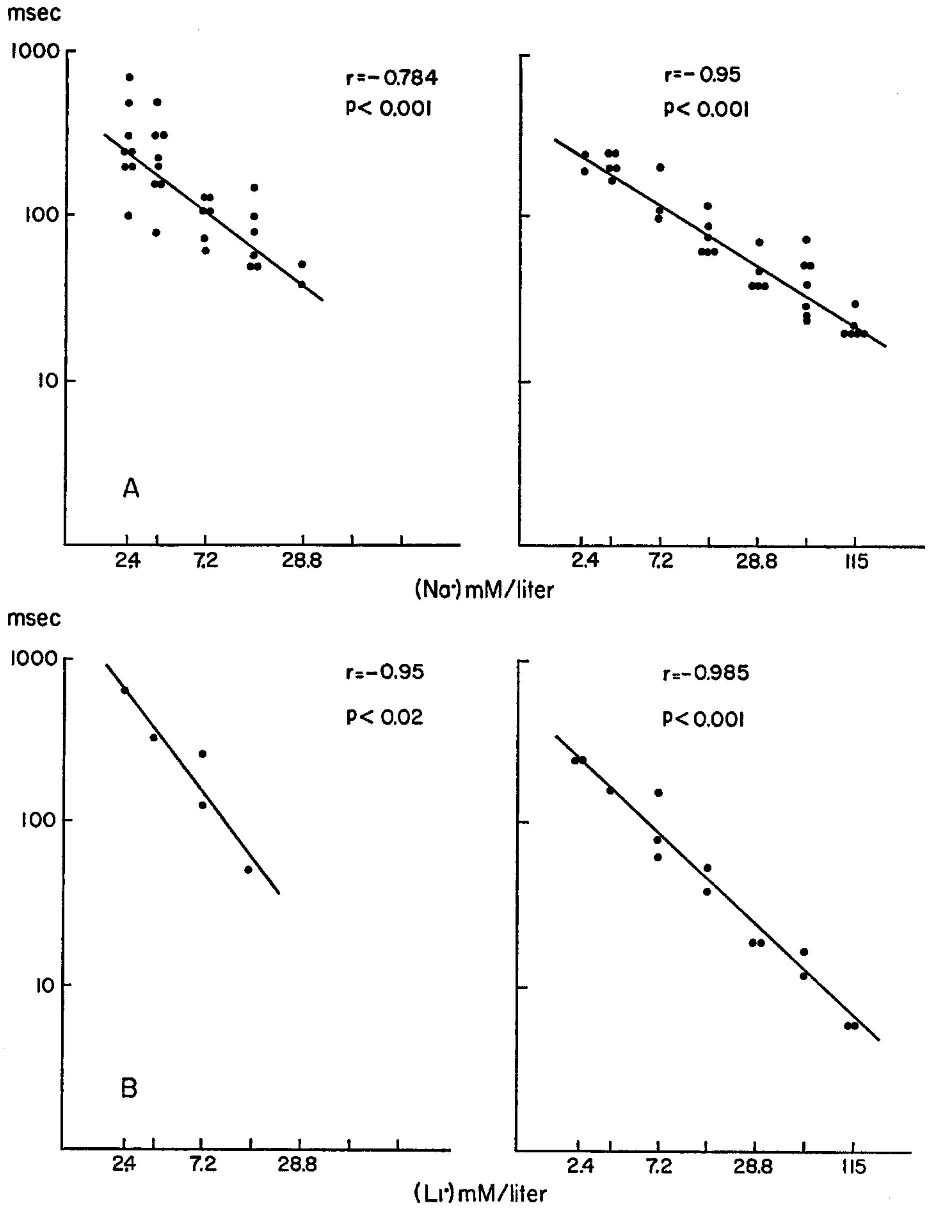

FIGURE 6. Relationship between AP duration and ion concentration in the outer side of the skin. In (*A*) log AP*t* has been plotted vs. log $[Na^+]_o$. In (*B*) log AP*t* has been plotted vs. log $[Li^+]_o$. In both cases the left correlations belong to AP*t* during repetitive activity and the right correlations to AP*t* of single AP's.

