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THE CONTRIBUTION OF THE ELECTROGENIC SODIUM–POTASSIUM PUMP TO THE ELECTRICAL ACTIVITY OF TOAD RODS

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SUMMARY

1. The membrane potential of rods in the isolated toad retina was recorded while changing the ionic composition of the extracellular medium.

2. Caesium (Cs^+) at a concentration of 1 mM was sufficient to completely block the sag from the peak to the plateau in the bright-flash voltage response.

3. In the presence of 10 mM-Cs^+ the bright-flash response increased in amplitude to about 90 mV, thus reaching an absolute membrane potential of between −110 and −135 mV. These responses consisted of an initial fast component of about 35 mV followed by a much slower component which could be as large as 50 mV.

4. At the peak of the initial fast component the rod membrane conformed closely to the behaviour of a K^+ electrode with a P_{Na}/P_K ratio of 0·023. On average the amplitude of the slow component was about 35 mV in the presence of 2·6 mM-K^+ and was reduced to about 25 mV in a K^+-free Ringer.

5. Addition of 100 μM-strophanthidin to the perfusate induced several reversible changes in the electrical activity of rods. The dark resting membrane potential depolarized by about 5 mV and the kinetics of the voltage response to dim flashes of light slowed down. The voltage sensitivity initially increased by about 30%, but the peak of the response to a bright flash of light was reduced by about 13 mV.

6. In rods treated with 10 mM-Cs^+ the slow component present in the bright flash response was abolished by strophanthidin with an apparent K_m of 3 μM.

7. The amplitude of the slow component decreased with a time lag of about 2 min when external Na^+ was reduced. A previous exposure of the retina to a Na^+-free Ringer solution for at least 3 min modified the voltage photoresponse in a way similar to that observed in the presence of 100 μM-strophanthidin.

8. When external Ca^{2+} concentration ($[Ca^{2+}]_o$) was increased from 2 to 5 mM the slow component decreased by about 30%. When $[Ca^{2+}]_o$ was reduced the slow component increased. A twofold increase was observed when $[Ca^{2+}]_o$ was lower than 10^{-4} M.

9. It is suggested that the slow component of the voltage response in the presence of external Cs^+ is caused by an electrogenic current driven by the Na^+–K^+ transport system, during a voltage-dependent block of external Cs^+ of some K^+ channels.

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INTRODUCTION

The light-modulated ionic current has recently been measured in toad rods as between 20 and 30 pA in darkness (Baylor, Lamb & Yau, 1979). Since this current is likely to be carried primarily by Na^+ ions (Korenbrot & Cone, 1972; Brown & Pinto, 1974; Capovilla, Cervetto, Pasino & Torre, 1981; Yau, McNaughton & Hodgkin, 1981) one must postulate the existence of an active transport mechanism capable of extruding sodium. A likely mechanism would be a Na^+–K^+ pump (Sillman, Ito & Tomita, 1969; Yoshikami & Hagins, 1970).

The Na^+–K^+ pump is, in general, electrogenic to some degree (Glynn & Karlish, 1975; Robinson & Flashner, 1979). Zuckerman (1973) has argued that in frog rods the Na^+–K^+ pump must be significantly electrogenic. While this idea is reasonable, it is not supported by the subsequent experiments of Hagins & Yoshikami (1975), who were unable to reproduce Zuckerman's results, and the degree of electrogenicity is at present uncertain. Assuming a Na^+–K^+ exchange of 3 to 2, as found in many other tissues (Glynn & Karlish, 1975, Thomas, 1972), the corresponding electrogenic current would contribute about 9·5 mV to the steady dark resting membrane potential (eqn. 9 in Thomas, 1972) assuming a ratio between P_{Na} and P_K of about 1 (Capovilla, Cervetto & Torre, 1980; Capovilla *et al.* 1981). Larger contributions are expected when light-sensitive channels and other shunting conductances are inactivated. In the presence of 2 mM-external Cs^+, which inactivates voltage- and time-dependent conductances (Fain, Quandt, Bastian & Gershenfeld, 1978), the voltage photoresponse may be as large as 70 mV and sometimes approaches an absolute peak membrane potential of −120 mV (Owen & Torre 1981*a*, Owen & Torre 1981*b*). The rising phase of these responses consists of an initial fast hyperpolarization of about 35 mV followed by a much slower wave, whose amplitude and time course depend on the concentration of the external Cs^+.

In this paper it will be shown that the late slow component of voltage photoresponses in Cs^+ is reversibly suppressed by 10–100 μM- strophanthidin, a cardioactive steroid known to block reversibly the Na^+–K^+ pump (Sachs, 1974; Eisner & Lederer, 1980), and is significantly reduced in a K^+-free medium. It is proposed that external Cs^+ blocks the voltage-sensitive channels that cause the relaxation from the initial peak and also a remaining K^+ conductance in a voltage-dependent manner. It is suggested that the slow component is caused by an electrogenic current driven by the Na^+–K^+ transport system and that this current gives a more significant contribution to the membrane potential during the slow component when shunting conductances are inactivated by Cs^+. Under these conditions the peak of the voltage response to a bright flash of light corresponds to absolute levels of membrane potential significantly more negative than the usual limiting value of E_K.

METHODS

Preparation

The experiments to be described were carried out on the isolated retina of the toad, *Bufo marinus* (supplied by Xenopus Ltd).

An animal selected for an experiment was first dark-adapted overnight and pithed under dim red light. Both eyes were immediately removed. With the aid of a dissecting microscope and infra-red

image converter, the posterior pole of the globe was cut off with a razor blade and cut into three or four segments avoiding the optic disk. The pieces of retina obtained from the two eyes were kept in complete darkness in oxygenated Ringer solution. One of these segments was placed in a small dish of Ringer solution and the retina was gently isolated from the sclera. The isolated retina was laid on a small piece of filter paper with the receptor side up. To increase mechanical stability an annular piece of filter paper was placed over the retina, with the hole over the portion of retina chosen for intracellular recording. The preparation was subsequently placed in a small volume perfusion chamber.

Perfusion

Six different solutions were tested in each experiment. Each one of the six reservoirs was connected by two six-way manifolds whose outputs were in turn connected to a two-way tap. With a flow rate of 2 ml/min a complete change of solution occurred within 15 s. The composition of the Ringer solution was (mM): Na^+, 132; K^+, 2·6; Ca^{2+}, 2; Mg^{2+}, 2; HCO_3^-, 22; Cl^-, 120·6; glucose, 5; buffered to pH 7·6 and oxygenated with 5 % CO_2–95 % O_2 (Cervetto, Pasino & Torre, 1977). The temperature was 18–20 °C. When Cs^+ was added to the Ringer solution an equimolar amount of Na^+ was removed. When external K^+ concentration was changed, osmolarity was preserved by equimolar change of Na^+. I am indebted to Drs S. Franklin & M. I. Sepulveda who with flame photometry measured a residual Na^+ concentration of 0·02 mM in nominal Na^+-free Ringer solution. Drs T. Rink and R. Tsien kindly measured residual calcium concentration in nominal Ca^{2+}-free Ringer. $[Ca^{2+}]_0$ was reduced to $1·7 \times 10^{-8}$ M with a Ca^{2+}-EGTA buffer kindly provided by Dr P. McNaughton. Strophanthidin (SIGMA) was dissolved by stirring in a saline solution.

Stimulation and recording

An optical bench of the Baylor & Hodgkin (1973) design was used to form a circular spot of 1000 μm diameter on the retina (Detwiler, Hodgkin & McNaughton, 1980; Torre & Owen, 1981). The intensity and spectral composition of stimuli were controlled using neutral density and narrow band interference filters as described by Baylor & Hodgkin (1973). Light intensity was measured with a calibrated silicon photodiode (United Detector Technology Inc.) placed at the position of the retina. The optical density of individual neutral density filters was measured using the same photodiodes. Intracellular micro-electrodes were drawn on a Brown-Flaming puller and when filled with 4 M-potassium acetate had a resistance of 150–350 MΩ.

Electrodes were connected to a high impedance negative capacitance pre-amplifier WPI. The reference electrode was connected to the bath by means of an Agar-3 M-KCl bridge. Changes in tip potentials following intracellular penetration were kindly checked by Dr C. Huang in the following way. A penetration of a muscle fibre was tried with a low resistance micro-electrode (10 MΩ) and with a high resistance micro-electrode pulled as described previously. The resting potential measured by the two electrodes on the same fibre was compared and no systematic difference was observed. The micro-electrode was centred on a spot of infrared light focused on the liquid surface, by observing it using a silicon vidicon camera (Akai Model VC-70) (W. G. Owen & V. Torre, in preparation). The micro-electrode was advanced with a hydraulic microdrive (Clark Medical Instruments). An FM tape recorder with band width of DC to 1250 Hz (Analog 7, Philips), was used to record intracellular potential, light stimulus and trigger pulse. A chart recorder was used to display the results of the experiment. Voltage responses were digitized and stored on a cartidge disk of a DEC PDP 11/10 computer. Responses were displayed on an X–Y plotter.

RESULTS

The blocking effect of external Cs^+

Voltage responses of toad rods to bright flashes of light consist of a fast hyperpolarizing transient of about 30 mV followed by a plateau of about 13 mV lasting for a few seconds (Cervetto *et al.* 1977). In the toad retina Fain *et al.* (1978) have shown that addition of 2 mM-Ca^+ to the extracellular medium completely blocks the relaxation of the voltage from the initial peak to the subsequent plateau.

Fig. 1*A* illustrates an experiment in which the concentration of external Cs^+ was

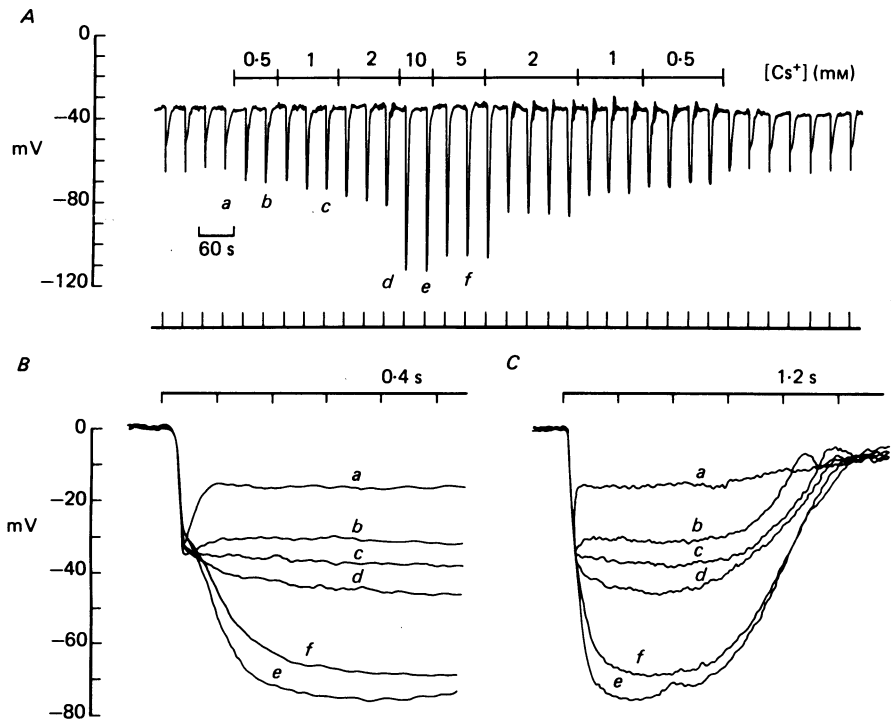

Fig. 1. Stoichiometry of the effect of external Cs^+. *A*, chart recording of the experiment. Upper bars indicate the time of solution changes; bottom trace is light monitor. Flash duration 50 ms, wave-length 498 nm, light intensity equivalent to 1400 Rh* (photoisomerization) per flash. *B*, *C*, responses labelled *a*, *b*, *c*, *d*, *e*, *f* in *A*, are shown on two different time scales. Observe the oscillations of membrane potential during the repolarization when external Cs^+ was progressively reduced from 10 to 1 mM. In *B* and *C* the dark membrane has been set equal to 0 mV.

progressively increased to 10 mM and subsequently decreased while recording intracellularly from a rod. The dark resting potential was hardly affected, but the amplitude of the voltage response to a flash of light (equivalent to about 1400 Rh* (photoisomerization)) increased considerably. In the presence of 10 mM-Cs^+ voltage photoresponses as large 90 mV were sometimes observed and absolute membrane potentials at the peaks of between -110 and -135 mV were measured.

