Net Charge Transport during Sodium-dependent Calcium Extrusion in Isolated Salamander Rod Outer Segments

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ABSTRACT The light-sensitive current and the current associated with the extrusion of internal Ca^{2+} in exchange for external Na^+ have been recorded from detached rod outer segments from the salamander retina by the use of the whole-cell voltage clamp technique. No significant current-carrying mechanisms are present in the outer segment membrane apart from the light-sensitive conductance and the Na:Ca,K exchange, and exchange currents can therefore be recorded directly without the use of subtraction procedures or pharmacological blockers. The charge moved by the exchange was studied by loading outer segments with a known amount of calcium and then recording the exchange current on return to a Na^+-containing solution. Calcium is not sequestered to any significant extent in a slowly exchanging internal store, as the charge recovered is unaffected if admission of the Na^+-containing solution is delayed for 40 s. The number of charges flowing into the cell in exchange for each Ca^{2+} ion extruded was found not to deviate significantly from one over a wide range of ionic conditions and membrane potentials. These results show that the stoichiometry of the exchange is fixed over a wide range of conditions, and that the size of the inward exchange current is therefore directly proportional to the rate of Ca^{2+} efflux through the carrier.

INTRODUCTION

Calcium enters the outer segment of rod photoreceptors through the light-sensitive channel (Hodgkin, McNaughton, and Nunn, 1985), and is expelled by an electrogenic exchange mechanism in which the efflux of calcium is linked to an influx of sodium (Yau and Nakatani, 1984; Hodgkin, McNaughton, and Nunn, 1985, 1987; McNaughton, Cervetto, and Nunn, 1986; Schnetkamp, 1986; Lagnado, Cervetto, and McNaughton, 1988). This mechanism is usually termed the Na:Ca exchange, although it has recently been demonstrated that K^+ is cotransported with Ca^{2+} in rods, so that the energy required for Ca^{2+} efflux is provided by both the Na^+ gradient and the K^+ gradient (Cervetto, Lagnado, Perry, Robinson, and McNaughton, 1989). In common with the exchange in cardiac muscle (Kimura, Miyamae, and Noma, 1987; Lipp and Pott, 1988; Bridge, Smolley, and Spitzer, 1990), the carrier generates

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an inward membrane current while extruding Ca^{2+}, and this property can be exploited to study the exchange using electrophysiological methods (Lagnado and McNaughton, 1990).

This paper describes experiments in which isolated rod outer segments under whole-cell voltage clamp have been used to investigate the average net charge transported by the Na:Ca,K exchange during a cycle in which a single Ca^{2+} ion is extruded. Previous measurements using suction pipettes to record the membrane current have found some variability in the apparent stoichiometry, with between 0.94 and 0.74 charges flowing into the outer segment in exchange for every Ca^{2+} ion extruded (Yau and Nakatani, 1984; Hodgkin et al., 1987). These observations raise the possibility that the stoichiometry of the exchange may vary depending on the conditions in which it is measured. An explicit test of this possibility is important both for understanding the mode of operation of this carrier, and for the design and interpretation of experiments that use the exchange current as a measure of the rate of Ca^{2+} transport. We have carried out measurements of the net charge transported by the exchange over a range of membrane potentials and external concentrations of Na^+, Ca^{2+}, and K^+. Although these various conditions caused the saturated exchange current to vary over a 40-fold range, there was no significant deviation from a coupling ratio of one charge entering per calcium extruded. The size of the exchange current can therefore be taken as a direct measure of the rate of Ca^{2+} extrusion over a range of conditions.

Preliminary reports of this work have been presented (Lagnado and McNaughton, 1987, 1988).

METHODS

Preparation

For each experiment a larval tiger salamander (*Ambystoma tigrinum*) was dark adapted for at least 1 h, decapitated, and pithed. The procedure for the mechanical dissociation of single rods from the retina (described by Hodgkin, McNaughton, Nunn, and Yau, 1984) also yields a large number of detached rod outer segments lacking the inner segment and the cell nucleus. The morphology of these outer segments is similar to that of outer segments of intact, functional rods. When the interior of these isolated outer segments is perfused with an appropriate internal solution through a whole-cell pipette the *I-V* relation of the light-sensitive current and the kinetics and sensitivity of the response to light flashes are similar to those of intact rods (Sather and Detwiler, 1987).