$p < 0.001$, $b = -0.585$, $n = 35$) (repetitive activity: $r = -84$, $p < 0.001$, $b = -0.46$, $n = 15$) (Fig. 6).

A covariance analysis showed that slopes for $[Na^+]_o$ and $[Na^+]_o$ + sucrose in single AP's were not significantly different ($Fb = 0.45$, $n = 70$, $p > 0.05$). Slope comparisons were performed with AP duration in single activity because correlation coefficients were of higher value than with repetitive AP's. The duration of single AP's is of a more reproducible nature than the AP's of the induced firing. From this analysis it was concluded that the effect of increasing $[Na^+]_o$ upon the AP*t* was not due to the increasing osmolarity of the outer solution. Results already published (7, 8) on the resting electrical properties of the skin have demonstrated the lack of influence of hypotonic solutions bathing the outside. Consequently from then on, when the ionic concentrations were varied in the outer chamber, osmolarity was not corrected.

EFFECT OF THE $[Cl^-]_o$ The influence of the $[Cl^-]_o$ upon AP*t* was investigated by replacing SO_4^{--} for Cl^- in the solutions. The inner chamber was filled with SO_4^{--} Ringer and the outer chamber with solutions of increasing $[Na^+]$. The effect of increasing $[Na^+]_o$ in single AP's was a decrease in AP*t*.

Plotting log AP*t* vs. log $[Na^+]_o$, a significant negative correlation was obtained ($r = -0.876$, $p < 0.001$, $n = 14$, $b = -0.50$). The slope does not differ statistically from the one obtained in the experiments with chloride as the anion ($Fb = 2.69$, $p > 0.05$, $n = 49$).

Repetitive activity in all respects similar to that obtained with chloride solutions was also obtained with these sulfate solutions.

Results were not evaluated because, as has been already mentioned, the analysis of the duration in single activity was considered more dependable. It was concluded that the shortening of the AP depends on the increasing $[Na^+]_o$ and not on the characteristics of the anion.

Effect of $[Li^+]_o$

The purpose of the $[Li^+]_o$ experiments was twofold: (*a*) to check sodium specificity on the production of AP's, and (*b*) since Li^+ is known to cross the outer barrier as easily as Na ions, but not the inner barrier (9), the production of AP's when Li^+ replaces Na^+ in the outer bath could be taken as a strong indication that the phenomenon originates at the level of the outer facing boundary of the skin. In these experiments the outside chamber was filled with solutions of variable concentrations of Li^+. In this condition the responses were qualitatively similar to those observed with variable $[Na^+]_o$ (Fig. 3 *b*).

Double logarithmic plotting showed a significant negative correlation both for single AP's and for AP's corresponding to repetitive firing (single AP:

$r = -0.985, p < 0.001, n = 15, b = -0.933$) (repetitive firing: $r = -0.95$, $p < 0.02$, $n = 5$, $b = -1.30$) (Fig. 6). The slope of single AP results was not parallel to the corresponding slope when Na ions were employed ($Fb = 24$, $p < 0.001$, $n = 50$). Repetitive activity could be obtained up to 14.4 mM Li^+. These results demonstrate that Li^+ can replace Na^+ in the outer bath in order to obtain action potentials, and they also give strong support to the assumption that some variation on the outward facing barrier produced by the current pulse is the cause of the AP's. This assumption is also supported by the fact that changing the sodium concentration in the inner chamber from 115 mM to zero mM the all-or-nothing phenomenon was not affected when Na^+ was replaced by sucrose. When all the Na ions in the inner chamber were replaced by K^+, there was an immediate change in the potential difference through the skin (e.g. from about 65 to 10 mv). In spite of this change of potential, AP's (with the same characteristics as observed before) could be evoked during the first 5–10 min. After this period AP's could be obtained only occasionally and then they were considerably altered.