Fig. 1*B* and *C* shows in greater detail the effect of different concentrations of extracellular Cs^+ on the shape of photoresponses illustrated in Fig. 1*A*. It is seen that 0·5 mM-Cs^+ (record *b*) reduces, but does not abolish, the sag from the peak to the plateau present in normal condition (record *a*). The sag is completely abolished by 1 mM-Cs^+ (record *c*), and with Cs^+ concentrations of 2, 5, 10 mM (record *d*, *f*, *e* respectively) a second slow hyperpolarizing component which may reach unusually negative values of membrane potential, appears.

This component, which is seen only in the presence of Cs^+, will be referred to as the slow or Cs^+-dependent component of the response. Its amplitude is defined as the potential difference between the initial peak and the peak of the slow component.

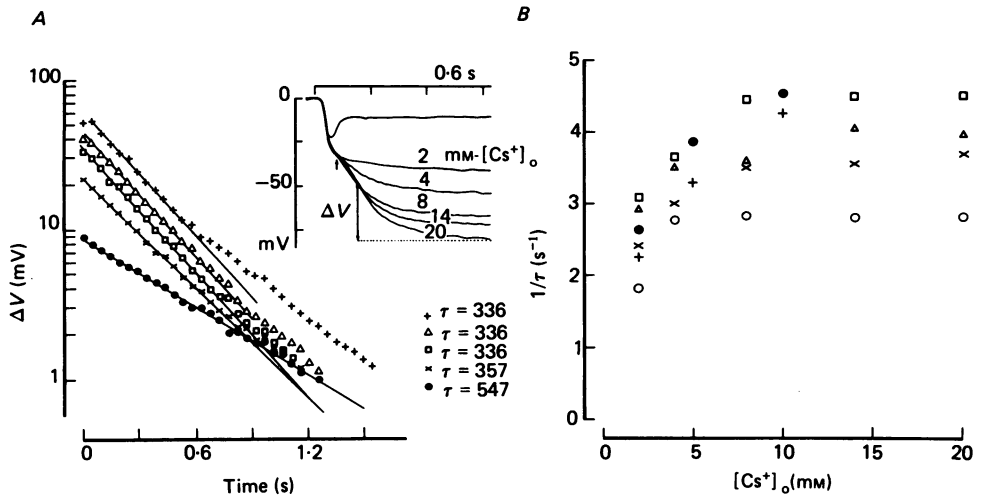

Fig. 2. Time course of the slow component of bright-flash responses in the presence of external Cs^+. *A*, time constant τ of the relaxation of the slow component ($V-V_{max}$) of responses shown in the inset. Responses were obtained in the presence of 2(●), 4(×), 8(□), 14 (△) and 20(+) mM-Cs^+. Time 0 coincides with time marked by the arrow in the inset. Light stimuli as in Fig. 1. *B*, dependence of $1/\tau$ on $[Cs^+]_o$. Collected data from six cells. In the inset the dark membrane potential (−43 mV) has been set equal to 0 mV.

Fig. 2*A* shows that the time course of the slow component can be described by a single exponential starting 250 ms after the onset of the light flash. The amplitude (ΔV) of the slow component *vs.* time is plotted on semilogarithmic axes for responses in the presence of 2, 4, 8, 14 and 20 mM-Cs^+. The time constant decreases as Cs^+ is increased from 2 to 8 mM; with further increases in Cs^+, the time constant approached a minimum value of about 330 ms. This is shown in Fig. 2*B* where the reciprocal of the time constant τ of the slow component is plotted against $[Cs^+]_o$.

It may be useful to present already at this stage the proposed explanation for the appearance of the slow component. It is suggested that the Na^+–K^+ pump is electrogenic and supplies a constant current. The contribution of this current to the membrane potential should become greater when the membrane resistance increases. It is proposed that Cs^+ ions have two separate effects: at concentrations of 1 mM or greater Cs^+ blocks within a few milliseconds the channels through which the current responsible for the sag from the peak to the plateau flows. This current is not carried by K^+ ions (Fain *et al.* 1978) and may have multiple components (Attwell & Wilson, 1980, Capovilla *et al.* 1981). In addition, at higher concentrations Cs^+ may cause an increase in membrane resistance, which would enhance the contribution of the electrogenic current to the membrane potential. The time course of the slow component may be the reflection of the development of this second blocking action of Cs^+.

This blocking action of Cs^+ is thought to be in some way voltage-dependent, because the slow component is observed when the membrane potential is more negative than −70 mV. In Fig. 2 it was observed that the development of the slow

component occurs with a single time constant which is suggestive of a simple first order process with α and β as rate constants:

$$\gamma_o + Cs^+ \underset{\gamma}{\overset{\alpha}{\rightleftharpoons}} \gamma_b, \quad (1)$$

where γ_o is an open channel and γ_b is a blocked channel. In a later section it will be shown that at the peak of the initial component of the response to a bright flash the membrane potential behaves as a K^+-electrode with a ratio $P_{Na}/P_K = 0{\cdot}023$. If it is assumed that this ratio reflects the properties of a single type of channel, then the slow component could be the manifestation of a time-dependent blocking action of Cs^+ of these channels.

From eqn. (1), it is expected that the time constant τ of the blocking action depends on $[Cs^+]_o$ as:

$$\frac{1}{\tau} = \beta + \alpha\ [Cs^+]_o. \quad (2)$$

The data of Fig. 2*A* show that between 2 and 8 mM, the relation between $1/\tau$ and $[Cs^+]_o$ is approximately satisfied by eqn (2). This observation suggest a value of 2 s^{-1} for β and a value of 0·2 s^{-1} mM^{-1} for α. At higher Cs^+ concentrations the time constant of the slow component should approach a minimum value determined by the membrane time constant itself.

Effect of K^+-free medium in the presence of Cs^+

At least two different mechanisms could explain the unusually negative membrane potentials reached by bright-flash responses in the presence of 10 mM-Cs^+. One possibility is that in the presence of Cs^+ the narrow space around the inner segments becomes depleted of K^+ during the photoresponse, leading to an increase of E_K. Alternatively it may be suggested that in the presence of Cs^+ conditions are such that the electrogenic activity of the Na^+–K^+ pump becomes more evident. These two hypotheses predict different results if K^+ is removed from bulk solution. If the extreme hyperpolarization is produced by a K^+ depletion it should be enhanced when extracellular K^+ is removed; if it is brought about by an electrogenic current driven by the Na^+–K^+ pump it should be reduced when extracellular K^+ is removed. Fig. 3*A* shows an experiment during which the pump has alternately been inactivated by a K^+-free medium and reactivated by return to 2·6 mM-K^+. During exposure to a K^+-free medium the amplitude of the voltage photoresponse decreased by about 10 mV (record *b* in Fig. 3*A*); Fig. 3*B* shows that this is due to a depression of the slow component. Subsequent reactivation of the pump by the return to 2·6 mM-K^+ in the extracellular medium restored the amplitude of the slow component (record *c* in Fig. 3).

These results are consistent with the idea that the Cs-dependent slow component of the voltage photoresponse is associated with the activity of a Na^+–K^+ pump. The presence of a residual slow component in a K^+-free medium may be explained if it is assumed that the pump may also be activated by extracellular Cs^+ (Eisner & Lederer, 1980).

Dependence of the two components on external K^+

Fig. 4*A* illustrates an experiment in which initially 10 mM-Cs^+ was added to the perfusate and subsequently $[K^+]_o$ was rapidly changed. In Fig. 4*B* the responses in

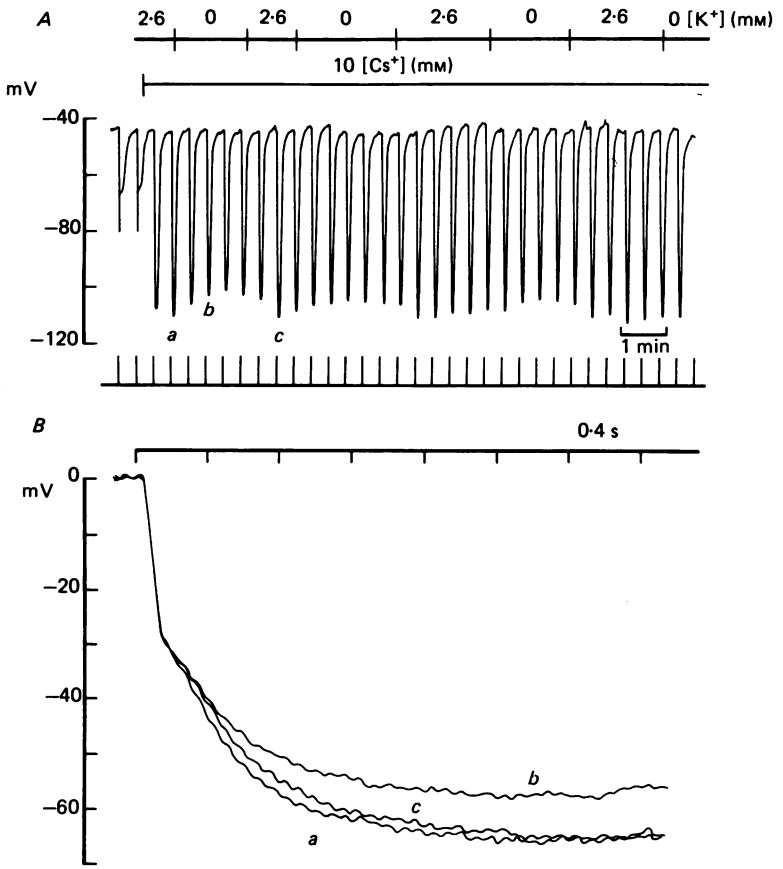

Fig. 3. Effect of a K^+-free Ringer solution on the photoresponse to a bright flash of light in the presence of 10 mM-external Cs^+. *A*, chart recording of the experiment. Upper bars indicate the time of solution changes; bottom trace is light monitor. *B*, responses labelled *a*, *b*, *c*, in *A* are shown in greater detail. Light stimuli as in Fig. 1. In *B* the dark membrane potential has been set equal to 0 mV.