Electrical Recording

Whole-cell pipettes were drawn from 100 μl borosilicate measuring capillaries (Farmalitalia Carlo Erba, Milan, Italy) pulled in two stages on a horizontal puller (BB-CH; Mecanex, Geneva, Switzerland). The internal diameter of the tip was typically 0.7–1.0 μm and the "bubble number" measured in methanol was 5.0–5.8 (Corey and Stevens, 1983). The pipette resistance when filled with intracellular solution was 3–10 MΩ. Fire polishing of the tip was not found necessary, nor were the shanks treated to reduce stray capacitance since only relatively slowly changing currents were recorded. Whole-cell recordings were made using a patch-clamp amplifier (model 8900; Dagan Corp., Minneapolis, MN) with a 10-GΩ feedback resistor in the headstage, and run at 1 kHz bandwidth. Seal resistances were between 1 and 50 GΩ, and the

access resistance (which was not compensated) was typically 10–40 MΩ. The voltage drop across a resistance of 40 MΩ will be <4 mV with even the largest exchange currents recorded in the present study (for example, see Fig. 5). Data were stored on magnetic tape and digitized for subsequent analysis using a PDP 11-73 minicomputer. Digital smoothing was performed in some cases by convolution with a Gaussian filter; the SD of the filter is given where appropriate in figure legends.

For mechanical stability the isolated outer segment was held end-on against the mouth of a suction pipette (internal tip diameter typically 7–9 μm). The suction pipette was filled with a Ringer solution containing 110 mM Li^+ and nominally zero Ca^{2+} (with no added EGTA) to prevent activation of the Na:Ca,K exchange, in either the forward or the backwards mode, in the area of membrane within the suction pipette mouth.

Solutions

Solutions bathing the outer segment were changed using the multi-pipe system described by Hodgkin et al. (1985), except that the pneumatic actuator was replaced by a stepping motor designed by the late Dr. Brian Nunn (see Hodgkin et al., 1987). Solution changes were typically complete in 50 ms.

The composition of normal Ringer was (in mM): 110 NaCl, 2.5 KCl, 1.6 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES, neutralized to pH 7.6 with tetramethylammonium hydroxide (6 mM). For Ca^{2+}-free or divalent-free solutions the $CaCl_2$ and $MgCl_2$ were omitted and 1 mM EGTA or 1 mM EDTA was added as appropriate. Low-Na^+ solutions were made by replacement of NaCl with LiCl, which does not activate the exchange (Hodgkin and Nunn, 1987). The high-Na^+ solution used to cause close-to-maximal activation of the exchange (see Fig. 5) contained 220 mM NaCl and no other cations; this solution was hypertonic and caused the outer segment to shrink noticeably, although the effects were fully reversible and caused no appreciable change in the light response recorded after returning to normal Ringer. This level of hypertonicity did not appear to affect the properties of the Na:Ca,K exchange because in a control experiment identical activation of the exchange was observed in an isotonic solution containing 110 mM NaCl and in a hypertonic solution containing 110 mM NaCl plus 110 mM LiCl.

The isotonic $CaCl_2$ solution used for introducing a Ca^{2+} load into the outer segment contained (in mM): 77.5 $CaCl_2$, 0.5 3-isobutyl-1-methylxanthine (IBMX), and 10 HEPES, pH 7.6. The phosphodiesterase inhibitor IBMX was included to keep open the light-sensitive channels, which would otherwise quickly close when the substantial rise in intracellular calcium inhibits the guanylate cyclase (Lolley and Racz, 1982; Hodgkin et al., 1985; Koch and Stryer, 1988).

The filling solution for the whole-cell pipette was Na^+-free, containing (in mM): 110 K-aspartate, 3 $MgCl_2$, 1 K_2ATP, 1 Li_2GTP, 0.06 3′-5′-cyclic GMP (cGMP), 0.02 bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate (BAPTA), and 10 PIPES, neutralized to pH 7.2 with KOH. In some experiments the cGMP was omitted, which reduced the size of the light-sensitive current but otherwise had no effect on the results reported. In later experiments the 20 μM of the calcium buffer BAPTA was also omitted, with no significant effect on the exchange currents.