Effect of $[Ca^{++}]_o$

The effect of Ca^{++} outer concentration upon the excitable characteristics of the skin was investigated by varying its concentration from zero to 16 mM in the outer solution. When Ca^{++} was omitted in the outside control Ringer the results were not constant. Some skins presented normal AP's while others showed only a graded response. The increase in $[Ca^{++}]_o$ from 1 to 16 mM resulted in a widening of the AP's. Total duration was increased three to four times. The effect of $[Ca^{++}]_o$ was also investigated with low $[Na^+]$ in the outside solution. In these experiments the $[Na^+]_o$ was 3.59 mM and $[Ca^{++}]_o$ was varied between zero and 16 mM. When calcium was omitted, AP's were usually present with the same characteristics as when $[Ca^{++}]_o$ was 1 mM. The increase in $[Ca^{++}]_o$ from 1 to 16 mM resulted in considerable widening of the AP's. The double logarithmic plotting of APt vs. $[Ca^{++}]_o$ showed a significant positive correlation for single AP's as well as for AP's corresponding to repetitive activity (single AP's: $r = 0.59$, $p < 0.001$, $n = 29$, $b = 77$) (repetitive activity: $r = 0.73$, $p < 0.001$, $n = 18$, $b = 0.95$).

In several experiments $[Ca^{++}]_o$ was increased to 32 mM; at this concentration AP's could be obtained only occasionally (Fig. 7).

Effect of $[K^+]_o$

These experiments were performed with 2.4 mM $[Na^+]$ on the outside and varying $[K^+]_o$ from zero to 16 mM. There was practically no effect in varying $[K^+]_o$ from zero to 8 mM. But when $[K^+]_o$ was varied between 8 and 16 mM, action potentials could not be obtained. The same behavior was observed when SO_4^{--} replaced Cl^- in both chambers.

Effect of Outward Pulses

The effect of outward pulses was investigated with control Ringer's as well as with low sodium solutions on the outside. In both conditions there is an increasing positive polarization on the inside that grows linearly with the current intensity when the pulse intensity is relatively low. For higher currents the voltage response is nonlinear but neither all-or-nothing response comparable to AP's obtained with inward currents nor repetitive activity could be obtained.

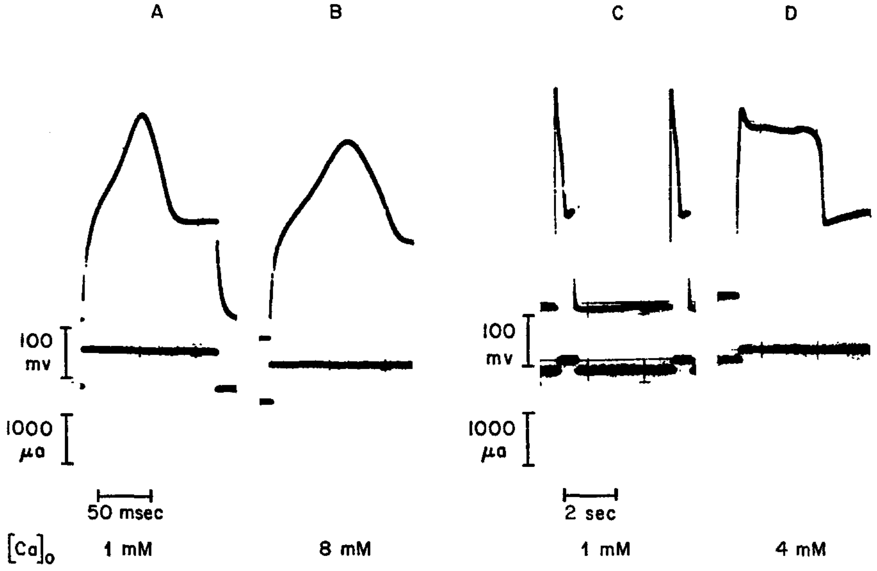

FIGURE 7. Typical effect of $[Ca^{++}]_o$ upon the duration of AP's. The increase in $[Ca^{++}]_o$ lengthens the AP when control Ringer's bathes the outer surface of the skin (records A and B). The effect is greater when $[Ca^{++}]_o$ is increased in a Ringer solution 2.4 mM in $[Na^+]_o$. Note that the ordinate scales are the same in the four records but the abscissa scale is 50 msec for records A and B while it is 2 sec for records C and D.