0, 0·5, 1·3, 2·6, and 5 mM-K^+ are illustrated in greater detail. The photoresponse in 0 mM-K^+ has the largest initial component and the smallest slow component (record *a*). When $[K^+]_o$ is increased the size of the initial component decreased while the slow component became larger. Results from several such experiments are summarized in Fig. 5, which shows the effect of changing $[K^+]_o$ in the presence of 10 mM-Cs^+.

Fig. 5*A* shows that the resting potential in darkness (open symbols) is almost independent of $[K^+]_o$, at concentrations below 10 mM, as expected from previous studies (Owen & Torre, 1981*a*; W. G. Owen & V. Torre, in preparation). The potential at the peak of the light response (filled symbols) when $[K^+]_o$ was raised beyond 5 mM behaves as a perfect K^+ electrode and the experimental points fall on a line with slope of 58 mV per decade. When $[K^+]_o$ is reduced below 10 mM, however, the response is more negative than predicted by this line. This is due to the addition of the slow component at $[K^+]_o$ less than 10 mM.

In Fig. 5*B* the amplitude of the slow component is plotted against $[K^+]_o$. Between

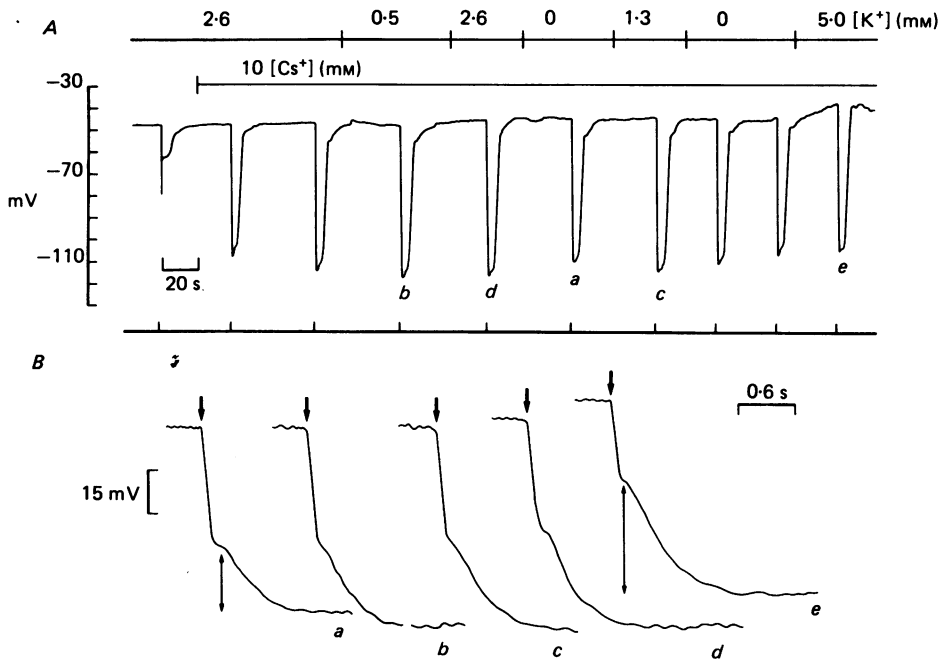

Fig. 4. Effect of external K^+ concentration on the photoresponse to a bright flash of light in the presence of 10 mM-external Cs^+. *A*, chart recording of the experiment. Upper bars indicate the time of solution changes. At the bottom trace is light monitor. *B*, responses labelled *a*, *b*, *c*, *d*, *e* in *A* shown in greater detail. The arrow indicates the onset of the light flash. Observe that the peak of the initial rapid hyperpolarization increases when $[K^+]_o$ is decreased and that the amplitude of the slow component is decreased when $[K^+]_o$ is reduced. Light stimuli as in Fig. 1.

0 and 5 mM-external K^+, K^+ increases the hyperpolarization, similar to its effect on the activation of Na^+–K^+ pump activity (Garrahan & Glynn, 1967). When $[K^+]_o$ is 10 mM or greater the slow component vanishes, leaving only the initial component of the light response which behaves almost as a K^+ electrode (see Fig. 6).

The membrane potential at the peak of the first component in the presence of external Cs^+ is plotted against $[K^+]_o$ in Fig. 6. The continuous line is given by the equation (Hodgkin & Horowitz, 1959; Hille, 1973)

$$V = 58 \text{ mV} \log_{10} \frac{[K^+]_o + \frac{P_{Na}}{P_K}[Na^+]_o}{[K^+]_i}. \quad (3)$$

with the values of $P_{Na}/P_K = 0.023$ and $[K^+]_i = 100$ mM, and is in reasonable agreement with the experimental points of Fig. 6.

If the electrogenic current of the Na^+–K^+ pump is responsible for the slow component of responses to bright light flashes in the presence of Cs^+, it is likely also to contribute to the amplitude of the initial component. In a later section it will be shown that the cardioactive steroid strophanthidin known to block the Na^+–K^+ pump, abolished the slow component and reduced by about 15 mV the amplitude of the initial component. The simplest way to estimate the contribution of the

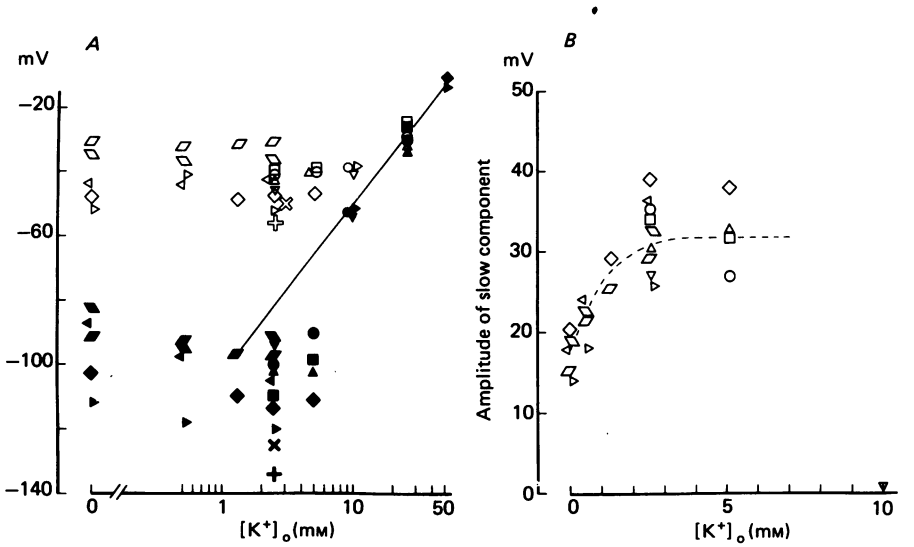

Fig. 5. Dependence on $[K^+]_o$ of dark membrane potential and absolute peak of the photoresponse to a bright flash of light in the presence of 10 mM-external Cs^+. *A*, open symbols: dark membrane potential, filled symbols: absolute peak potential of the light responses. Data from eleven cells. *B*, relation between amplitude of the slow component of the photoresponse and $[K^+]_o$. Identical symbols in *A* and *B* indicate the same cell. Light stimuli as in Fig. 1.

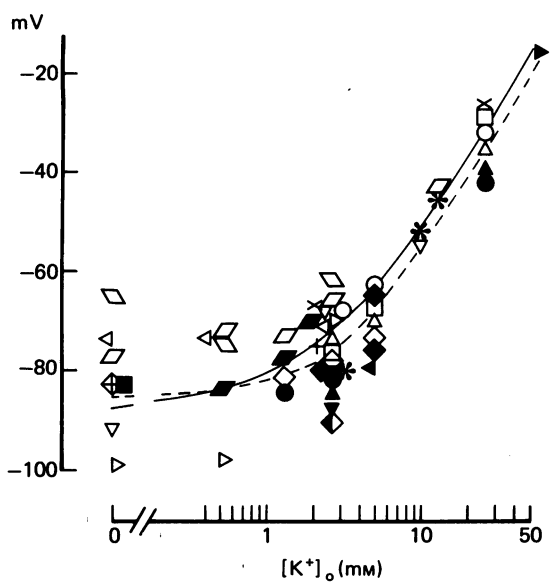

Fig. 6. Dependence on $[K^+]_o$ of the absolute peak of the initial fast component in the presence of external Cs^+. Collected data from nineteen cells. Open symbols refer to experiments with 10 mM-external Cs^+, filled symbols refer to experiments with 2 mM-external Cs^+, crosses and asterisks refer to experiments with 5 mM-external Cs^+. Continuous line drawn from eqn. (3), dashed line drawn from eqn. (5).

electrogenic current to the absolute level of the peak of the initial component is to correct eqn. (3) in the following way:

$$V = \frac{RT}{F} \ln \frac{[K^+]_o + \frac{P_{Na}}{P_K}[Na^+]_o}{[K^+]_i} + R_M I_p \quad (4)$$

where I_p is the electrogenic current of the Na^+–K^+ pump and R_M is the membrane resistance. The dependence of I_p on external $[K^+]_o$ can be assumed to be proportional to the dashed line drawn through the points of Fig. 5*B*. That is

$$V = \frac{RT}{F} \ln \frac{[K^+]_o + \frac{P_{Na}}{P_k}[Na^+]_o}{[K^+]_i} + f.\Delta V_p \quad (5)$$

where f is the normalized curve of Fig. 5*B* and ΔV_p is the maximal contribution of the electrogenic current to the membrane potential at the peak of the initial component. Since the amplitude of the initial component decreased by about 15 mV following addition of strophanthidin to the perfusate, ΔV_p can be assumed to be 15 mV. The dashed line in Fig. 6 has been obtained from eqn. (5) with the values of P_{Na}/P_K 0·023, $[K^+]_i$ = 65 mM.

Effect of strophanthidin in the presence of Cs^+

To test the idea that an electrogenic current from the Na^+–K^+ pump contributes to the photoresponse, it was useful to study the action of strophanthidin, which is known to block reversibly the Na^+–K^+-ATPase in many tissues (Sachs, 1974; Eisner & Lederer, 1980). Fig. 7*A* illustrates the effect of a brief exposure of the retina to 10 and 100 μM-strophanthidin, in the presence of 10 mM-Cs^+. The most striking effect was a large decrease in the size of the light response, while the dark resting potential decreased only slightly.

Almost the same effects were obtained using either 10 or 100 μM-strophanthidin. Recovery of the response to its initial size was obtained within 5 to 10 min after the removal of strophanthidin from the extracellular medium. Recovery was also complete, but slower when 100 μM-strophanthidin was used. It is clearly shown in Fig. 7*B* and 7*C* that the decrease of the amplitude of the voltage photoresponse is essentially due to a suppression of the slow component, which is drastically reduced in the presence of 10 μM-strophanthidin (record 14) and completely abolished by 100 μM-strophanthidin (record 30). These concentrations of strophanthidin also reduced the peak of the initial component by 10 and 20 mV, respectively.