RESULTS

Electrical Characteristics of Isolated Outer Segments

An example of a typical whole-cell recording from an isolated rod outer segment is shown in Fig. 1 *A*. Immediately after obtaining access to the cell interior the membrane current recorded under voltage clamp was zero, but an inward light-

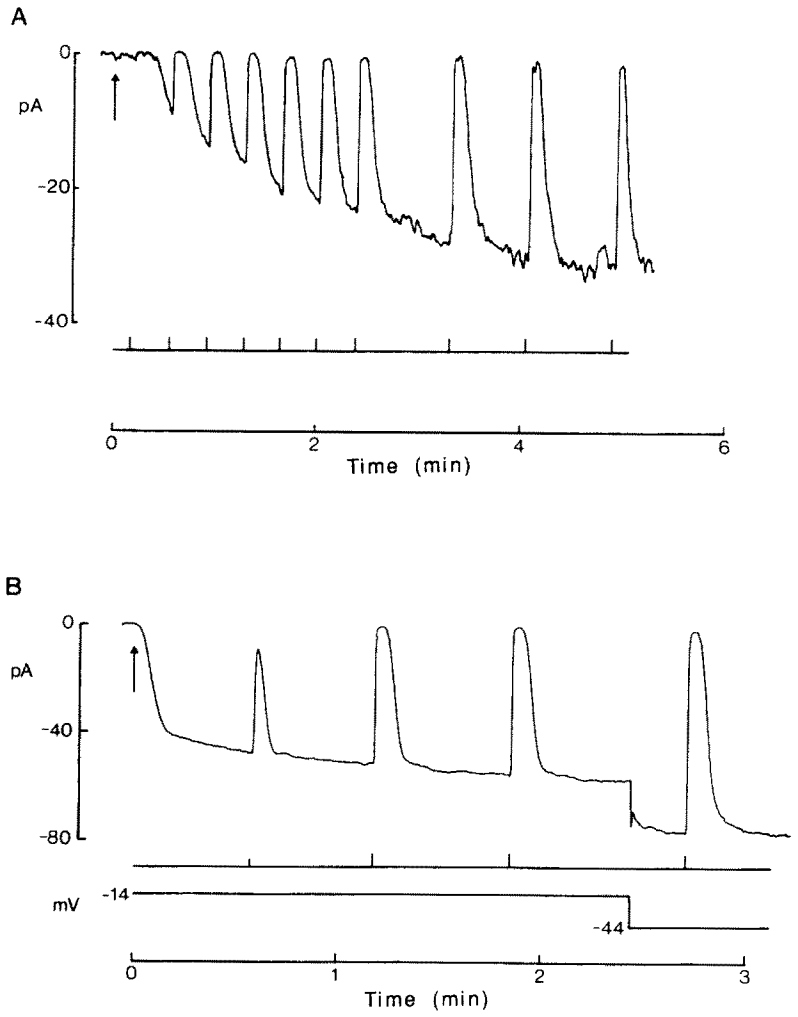


FIGURE 1. Development of the light-sensitive current in an isolated outer segment. (*A*) No cGMP in the patch solution. The light-sensitive current is zero on obtaining a whole-cell recording (marked by the arrow), and activates with a marked delay. The light monitor below the trace shows the timing of flashes delivering 340 photons μm^{-2}. V_m = −44 mV throughout. (*B*) In the additional presence of 60 μM cGMP in the patch solution the development of the light-sensitive current is accelerated (note the shorter time-scale) and the final level reached is greater. The first (subsaturating) flash delivered 197 photons μm^{-2}, and the subsequent flashes delivered 856 photons μm^{-2}. The holding potential is shown below the current trace. Traces in both *A* and *B* were smoothed by convolution with a Gaussian filter of SD = 200 ms.

sensitive current gradually developed and increased to normal levels. The initial lack of any light-sensitive current in the isolated outer segment suggests that it is depleted of cGMP, the internal transmitter that opens light-sensitive channels (Fesenko, Kolesnikov, and Lyubarski, 1985). The activation of a light-sensitive current in the

absence of cGMP in the patch pipette shows that the outer segment possesses an endogenous cyclase to convert GTP to cGMP (see also Sather and Detwiler, 1987).