Conductance Measurements

Up to this point similarities between repetitive firing of toad skin and pacemaker activities of normally excitable tissues were so striking that it was planned to investigate the evolution of the slope conductance during repetitive firing of the skin. The aim of these experiments was to find out how far the comparison of both phenomena could be carried on. A condition was sought in which AP's were long enough to permit measurements of slope conductance during the different phases of the repetitive action potentials. From Fig. 7 it may be seen that a preparation bathed with low Na-high Ca on the outside would fulfill these requirements. Hence a series of experiments was performed with 3.59 mM $[Na^+]_o$ and 8 mM $[Ca^{++}]_o$ on the outside using a small 50 msec continuous square wave pulse superimposed on the main pulse. With this setup, the width of the tracing is an inverse function of the slope conductance.

In Fig. 8 it may be seen that the slope conductance has a minimum value at, and immediately following the spike and also after the descending limb of the AP's, presenting a general aspect similar to that obtained by Weidmann (10) in sheep Purkinje fibers.

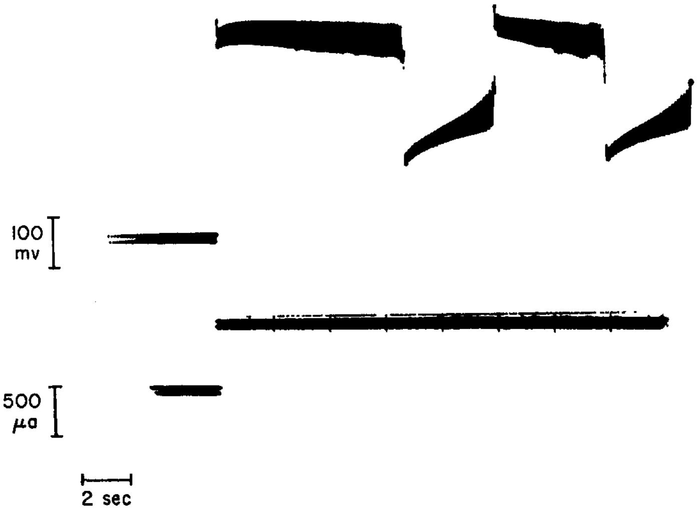

FIGURE 8. Record illustrating the evolution of slope conductance when a 50 msec continuous square wave pulse is superimposed on the main pulse. There is a steady widening of the tracing after the spike and immediately after the descending limb; this corresponds to the evolution of slope resistance. The intensity of the small pulse is 7 μa cm^{-2}.

DISCUSSION

The results reported in this paper demonstrate that under certain experimental conditions toad skin is able to present some reactions typical of excitable tissues, namely action potentials and repetitive firing. The AP's elicited when control Ringer's solutions bathed both sides of the skin have approximately the same characteristics already described for frog skin (3, 4) and undoubtedly they represent the same phenomenon. Small differences may be attributed to species differences. Action potentials produced with low sodium outer concentration differ mainly in their longer duration from those obtained at high sodium outer concentration. The regression line obtained when log AP*t* was plotted vs. log $[Na^+]_o$ shows that the duration of the AP's is a potential function of the sodium outer concentration.

Repetitive activity seems to represent a particular expression of excitability that can be evoked when low $[Na^+]$ bathes the outside of the skin and a long inward current pulse is passed through it. The reason why repetitive firing can be produced only in low $[Na^+]_o$ or $[Li^+]_o$ conditions can be explained by taking into account: (*a*) that with control Ringer's on the outside the skin resistance is relatively low and the pulse intensity must be accordingly high; these intensities cannot be maintained because the skin is damaged irreversibly and (*b*) the refractory period is very long (5–10 sec).