In many experiments it was observed that during the Cs-dependent photoresponse the membrane potential showed fluctuations in the depolarizing direction. A typical example is shown in Fig. 7 (record 24). These fluctuations disappeared when the membrane potential was depolarized by the addition of strophanthidin or K^+ to the perfusate. Recording the membrane current of an isolated rod with a suction electrode it is possible to observe similar bursts on top of bright flash responses if 10 mM-Cs^+ is added to the perfusate (A. L. Hodgkin, P. A. McNaughton & K. W. Yau, personal communication). Tentatively it is proposed that these bursts are due to reversible dielectric breakdown (Benz & Conti, 1981) caused by a prolonged polarization of the membrane below −110 mV.

Similar experiments with different concentrations of strophanthidin indicated an affinity of 3 μM for its action in blocking the slow component of the response; this is at least an order of magnitude lower than the affinity of the Na^+–K^+-ATPase in other tissues in *Bufo marinus* (Robinson, 1970) and *Bufo bufo* (Repke, Est & Portius, 1965).

The blockage of the Cs^+-dependent slow component of bright flash responses by

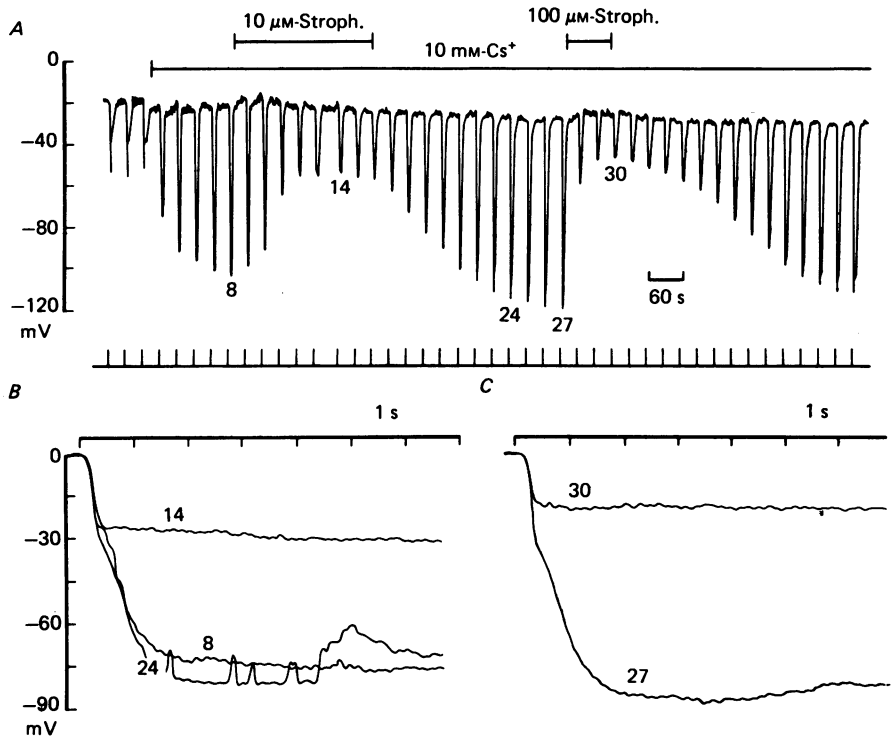

Fig. 7. Effect of strophanthidin (stroph.) on the photoresponse to a bright flash of light in the presence of 10 mM-external Cs^+. *A*, chart recording of the experiment. Upper bars indicate the time of solution changes; bottom trace is light monitor. Light stimuli as in Fig. 1 with the exception of the flash precedent the one labelled 14 where a light intensity equivalent to 5800 Rh*/flash was used. *B*, effect of 10 μM-strophanthidin on the slow component. The slow component (see record 8) was drastically reduced by 10 μM-strophanthidin (see record 14). The bursts present in record 24 are thought to be reversible dielectric breakdown. *C*, effect of 100 μM-strophanthidin on the slow component. The slow component (see record 27) was completely abolished by 100 μM-strophanthidin (see record 30). In *B* and *C* the dark membrane potential has been set equal to 0 mV.

strophanthidin supports the idea that there is a direct link between this voltage component and the activity of the Na^+–K^+ pump. However it is possible that a substantial accumulation of K^+ in the extracellular medium, due to blockage of the pump, could account for the effects of strophanthidin. To rule out this possibility the effects of strophanthidin were examined in conditions in which relative changes of K^+ around the inner segments were minimized.

If the concentration of K^+ in the bulk solution is raised to 10 mM, the relative contribution of K^+ leaking out from retinal cells to the concentration of K^+ around the inner segments is expected to be reduced. Fig. 8*A* illustrates such an experiment. When 100 μM-strophanthidin was applied to a retina pre-treated with Cs^+ and high K^+, the absolute peak of the voltage response became more positive by about 20 mV and the slow component was completely abolished. For this result to be due solely to an extracellular K^+ accumulation, $[K^+]_o$ would have to increase to about 20 mM (see Fig. 6).

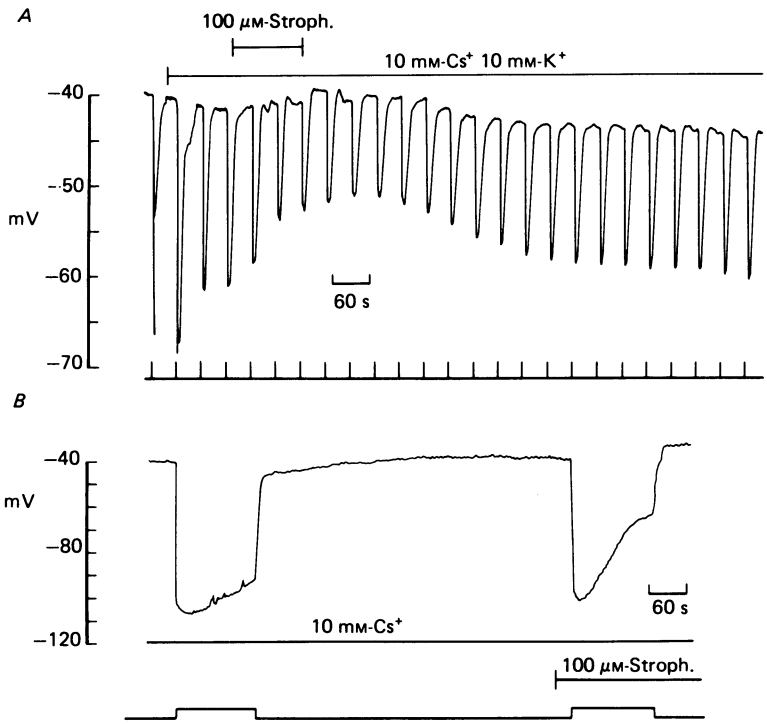

Fig. 8 *A*, effect of 100 μM-strophanthidin on the photoresponse to a bright flash in the presence of 10 mM-external Cs^+ and of 10 mM-external K^+. Upper bars indicate the time of solution changes, bottom trace is light monitor. Light stimuli as in Fig. 1. *B*, effects of strophanthidin on the photoresponse to a steady light in the presence of 10 mM-external Cs^+. Lower bars indicate the time of solution changes, bottom trace is light monitor. Steady light equivalent to 9800 Rh*/s.

One way to minimize K^+-leakage from photoreceptors is to reduce the driving force for K^+ efflux by hyperpolarizing the rod membrane with a steady bright light. Fig. 8*B* illustrates the protocol of an experiment in which a retina treated with 10 mM-external Cs^+ was exposed to 100 μM-strophanthidin during a steady bright light. First the voltage response of the rod to the steady light was observed in the absence of the Na^+–K^+ pump blocher. Subsequently, after several minutes of darkness the rod was again exposed to the same light stimulus, immediately after adding 100 μM-strophanthidin to the perfusate. Even in this case, the membrane potential, after the initial peak hyperpolarization, depolarized by about 35 mV.

Effects of strophanthidin in the absence of external Cs^+

Fig. 9 illustrates the effects of 100 μM-strophanthidin on the voltage photoresponse to bright flash of light (equivalent to about 1400 Rh*). As already shown by Oakley, Flaming & Brown (1979), addition of strophanthidin caused an immediate decrease in the peak of the photoresponse (compare records *a* and *b* in Fig. 9).

Subsequently the response became slower (see record *c* in Fig. 9*C*). After returning the perfusate to the usual physiological extracellular medium the amplitude of the

light response recovers within 5 min, but it may be necessary to wait about 15 min before the kinetics of the photoresponse return to their original state.

The results of the experiment shown in Fig. 9 are consistent with the hypothesis that an electrogenic Na^+–ΔK^+ pump may contribute to the shaping of the voltage response also in physiological conditions. If this is the case, the electrogenic current should give a large contribution to the voltage when the membrane resistance is high. It can be seen that strophanthidin strongly suppresses the peak of the light response, when the membrane resistance is high, but has relatively little effect on membrane potential in darkness or during the plateau phase of the response, when membrane resistance is lower (Fain *et al.* 1978; Copenhagen & Owen, 1980).

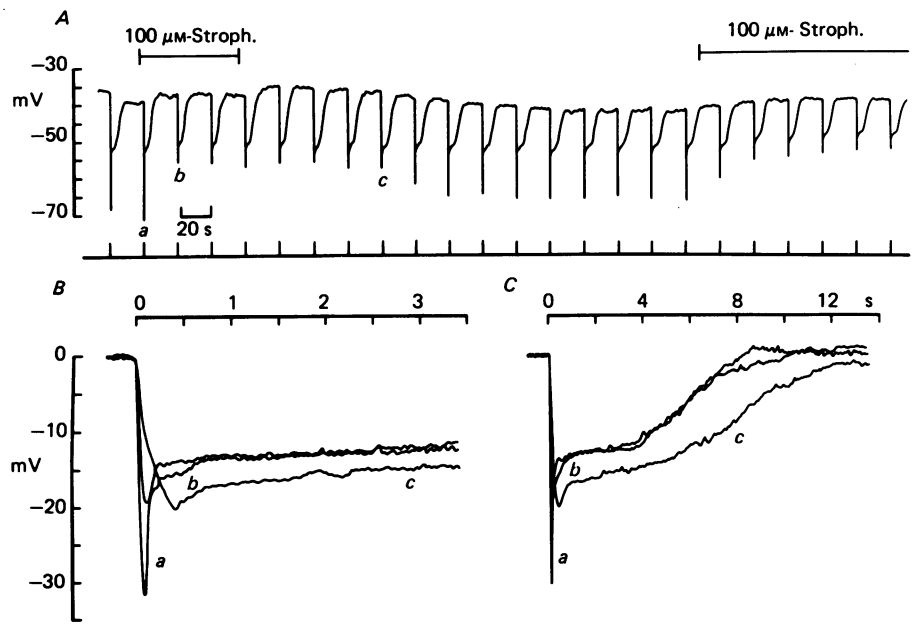

Fig. 9. Effect of 100 μM-strophanthidin on the photoresponse to a bright flash of light. *A*, chart recording of the experiment, upper bars indicate the time of application of 100 μM-strophanthidin, bottom trace is light monitor. *B*, *C*, responses labelled *a*, *b*, *c*, in *A* are shown at two different time scales. Light stimuli as in Fig. 1. Dark membrane potential has been set equal to 0 mV in *B* and *C*.