Fig. 1 *B* shows that the presence of 60 μM cGMP in the patch solution accelerates the rate at which the light-sensitive current develops in an isolated outer segment (note the shorter time-scale relative to that in Fig. 1 *A*). In this example the time to half-activation of the light-sensitive current ($t_{1/2}$) was ~6.5 s, whereas in Fig. 1 *A*, in the absence of exogenous cGMP, it was ~85 s. In 35 experiments in which 60 μM cGMP was present in the patch pipette, $t_{1/2}$ was 11.5 ± 1.5 s, and in 26 experiments in which cGMP was absent, $t_{1/2}$ was 51.2 ± 6.0 sec. There was no systematic difference in the pipette access resistance in these two conditions, so it appears that the rate at which cGMP diffuses into the outer segment is considerably greater than the rate at which it can be synthesized by the endogenous guanylate cyclase. This may be because the outer segment is initially loaded with Ca^{2+}, which is known to inhibit the cyclase (Lolley and Racz, 1982; Koch and Stryer, 1988).

The mean amplitude of the light-sensitive current with 1 mM ATP and 1 mM GTP in the patch solution was 35 ± 3 pA (mean ± SEM, n = 18) in the 5–15-min period after obtaining a whole-cell recording with the membrane clamped at −44 mV. In the additional presence of 60 μM cGMP the current at −44 mV was 88 ± 10 pA (n = 10). This concentration of cGMP was included in the patch solution in the majority of the experiments to be described, because as well as increasing the light-sensitive current and therefore facilitating the Ca^{2+} loading of the outer segment, exogenous cGMP appeared to prolong cell survival and to counteract the gradual decrease in the light-sensitive current normally observed during a whole-cell recording.

Fig. 1 shows that there is negligible current flow at the plateau of a saturating light response, reflecting the high input resistance of the isolated outer segment when the light-sensitive channels are shut (Baylor and Lamb, 1982; Baylor and Nunn, 1986). In keeping with this idea, a 30-mV hyperpolarization (Fig. 1 *B*) caused a negligible shift in the current measured at the plateau of the light response. The apparent input resistances in the light of the outer segments shown in Fig. 1, *A* and *B*, were typical, being 10 and 12 GΩ, respectively. It is likely that the input resistance of the outer segment in bright light is considerably higher than these figures and that the major contribution to the apparent input resistance results from the imperfect seal between the membrane and the glass of the patch pipette. An upper limit to the light-insensitive conductance of the outer segment can be obtained from the highest input resistance measured in bright light, which was 45 GΩ, corresponding to a conductance of 22 pS, or less than the conductance of many single channels. This estimate is similar to that obtained by Baylor and Nunn (1986), and supports the idea that the light-sensitive channel is the only ionic channel open in the outer segment membrane under normal conditions.

Isolation of the Na:Ca,K Exchange Current

The method used to introduce a known Ca^{2+} load into the outer segment and measure the Na:Ca,K exchange current is similar to that adopted by Hodgkin et al. (1987) (Fig. 2 *A*). The outer segment was loaded with Ca^{2+} by exposure to an isotonic $CaCl_2$ solution containing 0.5 mM IBMX to hold the light-sensitive channels open. A bright light terminated the Ca^{2+} influx, and the outer segment was then transferred