Both conditions are against the possibility of repetitive firing. The inverse situation prevails with low $[Na^+]_o$. Nevertheless it cannot be ruled out that eventually, circumstances prevailing in the site of AP production would forbid repetitive activity even if the high intensity pulse could be maintained.

Site of Response

Since the transient response is so closely related to variations in the composition of the outer bathing solution, mainly $[Na^+]$ or $[Li^+]$, we have to discuss briefly the existence of an external barrier and its localization within the epithelium.

It has already been mentioned that Koefoed-Johnsen and Ussing (1) demonstrated that there is a sodium-selective barrier facing the outer bathing solution. Cereijido and Curran (11), Whittembury (12), and Biber et al. (13) have reported that with low $[Na^+]_o$ the largest portion of the skin resistance is located at the outer barrier.

There is as yet no general agreement upon the actual localization of this barrier within the skin structures. In the first model proposed by Koefoed-Johnsen and Ussing (1) it was localized on the outer aspect of the stratum germinativum. Later results suggested that its localization should be moved outwards (14, 15). Kidder, Cereijido, and Curran (16) postulated that the sodium barrier was located near or at the anatomical outer surface of the skin; this would explain the time evolution of potentials when sodium outside was increased. Cereijido et al. (17) found that the specific permeability to sodium of the outer membrane is a function of the concentration of sodium in the outer solution. Thus the outer anatomical border of the skin seems to be the seat of a variable sodium conductance which gives rise to a sodium-dependent electrical potential. Chowdhury and Snell (18) found a continuous voltage profile from the outside to the inside of the epithelial layer which would represent a distributed resistance (or voltage-generating elements) and not an unique external barrier. Nevertheless, the electrical characteristics of this barrier assembly could be modified by varying $[Na^+]_o$, although some influence could also be observed by varying the internal $[K^+]$ (19). We can summarize the preceding statements by saying that even when there is some controversy about the existence of an *unique* barrier there is complete agreement about the influence of $[Na^+]_o$ upon its electrical characteristics. The strong dependence of the AP characteristics on the ionic composition of the outside solution supports the concept that the outward facing boundary has to be involved in its production. Besides, the almost complete independence with reference to the composition of the inside solution points in the same direction.

Mechanism of the Action Potentials

Finkelstein (4) has postulated that the AP in frog skin is a consequence of the current flowing through a time-variant resistance element that must be localized at the outer membrane level. Based on chord conductance measurements, he concluded that *at least* the sodium resistance must increase during the AP. Even when no direct measurements were performed in our experiments, it was observed that when the external resistance in the current circuit was omitted or had a low value, the current pulse tended to diminish during the AP. This indirect evidence seems to indicate for toad skin also that the response must be produced at least by a transient increase in resistance of the outward facing boundary. The skin action potential would then be characterized by a transient decrease in sodium conductance, which is opposite to the changes of conductance measured in nerve and other excitable tissues.

The outer barrier has been compared (2) to an excited plasma membrane on account of its polarization and its sodium conductance. This similarity seems to be true for $[\mathrm{Na}^+]_o$ higher than 0.34 mM (13). In the experiments here reported, the lower $[\mathrm{Na}^+]_o$ investigated in which AP could be obtained was 0.5 mM, that is at a sodium outer concentration at which the outer barrier can be compared to an excited plasma membrane. In order to explain the production of all-or-nothing responses we have to look for a mechanism capable of switching off sodium conductance. The effect of an inward pulse through the outer barrier must be a positive polarization on its outer aspect that is opposite to its polarization in open conditions. The effect of this new polarization upon sodium conductance on the outer barrier is not known. However, some properties demonstrated in excitable plasma membranes *may be tentatively* attributed to the outer barrier. In Purkinje fibers, Weidmann (10) showed that an inward current pulse given through a microelectrode during the plateau of an action potential could produce a quick repolarization. Cranefield and Hoffman (20) obtained all-or-nothing repolarization in papillary muscles by employing large external electrodes. It seems necessary for the current to repolarize a large area to produce the effect (21). Under the conditions in which toad skin was mounted, an evenly distributed density current through it was to be expected. Probably the same would be the case for the positive polarization on the outside aspect of the outer barrier. If we could ascribe to this barrier the same properties as those demonstrated in Purkinje and ventricle fibers, the effect of this polarization when high enough, would be to switch-off sodium chord conductance (gNa). In Purkinje and ventricle fibers, the effect of the inward pulse is a repolarization to a level of predominant gK. In frog skin the AP is characterized by a switch-off of the sodium conductance to a new level of nondetermined predominant conductance. AP in toad skin would then actually represent a transition to a state that can be compared (at least for our purpose) to the resting state of a plasma membrane. If this comparison holds, the outer barrier of the skin in open normal conditions would represent a state similar to a long-lasting plateau in Purkinje or ventricle tissue. As has already been mentioned, the slope conductance showed an evolution similar to that determined in Purkinje fibers. Slope conductance seems to be time- and voltage-dependent. Finkelstein (4) also found a marked increase in the slope conductance during the spike in frog skin with Ringer's solution on the outside.