Effects of strophanthidin on voltage sensitivity and the kinetics of responses to dim flashes of light

Fig. 10 illustrates the effect of 100 μM-strophanthidin on the voltage responses to dim light flashes at different times: in control Ringer solution (*A*), during the first phase of the action of strophanthidin (*B*) and after about 6 min (*C*). During the first phase the flash sensitivity increased by about 30% and the time-to-peak of the voltage response was delayed about 300 ms (Fig. 10*B*). At later times the sensitivity decreased and the time course of the photoresponse became remarkably slow (Fig. 10*C*). The rod recovered its normal sensitivity and kinetics after about 17 min of perfusion with control medium. Subsequently $[K^+]_o$ was increased to 10 mM and the

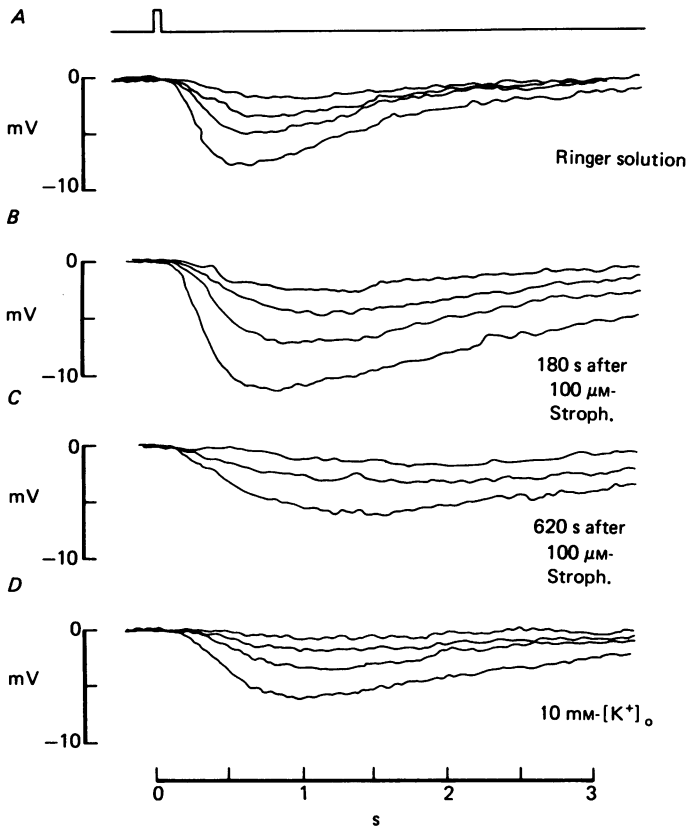

Fig. 10. Sensitivity and kinetics changes in the response to dim flashes of light in the presence of 100 μM-strophanthidin. *A*, responses to dim flashes of light in Ringer (1·3, 2·5, 5·9 and 12·1 Rh*/flash, respectively). *B*, same as in *A* but after 180 s following addition of 100 μM-strophanthidin to the perfusate. *C*, same as in *B*, but after 620 s following addition of 100 μM-strophanthidin to the perfusate (the response to 12·1 Rh*/flash not shown). *D*, responses of the same cell after complete recovery (18 min later) in the presence of 10 mM-K^+. Observe that time courses of responses in *B* and *D* are similar but flash sensitivity in *B* is much higher than in *D*. Data smoothed with a digital filter with $N = 3$ as described in Torre & Owen (1981). Flash duration 50 ms; wave-length 498 nm.

responses shown in Fig. 10*D* were recorded. It is clear that the increase in sensitivity observed immediately after addition of strophanthidin (Fig. 10*B*) cannot be explained by K^+ accumulation around the inner segments since an increase in $[K^+]_o$ causes a decrease in sensitivity (Fig. 10*D*).

Effects of prolonged exposure to strophanthidin

In most of the experiments so far described the retina was exposed to strophanthidin for only a very few minutes. However in three experiments, such as one from which the records of Fig. 10 were obtained, the retina was perfused continuously with a medium containing 100 μM-strophanthidin for 15 min or longer. The major effects of strophanthidin occurred during the first 2 or 3 min, following which only slow further changes were observed; the dark resting potential depolarized on average by about

11 mV and the maximal amplitude of the photoresponse decreased from 12 mV to about 8 mV. This slow trend might be associated with the loss of internal K^+ and the accumulation of internal Na^+, while the initial effect of strophanthidin is likely to be mediated by the rapid abolition of the electrogenic current.

Effect of low $[Na^+]_o$ in the presence of Cs^+

In the previous sections it was shown that the effect of varying $[K^+]_o$ was compatible with a direct contribution of an electrogenic current from the Na^+–K^+ pump to the membrane potential. The activity of the Na^+–K^+ pump is stimulated by external K^+ and by internal Na^+ (Garay & Garrahan, 1973) and in the following sections it will be shown that the amplitude of the slow component is related to the concentration of internal $[Na^+]_i$. Depletion of internal Na^+ was achieved by decreasing $[Na^+]_o$ or increasing $[Ca^{2+}]_o$. An increase in intracellular Na^+ was obtained by removing Ca^{2+} from the bathing medium, which causes an increase in the inward dark Na^+ current (Yau *et al.* 1981).

Fig. 11 illustrates the effect of reducing $[Na^+]_o$ from 132 mM to 80 and 50 mM, in the presence of 10 mM-Cs^+, on the response to a bright flash. The first effect of Na^+ removal was a rapid hyperpolarization of the dark potential followed by a pronounced decrease in the peak of the light response. These effects were more pronounced when $[Na^+]_o$ was reduced to 50 mM. Figs. 11*B* and 11*D* show that the decrease in the size of photoresponse observed after the initial hyperpolarization of the dark potential reflects a suppression of the slow component (see record 7 and 22). When $[Na^+]_o$ was restored to the control level the dark membrane potential quickly recovered, with a transient overshoot, but recovery of the slow component (see Figs. 11*C* and 12*E*) of the light response to its original amplitude took about 2 min. The observation that the amplitude of the slow component is not immediately affected by changes of $[Na^+]_o$, whereas the dark membrane potential is, suggests that the changes in the slow component are due to changes in the intracellular concentration of Na^+.

When $[Na^+]_o$ was reduced to 22 mM the slow component was almost abolished and the total duration of the light response was prolonged by 1 or 2 s. The dependence of the amplitude of the slow component on $[Na^+]_o$, between 22 and 132 mM, was almost linear. If the size of the slow component is related to the activity of the Na^+–K^+ pump and if the activity of the pump is linearly related to $[Na^+]_o$ (see next section), then the above observation suggests that when $[Na^+]_o$ is reduced in the steady state $[Na^+]_i$ decreases by approximately the same factor.

The kinetics of $[Na^+]_i$

In the previous section it was proposed that the effect of a decrease in $[Na^+]_o$ on the amplitude of the slow component was mediated by changes in $[Na^+]_i$. This hypothesis requires that a sizeable loss of internal Na^+ occurs within 1 or 2 min after reducing $[Na^+]_o$. The dark photocurrent $\bar{I}_{Na}$ is about 30 pA (Baylor *et al.* 1979), and the intracellular volume is approximately $1{\cdot}5 \times 10^{-12}$ l (Cervetto *et al.* 1977); therefore internal Na^+ is replaced at a rate of 0·2 mM/s. As a consequence, when external Na^+ is removed changes in the range of a few millimolar may be expected within a minute. These changes may affect the value of E_{Na} if the normal dark value of $[\overline{Na^+}]_i$ is low,

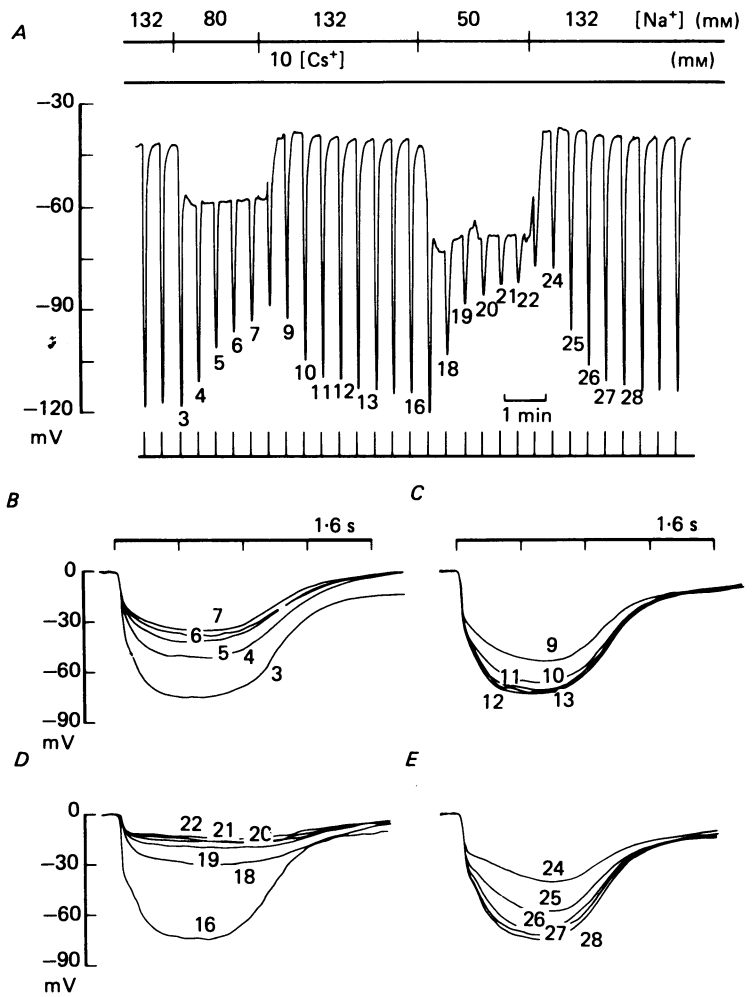

Fig. 11. Effect of low $[Na^+]_o$ on the photoresponse to a bright flash in the presence of 10 mM-external Cs^+. *A*, chart recording of the experiment. Upper bars indicate the time of solution changes; bottom trace is light monitor. Light stimuli as in Fig. 1. *B*, responses recorded when $[Na^+]_o$ was reduced from 132 to 80 mM. Observe that the decrease in the amplitude of the slow component occurred subsequent to the hyperpolarization of the dark membrane potential. *D*, responses recorded when $[Na^+]_o$ was reduced from 132 to 50 mM. *C* and *E*, recovery of the slow component of the light response following return to 132 mM-external Na^+. Numbers by each response refer to the corresponding responses in *A*.

that is about 10–20 mM. Given the small dimensions of a rod, it is extremely difficult to measure $[Na^+]_i$ with a Na^+-sensitive electrode. An alternative method to estimate $[Na^+]_i$ is to analyse the rate of change of internal Na^+ and its reflection on the Na^+–K^+ pump activity.