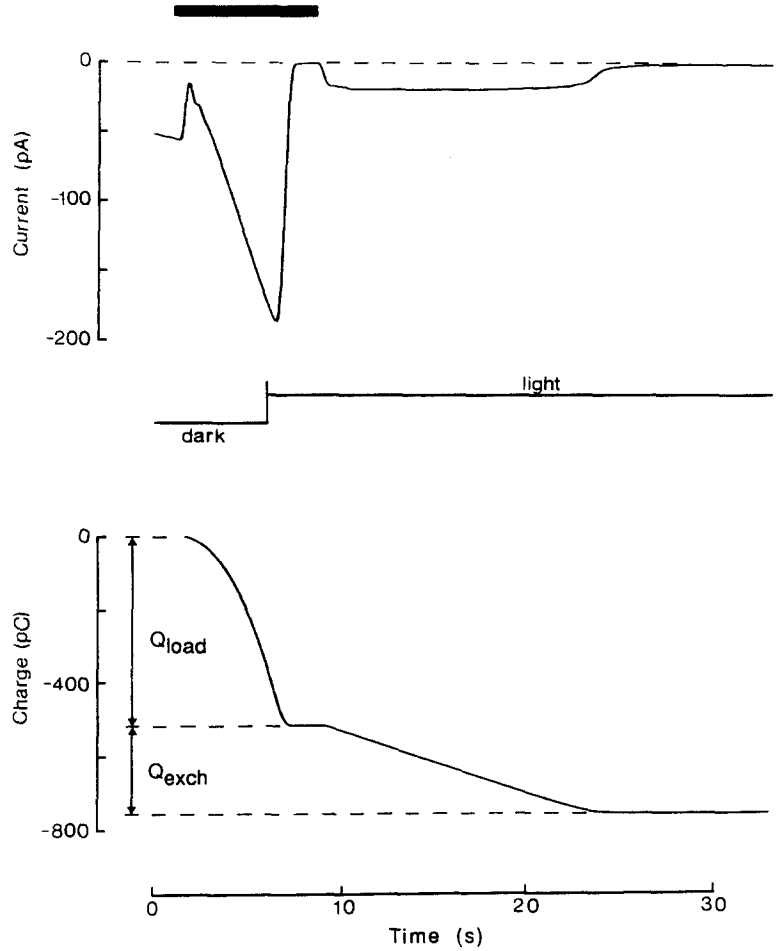


FIGURE 2. The method used to estimate the net charge movement through the Na:Ca,K exchange. The upper panel shows the membrane current, with light monitor trace below. The light-sensitive current in normal Ringer was ~55 pA. The timing of the exposure to 77.5 mM $CaCl_2$ and 0.5 mM IBMX is shown by the black bar. After this exposure, the rod was immediately returned to normal Ringer to activate Ca^{2+} efflux through the exchange. The lower panel shows the integral of the membrane current, calculated relative to the zero-current level (dashed line in the upper panel). V_m = −14 mV throughout. The light flash delivered 1,900 photons μm^{-2} and the continuous light step delivered 613 photons $\mu m^{-2} s^{-1}$. 0 BAPTA in patch solution. Further details in text.

to a normal Ringer solution, activating an inward current caused by the electrogenic exchange of external Na^+ for internal Ca^{2+} (Yau and Nakatani, 1984; Hodgkin et al., 1987; Lagnado et al., 1988; reviewed by Lagnado and McNaughton, 1989). The exchange current remained saturated at −19 pA for ~9 s in this experiment, and then declined to zero as the Ca^{2+} load was removed. Note that the membrane current in bright light in both isotonic calcium solution and Ringer are equal to zero; because

of the high resistance of the seal between the whole-cell pipette and the outer segment, no subtraction procedure is necessary to remove junction currents caused by the different mobilities of the ions in the two solutions.

The mean value of the saturated exchange current was 12.5 ± 1.3 pA in normal Ringer solution at a membrane potential of −14 mV (Table I), which is close to the value of 20 pA measured in intact rods with a suction pipette (Hodgkin et al., 1987). Part of the difference can be explained by the dependence of exchange current on membrane potential, which would have been about −55 mV for an intact rod in bright light (Baylor and Nunn, 1986).