In order to understand the mechanism of action of the different cations on the outer barrier, we have to assume that the known influence of Na (17), Ca (22), and K (23) upon the resting properties of the skin is due to the presence at this location of specific anionic binding sites. Considering that the phenomenon we are dealing with represents a transition between two different and characteristic conductances of the outer barrier (the normal state with a predominant *g*Na and the transient with a low *g*Na) we have to speculate on the effects of these ions, acting upon the *stability* of both states of conductance, when they are fixed to the outer aspect of the barrier.

Most of the experimental results become logical assuming that $[Na^+]_o$ is instrumental in the tendency of the skin to be fixed at the level of a predominant *g*Na while $[Ca^{++}]_o$ allows the skin to leave the normal state more easily and increases the possibility of remaining in the transient state.

When $[Na^+]_o$ is high the barrier would be stabilized and it would be difficult to change its conductance properties except for a brief interval. As a consequence AP's under this condition, are of short duration and associated with a long-lasting refractory state. When $[Na^+]_o$ is low, the normal state would not be so stabilized and the electrical characteristics of the skin could be changed more easily. As a corollary, action potentials are expected to be longer (and even repetitive) helping to explain the negative relationship between AP*t* and $[Na^+]_o$ illustrated in Fig. 6.

The considerations given above for $[Na^+]_o$ can be extended to the action of $[Li^+]_o$. Even the fact that the regression line (Fig. 6) for Li^+ is significantly steeper than that for Na^+ seems to indicate that the effect of Li^+ is quantitatively different from the effect of Na^+ and probably related to the fact that the outer border would prefer Li^+ to Na^+ (23).

The stronger effect of $[Ca^{++}]_o$ in lengthening the action potential when $[Na^+]_o$ is low (Fig. 7) is to be expected considering the assumptions stated above. The abolition of AP's in the experiments carried out raising $[K^+]$ in an external solution containing 2.4 mM $[Na^+]$ cannot be explained satisfactorily at the present time. However, some effects of this ion are to be expected since it has been reported (23) that $[K^+]_o$ can mimic, although poorly, the effect of $[Na^+]_o$ on the resting electrical potential of frog skin.

The effect of the replacement of Na^+ by K^+ in the *inner* chamber upon the AP's can be explained assuming that during the first 5–10 min, K^+ exerts its action mainly on the inner barrier. During this period the action potential evoked by an inward pulse has the same characteristics with regard to shape, duration, and threshold as before the replacement, in spite of the fall in the potential difference through the skin. After this 5–10 min period, the inner K^+ would act on the outer barrier and consequently the mechanism of AP production would begin to deteriorate until only occasional and distorted AP's could be obtained.

The possibility of unmasking excitable properties in toad skin, similar in many respects to those obtained in excitable tissues, in addition to the results reported by Finkelstein in frog skin and toad bladder, supports the presumption that normally transporting epithelia share common structural patterns with plasma membranes of excitable tissues. It seems very likely that a condition could be found in which the same phenomenon could be evoked in other types of transporting epithelia, such as intestinal mucosa, renal tubuli, etc.

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