The rate of change of internal sodium ($[Na^+]_i$) is regulated by the following equation

$$[Na^+]_i = \frac{I_{Na}}{vNq} - J([Na^+]_i) \tag{6}$$

where I_{Na} is the passive Na^+ influx, v the rod volume, N the Avogadro number, q the electron charge and J is the active Na^+ efflux driven by the Na^+–K^+ pump. The results of Fig. 5 and 6 suggest that at the peak of bright-flash responses the Na^+ current is negligible and therefore I_{Na} may be assumed equal to the photocurrent. In first approximation the activity of the Na^+–K^+ pump is assumed to be proportional to $[Na^+]_i$ (Eisner & Lederer, 1980).

$$J([Na^+]_i) \sim \frac{[Na^+]_i}{\theta}, \tag{7}$$

where θ is a constant. Therefore, if at time 0 Na^+ is completely removed from the perfusate, $[Na^+]_i$ will decline exponentially:

$$[Na^+]_i = \overline{[Na^+]}_i e^{-t/\theta}. \tag{8}$$

If θ is known, then

$$\overline{[Na^+]}_i = \frac{\bar{I}_{Na}\theta}{vNq}. \tag{9}$$

Eqn. (9) shows that if the rate of decay of $[Na^+]_i$ in Na^+-free Ringer is known, it is possible to obtain an estimation of $\overline{[Na^+]}_i$.

Fig. 12 shows experiments in which the retina was exposed to a Na^+-free Ringer solution. In *A*, with no caesium present, removal of Na^+ from the perfusate caused the dark membrane potential to hyperpolarize to about −80 mV. When $[Na^+]_o$ was restored the first light responses (see record *a* in *B*) had a much smaller peak than in the control responses (see record *b* in *B*). The shapes of these responses were very similar to those observed immediately after addition of 100 μM-strophanthidin (compare with record *b* in Fig. 9). When the same experiment was repeated in the presence of 10 mM-external Cs^+ the dark membrane potential initially hyperpolarized to about −115 mV, far beyond the level reached in the absence of Cs^+ (compare *A* and *C*) but then it depolarized back to a level of about −80 mV within about 90 s. In the presence of Cs^+ the initial large hyperpolarization of the dark membrane potential and the subsequent partial recovery observed could be caused by the electrogenic activity of the Na^+–K^+ pump and its inactivation following a decrease in $[Na^+]_i$. To test this hypothesis the same experiment was repeated in the presence of 100 μM-strophanthidin (see *C*). Under these conditions when $[Na^+]_o$ was reduced the dark resting potential hyperpolarized to about −80 mV, without the initial hyperpolarizing overshoot.

In the presence of Cs^+ the first light response in 100 μM-strophanthidin (record 26 in *E*) was very similar to the first response observed during recovery from exposure to Na^+-free Ringer (record 21 in *D*). This observation suggests that the previous exposure to a Na^+-free Ringer was sufficient to drastically decrease $[Na^+]_i$ and therefore to block the Na^+–K^+ pump. As already mentioned (see Fig. 7 and 9) after exposure for a few minutes to strophanthidin the kinetics of photoresponses slowed down (see record 36 in *E*). The progressive reappearance of the amplitude of the slow component after exposure to a Na^+-free Ringer and after strophanthidin was very similar (compare *D* and *E*). However the recovery from exposure to strophanthidin was slower (see Fig. 12*C*).

In another series of experiments the effect of different periods (1, 2, 3, 4 and 5 min)

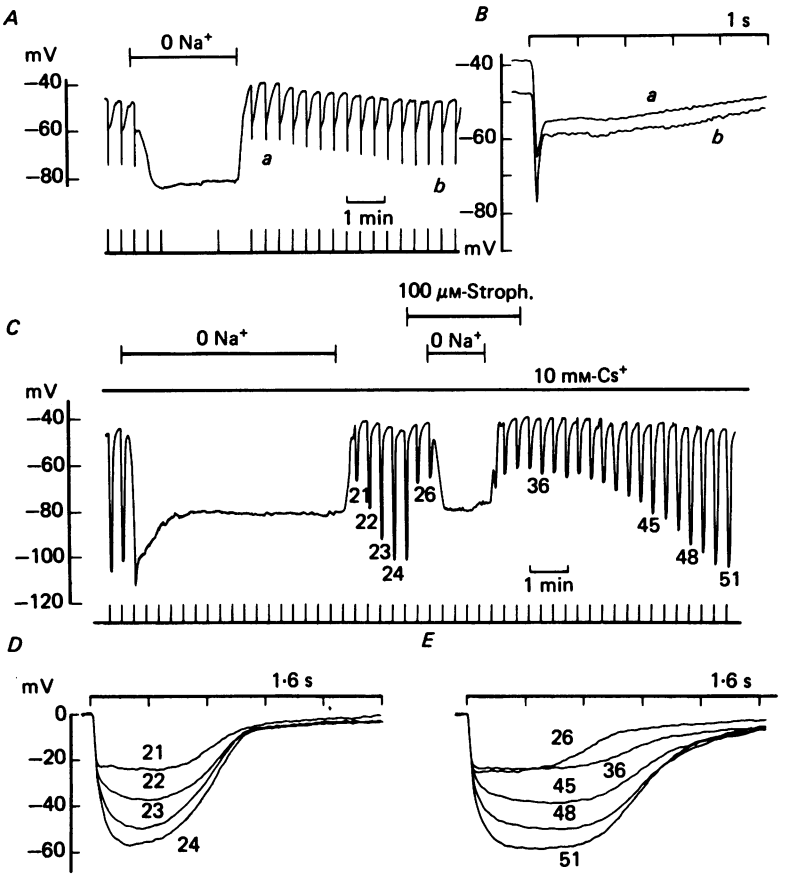

Fig. 12. Effect of Na^+-free Ringer solution on the bright flash response. *A*, exposure to Na^+-free Ringer in absence of external Cs^+. Chart recording of the experiment. Upper bars indicate the time of solution changes. Bottom trace is light monitor. Observe the rebound of the dark membrane potential after the exposure to 0 extracellular Na^+. *B*, responses labelled *a* and *b* in *A* shown in greater detail. *C*, exposure to Na^+-free Ringer in the presence of 10 mM-Cs^+. Upper bars indicate the time of solution changes. Bottom trace is light monitor. *D*, recovery from exposure to Na^+-free Ringer. Responses labelled 21–24 in *C* shown in greater detail. *E*, recovery from exposure to 100 μM-strophanthidin in the presence of 10 mM-Cs^+. Responses labelled 26, 36, 49, 48, 51 in *D* shown in greater detail. Observe that responses 21 and 26 are identical. Light stimuli as in Fig. 1. In *D* and *E* the dark membrane potential has been set equal to 0 mV.

of exposure to a Na^+-free Ringer solution in the presence of 10 mM-Cs^+ were compared. When the retina was exposed to a Na^+-free Ringer for at least 150 s the slow component of the first response to a bright flash of light after returning to the normal $[Na^+]_o$ was completely blocked. In general, blocking of the slow component occurred with $[Na^+]_o$ was restored to its normal value after the dark membrane potential had reached a steady level. This last observation suggests that the relaxation of the dark membrane potential in a Na^+-free Ringer, best observed in the presence of 10 mM-Cs^+, is indeed caused by the inactivation of Na^+–K^+ pump following a fall of $[Na^+]_i$.

Fig. 13 shows the time constant of the recovery of the dark membrane potential

from the initial peak hyperpolarization to a steady state, during exposure to Na^+-free Ringer in the presence of caesium, in four different cells. The time constant was typically about 40 s but in some cells was as fast as 24 s. Assuming that a good estimation of θ in eqn. (9) is indeed this time constant, then from eqn. (9), using a value of 30 pA for $\bar{I}_{Na}$ and $1{\cdot}5\ 10^{-12}$ l for v, a value of $\overline{[Na^+]}_i \sim 8$ mM is obtained. The value of the free intracellular space in a rod is expected to be different from its volume because of the existence of various intracellular structures; therefore the real value of $\overline{[Na^+]}_i$ is expected to be slightly larger than the above estimation.

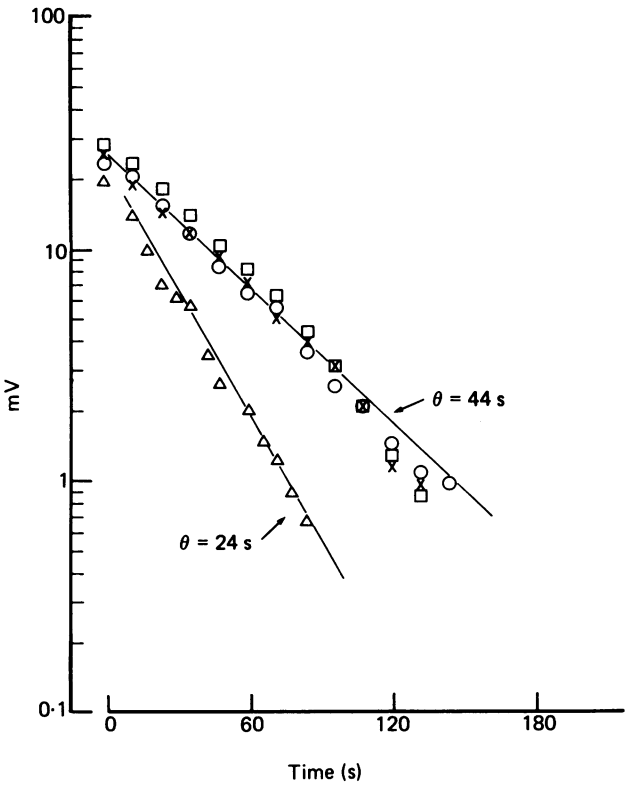

Fig. 13. Recovery of the dark membrane ($V_{ss} - V$) potential after initial hyperpolarization to V in Na^+-free Ringer in the presence of 10 mM-Cs^+. V_{ss} is the steady-state dark membrane potential. Time 0 coincides with the onset of the first light flash that did not evoke a light response. The lines were drawn through the points by eye. Data from four cells.

The above derivation of the value of $\overline{[Na^+]}_i$ depends on the assumption that the pump activity pump is proportional to $[Na^+]_i$. However for small values of $[Na^+]_i$ the pump activity seems dependent on $[Na^+]_i^n$ with $n = 3$ (Garay & Garrahan, 1973). Therefore in a Na^+-free Ringer, the internal Na^+ would decrease following the eqn.

$$[Na^+]_i = -\frac{[Na^+]_i^n}{\theta}. \tag{10}$$

That when $n \geqslant 2$ has the solution

$$[Na^+]_i = \left(\frac{1}{\frac{t}{\theta(n-1)} + \overline{[Na^+]}_i^{1-n}}\right)^{\frac{1}{n-1}}, \tag{11}$$

and the pump activity would fall as:

$$\left(\frac{1}{\frac{t[\overline{Na^+}]_i^{n-1}}{\theta(n-1)}+1}\right)^{\frac{n}{n-1}}. \tag{12}$$

Now let θ be the time at which the pump activity has decreased by $(\frac{1}{2})^{n/n-1}$ then

$$\bar{\theta} = \frac{\theta(n-1)}{[\overline{Na^+}]_i^{n-1}}. \tag{13}$$

From eqns. (11) and (13) is obtained

$$\overline{[Na^+]}_i = \frac{\bar{I}_{Na}}{9Nv}\frac{\theta}{n-1}. \tag{14}$$

Using eqn. (14) with $n = 2$ and $n = 3$, and assuming that the relaxation of the voltage potential shown in Fig. 13 represents the inactivation of the Na^+–K^+ pump, the value of $[Na^+]_i$ is 13 and 7 mM respectively. Therefore the previous estimation of about 8 mM was not critically dependent on the value of n. However the exponential decay shown in Fig. 13 supports a value of n equal to 1 in eqn. (10).