Estimating the Net Charge Movement

The net charge moved by the Na:Ca,K exchange during the extrusion of a single Ca^{2+} ion, q', was obtained as shown in Fig. 2 *B*, using the assumption that all of a Ca^{2+} load introduced into the outer segment is removed by the activity of the Na:Ca,K exchange (see below). The amount of Ca^{2+} introduced into the outer segment during the exposure to isotonic $CaCl_2$ was calculated by integrating the

TABLE I

Mean Values of the Saturated Na:Ca,K Exchange Current, j_{sat}, in 110 mM Na^+ at a Membrane Potential of −14 mV

$[Ca^{2+}]$	$[Mg^{2+}]$	$[K^+]$	j_{sat}	n
mM	*mM*	*mM*	*pA*	
1	1.6	2.5	12.5 ± 1.3	23
0.001	1.6	2.5	21.6 ± 4.9	3
0	1.6	2.5	29.8 ± 2.3	9
0	0	0	60.9 ± 6.6	7

The external ionic conditions are shown in the first three columns. Calculated values are mean ± SEM. n refers to the number of different outer segments in which each estimate was made.

light-sensitive current. The total charge (Q_{load}) in this exposure was 512 pC, equivalent to 1.6×10^9 Ca^{2+} ions in a cytoplasmic volume of 1 pl. The total charge entering the outer segment during the activation of the exchange (Q_{exch}), measured by integrating the exchange current, was 245 pC. The exchange must remove both the resting Ca^{2+} load present in the outer segment in darkness at the start of the experiment and the incremental load introduced by exposure to isotonic $CaCl_2$. The component of Q_{exch} attributable to extrusion of the resting Ca^{2+} load can be calculated from the integral of the exchange current observed after delivering a saturating flash in Ringer (see, for instance, Fig. 3 of Hodgkin et al., 1987) and in this example was 2 pC; the corrected value of Q_{exch}, attributable to the charge transferred due to the removal of the Ca^{2+} load, was therefore 243 pC. The charge moved per calcium ion extruded, q', is equal to $2Q_{exch}/Q_{load}$, and is therefore 0.95 in this experiment. The mean value of q' under these conditions (Ringer at −14 mV) was 0.98 ± 0.03 (see Table II).

Assumptions of the Method Used for Measuring Exchange Stoichiometry

The validity of the method used to estimate q' depends on several assumptions (see below), one of the most important being that all of the Ca^{2+} load is removed by the Na:Ca,K exchange. The possibility of Ca^{2+} sequestration within the outer segment, or of Ca^{2+} removal by a Na^+-independent extrusion process, was tested by the type of experiment shown in Fig. 3. A Ca^{2+} load was introduced into an outer segment using a protocol similar to that in Fig. 2, but after shutting off the Ca^{2+} influx the outer segment was transferred to a solution containing 110 mM Li^+, 0 Ca^{2+}. The extrusion of the Ca^{2+} load through the Na:Ca,K exchange was then activated by returning to normal Ringer solution. The value of q' obtained in this experiment was 1.04, which is not significantly different from the value of unity found when the load was extruded immediately (see Table II). We conclude that the calcium load remains within the outer segment in the absence of external Na^+, and is neither sequestered into an inaccessible store nor extruded from the outer segment by a Na_o-independent mechanism, nor does calcium diffuse to any appreciable extent into the whole-cell pipette. Similar results were obtained in three other cells, in which the exchange was disabled for 20–40 s after the introduction of the Ca^{2+} load, and no difference was observed when using isotonic $CaCl_2$ rather than isotonic $LiCl_2$ to disable Ca^{2+} efflux through the exchange. We conclude that in the conditions of our experiments the time constant of Ca^{2+} loss through mechanisms other than the Na:Ca,K exchange is >100 s.

The method of estimating the net charge movement through the Na:Ca,K exchange depends in addition on the following assumptions:

The current in isotonic $CaCl_2$ is carried only by Ca^{2+} ions. The principal cation in the patch pipette solution is K^+, which has been shown to pass through the light-sensitive channel (Capovilla, Cervetto, and Torre, 1983; Hodgkin et al., 1985). The outward K^+ flux is, however, likely to be negligible because the light-sensitive channel appears to behave as a single-file pore with a permeability to Ca^{2+} at least 25 times that for monovalent cations (Hodgkin et al., 1985; Zimmerman and Baylor, 1986; Menini, Rispoli, and Torre, 1988).