Effect of low $[Ca^{2+}]_o$ in the presence of Cs^+

When $[Ca^+]_o$ is decreased the size of the dark photocurrent increases substantially providing a method of loading the rod with Na^+. If $[Na^+]_i$ increases, the activity of the Na^+–K^+ pump should be increased, and according to the proposed hypothesis the amplitude of the Cs^+-dependent slow component of responses to bright flashes should also increase. Fig. 14*A* illustrates an experiment in which the retina, while in the continued presence of 10 mM-Cs^+, was exposed for two different periods to a nominally Ca^{2+}-free Ringer solution. The concentration of residual Ca^{2+} was about 30 μM. When Ca^{2+} was removed from the perfusate the dark membrane potential depolarized by about 40 mV and the amplitude of the photoresponse increased to about 120 mV. In Fig. 14 *B* responses recorded in 2 mM- and 30 μM-external Ca^{2+} are compared. In low $[Ca^{2+}]_o$ the slow component increased twofold in amplitude and became slightly faster. The first effect can be explained if we assume that an increase of $[Na^+]_i$ stimulates the activity of the pump, while the second effect is likely to be due to an increase of the membrane conductance caused by the Ca^{2+} removal. When the normal level of $[Ca^{2+}]_o$ was restored the dark membrane potential quickly hyperpolarized to a level more negative than the original value and the light response was smaller and slower than before exposure to low Ca^{2+}. After a few minutes both dark potential and light response returned to their control values. These effects were dependent on the duration of the exposure to low $[Ca^{2+}]_o$ as shown in Fig. 14*A*. The process of the recovery of the normal light response is illustrated in Fig. 14*C*.

Similar phenomena were first observed by Yau *et al.* (1981), recording the membrane current with a suction electrode. They explained the transient depression of the light response following return to normal $[Ca^{2+}]_o$ as being caused by the accumulation of internal Na^+ during the exposure to low $[Ca^{2+}]_o$. As the excess of Na^+ is subsequently pumped out the normal response is restored. As a consequence of the Na^+ loading, a larger contribution of the Na^+–K^+ pump to the dark membrane potential may also be expected. It will be shown below that a large fraction of the hyperpolarizing rebound observed upon returning to the normal level of $[Ca^{2+}]_o$ is strophanthidin-sensitive. The amplitude of the slow component increased about twofold when $[Ca^{2+}]_o$

was reduced from 2 mM to 100 μM, but further reduction of $[Ca^{2+}]_o$ did not produce any additional effect. When $[Ca^{2+}]_o$ was increased to 5 mM the amplitude of the slow component decreased by about a third. In experiments similar to that illustrated in Fig. 14, when $[Ca^{2+}]_o$ was reduced up to about 10 μM, the size of the photoresponse to a bright flash reached a steady amplitude of about 115 mV. These responses consisted of an initial component of about 50 mV followed by a slow component of

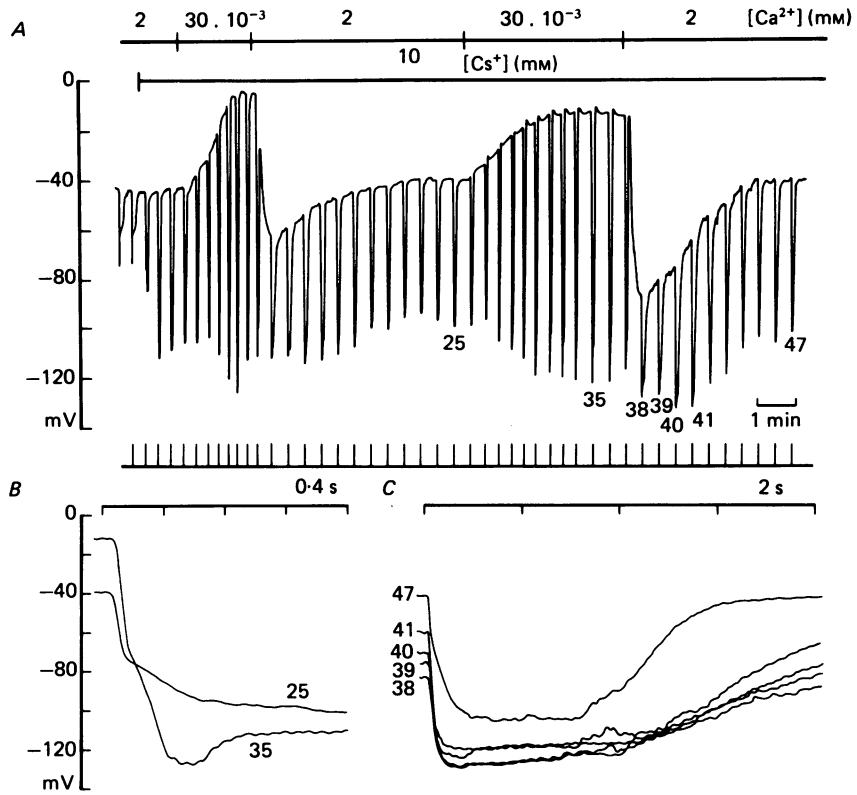

Fig. 14. Effect of low Ca^{2+}-Ringer on the photoresponse to a bright flash in the presence of 10 mM-external Cs^+. *A*, chart recording of the experiment. Upper bars indicate the time of solution changes; bottom trace is the light monitor; light stimuli as in Fig. 1. *B*, effect of low $[Ca^{2+}]_o$ on the amplitude of the slow component. Responses labelled 25 and 35 in *A* are shown in greater detail. *C*, recovery from exposure to low $[Ca^{2+}]_o$. Responses labelled 38, 39, 40, 41 and 47 in *A* are shown in greater detail.

about 65 mV (see record 35 in Fig. 14*B*). According to the hypothesis previously proposed the increase in the size of the slow component is due to an increase in the pumping activity caused by an increase in $[Na^+]_i$. In a previous section $[K^+]_i$ was estimated to be between 65 and 100 mM and $[Na^+]_i$ to be about 10 mM. Therefore a twofold increase in the size of the slow component may be explained by an increase on $[Na^+]_i$ of 10–20 mM. In conditions of osmotic equilibrium $[K^+]_i$ is expected to decrease by the same amount. Therefore the value of E_K is expected to decrease only by a few millivolts. In fact the absolute level of the initial component, which behaves

almost as a K^+ electrode (see Fig. 6), depolarized only by about 7 mV when $[Ca^{2+}]_o$ was decreased to 30 μM (see Fig. 14*B*).

If $[Ca^{2+}]_o$ was reduced to about 10^{-8} M with a Ca^{2+}-EGTA buffer the electrical activity of the rod was profoundly affected. Such an experiment is illustrated in Fig. 15. In this case after the dark membrane potential had depolarized to approximately 0 mV, the maximal amplitude of the light response started to decline and its duration

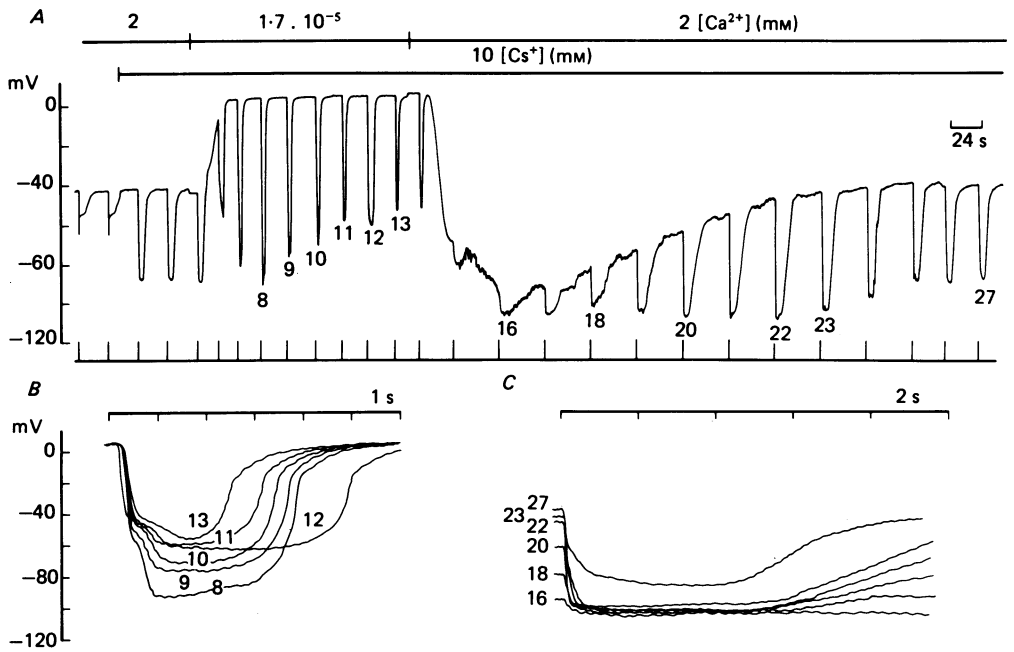

Fig. 15. Effect of very low $[Ca^{2+}]_o$ on the photoresponse to a bright flash of light in the presence of 10 mM-Cs^+. *A*, chart recording of the experiment. Upper bars indicate the time of solution changes. Bottom trace is light monitor. Light stimuli as in Fig. 1 with the exception of response labelled 12, where the light intensity was four times as bright as the other stimuli. *B*, decrease and shortening of responses in the presence of $1{\cdot}7 \times 10^{-8}$ M-Ca^{2+}. Responses labelled 8–13 in *A* are shown in greater detail. *C*, recovery from exposure to $1{\cdot}7 \times 10^{-8}$ M-external Ca^{2+}. Responses labelled 16, 18, 20, 22, 23 and 27 in *A* are shown in greater detail.

became progressively shorter (see record 8, 9, 10, 11, 13, in Fig. 15*B*). Increasing the light intensity by a factor of four prolonged the duration of the photoresponse but did not significantly increase its amplitude (see record 12 in Fig. 15*B*). When $[Ca^{2+}]_o$ was increased back to 2 mM the dark membrane potential transiently hyperpolarized by more than 100 mV and the photoresponse became very small. Recovery of the dark potential and light response to control levels occurred during the following 5 min. The kinetics of the recovery are illustrated in Fig. 15*C*.

The progressive decrease in the amplitude of the photoresponse in $1{\cdot}7 \times 10^{-8}$ M-external Ca^{2+} was associated with a positive shift in the peak of the initial component (record 8–13 Fig. 15*B*). After 3 min of exposure to $1{\cdot}7 \times 10^{-8}$ M-Ca^{2+} the peak of the initial component depolarized by about 35 mV from its usual level in 2 mM-Ca^{2+}.