There is a negligible Ca^{2+} influx other than through light-sensitive channels. In some preparations with a poor seal between the whole-cell pipette and the membrane values of q' greatly in excess of one charge exchanging for one Ca^{2+} were observed. Typically in such preparations a net charge transfer of close to one charge per Ca^{2+} extruded was observed at the start of the experiment when the seal resistance was high, and as the seal deteriorated to 1 GΩ or less the apparent stoichiometry changed in a direction consistent with an increasing electrically silent entry of Ca^{2+} (presumably as $CaCl_2$) during the exposure to isotonic calcium solution. In contrast, higher seal resistances (3–45 GΩ) gave consistent values of the exchange stoichiometry, and so to give a margin of safety a lower limit of 5 GΩ was set on the acceptable seal resistance. About 50 measurements made when the seal resistance was below 5 GΩ were rejected.

There is no Ca^{2+} efflux during the loading period. The absence of Na^+ in the external solution will prevent any Ca^{2+} efflux through forward Na:Ca,K exchange. The possibility of Ca^{2+} efflux through an ATP-driven pump cannot be discounted, but will make a negligible contribution in the conditions of the present experiments,

as it is estimated that the maximum Ca^{2+} current through such a pump is <0.4 pA (McNaughton et al., 1986), so that the maximum efflux during a typical 5-s exposure is 2 pC compared with a typical load of 300–400 pC. Further evidence against a significant Na^+-independent transport across the outer segment membrane is presented above (Fig. 3).

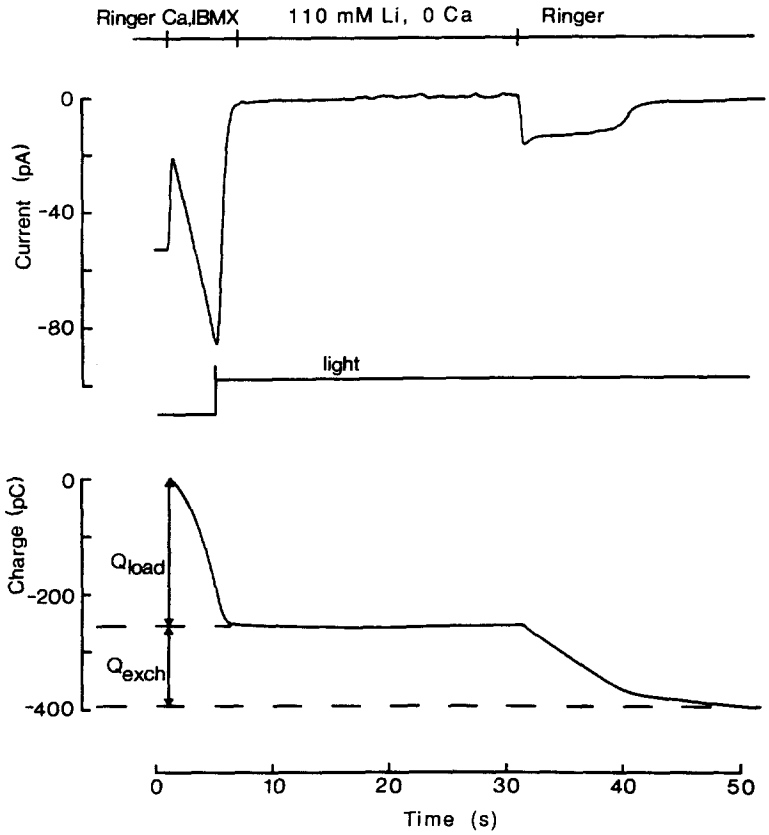
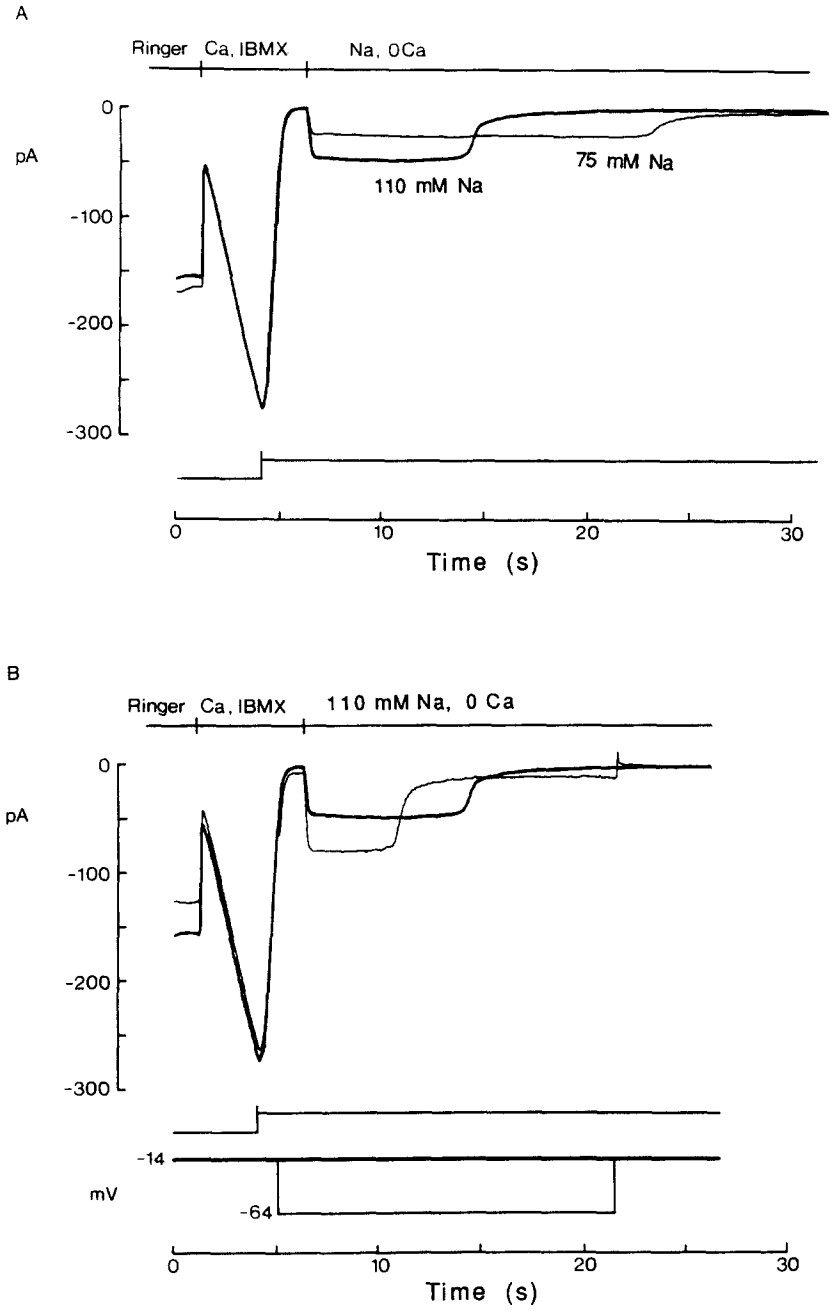