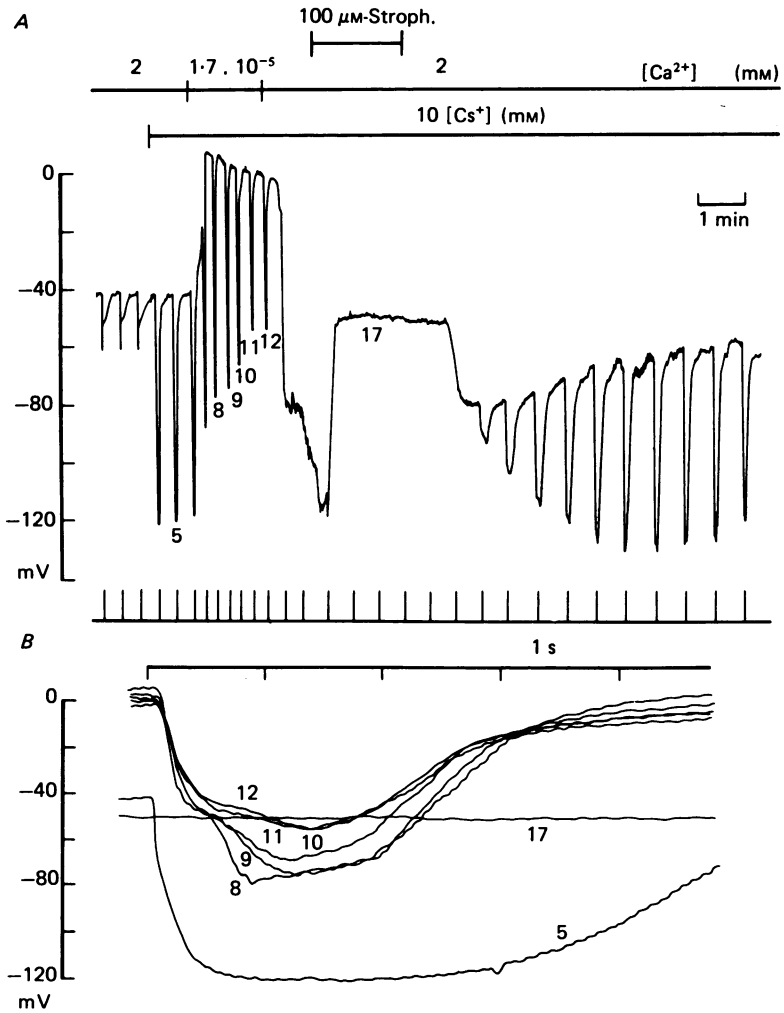

Fig. 16. Effect of strophanthidin on the recovery from exposure to $1{\cdot}7 \times 10^{-8}$ M-Ca^{2+}. *A*, chart recording of the experiment. Upper bars indicate the time of solution changes, bottom trace is light monitor. Light stimuli as in Fig. 1. *B*, effect of 100 μM-strophanthidin on the electrical activity after exposure to $1{\cdot}7 \times 10^{-8}$ M-Ca^{2+}. Responses labelled 5, 8–12, 17 in *A* shown in greater detail. Observe the complete blockage of light response in the presence of strophanthidin at a membrane potential (see record 17) close to the peak of the initial component of responses (see record 8–12) in $1{\cdot}7 \times 10^{-8}$ M-Ca^{2+}.

According to the notion that the absolute level of the initial component is a good indicator of E_K (see page 322) $[K^+]_i$ must have decreased to one fourth of its original value.

Fig. 16*A* illustrates an experiment whose protocol was similar to that shown in Fig. 15. In this experiment, however, after the dark membrane potential had hyperpolarized to about 120 mV following the return from low to normal $[Ca^{2+}]_o$, 100 μM-strophanthidin was added to the perfusate. Within a few seconds the dark membrane potential depolarized by 60 mV and the light response was completely

blocked. After removal of strophanthidin the rod recovered its normal electrical activity. This experiment indicates the existence of a large contribution of the electrogenic current of the Na^+–K^+ pump to the dark membrane potential after the exposure to a Ringer solution with very low external Ca^{2+}. Strophanthidin also stopped the recovery of responsiveness, substantiating the hypothesis of a blockage by internal Na^+ of light-sensitive channels (A. L. Hodgkin, P. A. McNaughton & K. W. Yau in preparation). Fig. 16*B* shows that the membrane potential in the presence of strophanthidin (record 17) is very close to the level of the peak of the initial component in low $[Ca^{2+}]_o$ (compare with record 8–12). Therefore if this membrane potential is taken as an indication of E_K, $[K^+]_i$ is about 30 mM.

DISCUSSION

Depletion and accumulation extracellular K^+

The access of the bulk solution to the narrow extracellular space around the inner segments of the photoreceptors may be impeded by the outer limiting membrane and it is legitimate to consider the possibility of transient depletion and accumulation of K^+ in this space (Oakley *et al.* 1979). In the presence of at least 2 mM-external Cs^+ it is common to observe absolute peak voltage photoresponses between -110 and -135 mV. At the peak of photoresponses to bright flashes of light the Na^+ permeability is very low and if the ratio between P_{Na} and P_K is about 0.023 (see Fig. 6) even with complete removal of $[K^+]_o$ the passive membrane potential could not reach values more negative than -100 mV. To explain the observed hyperpolarization beyond this value it is proposed that the membrane potential is not set entirely by passive ionic fluxes, but receives an additional contribution from the electrogenic current produced by the Na^+–K^+ pump.

The action of external Cs^+

Extracellular Cs^+ at concentrations up to 20 mM does not affect the dark membrane potential of toad rods (see Figs. 1 and 2) and has very small effects on the shape of responses to dim flashes of light (Fain *et al.* 1978; Torre & Owen, 1981). However in the presence of at least 1 mM-external Cs^+ the sag from the peak to the plateau of bright flash responses is completely blocked (see record *C* in Fig. 1), and when $[Cs^+]_o$ is further increased a slow, hyperpolarizing component appears. The amplitude of the slow component increases with $[Cs^+]$ and in the presence of 10–20 mM-external Cs^+ it can be as large as 50 mV. As already suggested these effects can be best explained by a dual action of Cs^+. At concentrations of at least 1 mM-Cs^+ blocks within a few milliseconds the channels through which the current responsible of the sag from the peak to the plateau flows. These channels are likely to be closed at the resting membrane potential, because Cs^+ does not affect the electrical activity of the rod at this membrane potential. Following the membrane hyperpolarization caused by a bright flash of light these channels open up and can be blocked by Cs^+.

A second action of Cs^+ was also proposed. This action is a time-dependent blockage of some additional channels, whose permeability ratio between Na^+ and K^+ is about 0·023. These channels are likely to be K^+ channels; this action of Cs^+ would be similar to that in other preparations (Hagiwara, Miyazaki & Rosenthal, 1976; Bezanilla &

Armstrong, 1972). This blockage may be voltage-dependent and may be also influenced by $[K^+]_o$ (Ciani, Krasne & Hagiwara, 1980; Coronado & Miller, 1979), because the slow component disappears when $[K^+]_o$ is increased above 10 mM. It is proposed that a constant electrogenic current associated with the Na^+–K^+ pump becomes evident during a time dependent blockage by Cs^+ of some K^+-channels, and that this is the origin of the slow component present in responses to bright flashes of light.

The electrogenic current

In the previous sections experimental evidence in favour of the existence of a sizeable electrogenic current associated with the Na^+–K^+ pump have been presented. The Cs^+-dependent slow component of the bright-flash response was quickly blocked by strophanthidin with a K_m of about 3 μm, and was also decreased when K^+ was removed from the perfusate. Moreover, the amplitude of the slow component increased when the rod was loaded with Na^+ and decreased when internal Na^+ was depleted. All these observations strongly suggest a direct link between the slow component of the photoresponse and the activity of the Na^+–K^+ pump. Assuming that the dark membrane depolarization observed after addition of strophanthidin to the perfusate was entirely due to the blockage of this current, it is possible to estimate its magnitude. From Thomas (1972) the contribution of the Na^+–K^+ pump to the membrane potential ΔV_p in the steady state is given by (Mullins & Noda, 1963)

$$\Delta V_p = \frac{RT}{F} \ln \frac{1}{r} \frac{r[K^+]_o + \frac{P_{Na}}{P_K}[Na^+]_o}{[K^+]_o + \frac{P_{Na}}{P_K}[Na^+]_o}. \tag{15}$$

Where r is the number of Na^+ pumped out per K^+ pumped in. Since in darkness $P_{Na} \sim P_K$ (Capovilla *et al.* 1980, Capovilla *et al.* 1981) eqn. (15) becomes

$$\Delta V_p \sim \frac{RT}{F} \ln \frac{1}{r}. \tag{16}$$

If the average dark membrane depolarization caused by strophanthidin is 5 mV, a value of 1·2 is obtained for r. That is, 6 Na^+ are exchanged for 5 K^+. The effect of strophanthidin on the bright flash responses suggests that the electrogenic current contributes about 15 mV to the peak of the voltage response to a bright flash of light.

The intracellular levels of K^+ and Na^+

The data shown in Fig. 6 show that at the peak of the initial component of the bright flash response in the presence of Cs^+ the membrane behaves as a K^+-electrode with a P_{Na}/P_K ratio of 0·023 with a value of 100 mM for $[K^+]_i$. When the electrogenic current of the Na^+–K^+ pump is considered, the data of Fig. 6 indicate a slightly lower value for $[K^+]_i$ of about 65 mM. However this estimation was based on the hypothesis that only K^+ and Na^+ currents contribute to the determination of the membrane potential at that time. If additional current for instance carried by Cl^-, Ca^{2+} and Mg^{2+} are present, the value of $[K^+]_i$ is expected to increase. Therefore a good estimation for $[K^+]_i$ is between 70 and 105 mM.

The experiments described in Fig. 11 indicate that when $[Na^+]_o$ is reduced a substantial decrease of $[Na^+]_i$ may occur within 2 or 3 min. When $[Ca^{2+}]_o$ was decreased to about 10^{-5} M, the amplitude of Cs^+-sensitive component increased by a factor of two; if this increase was indeed due to a stimulation of the activity of the Na^+–K^+ pump, the level of $[Na^+]_i$ may be expected to have increased almost proportionally. When $[Ca^{2+}]_o$ was further reduced the results shown in Figs. 15 and 16 suggest a dramatic exchange of K^+_i for Na^+_o.

The experiments reported in Fig. 12 indicate that exposure of the retina to a Na^+-free Ringer for at least 150 s is likely to cause a substantial loss of internal Na^+. On the basis of this observation and from the arguments outlined on page 331 the value of $[Na^+]_i$ was estimated to be between 8 and 16 mM; these values of $[Na^+]_i$ give an equilibrium potential for Na^+ in the range of +50 mV, whereas the reversal potential of light-sensitive current is around +5 mV (Bader, MacLeish & Schwartz, 1979). Since the photocurrent in physiological condition is essentially carried by Na^+ (Capovilla *et al.* 1981, Yau *et al.* 1981), there is an apparent contradiction.

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