FIGURE 3. The effect of blocking the Na:Ca,K exchange on a Ca^{2+} load in the outer segment. The upper panel shows membrane current and the lower panel its integral. The outer segment was exposed to isotonic $CaCl_2$, 0.5 mM IBMX (1–7 s) and to 110 mM Li^+, 0 Ca^{2+} (7–31 s), and was then returned to normal Ringer. $V_m = -14$ mV throughout. Light flash (2,600 photons μm^{-2}) and continuous light step (613 photons μm^{-2} s^{-1}) switched on at 5 s. $Q_{load} = 262$ pC; $Q_{exch} = 137$ pC; $q' = 1.04$. The saturated exchange current in Ringer was 13 pA. The small relaxation at the beginning of the exchange current may be an artifact caused by the solution change, and was not observed in other experiments. 0 BAPTA in patch solution. Further details in text.

There is negligible Ca^{2+} influx through reversed Na:Ca,K exchange during loading. The strongest evidence against this possibility is provided by the demonstration that Ca^{2+} influx through the exchange depends absolutely on the presence of external K^+ (Cervetto et al., 1989). K^+ was not included in the isotonic $CaCl_2$ solution, thus blocking reversal of the exchange.


A
Ringer
Ca, IBMX
Na, 0Ca
0
pA
-100
-200
-300
110 mM Na
75 mM Na
0
10
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30
Time (s)
B
Ringer
Ca, IBMX
110 mM Na, 0 Ca
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pA
-100
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Time (s)

FIGURE 4.

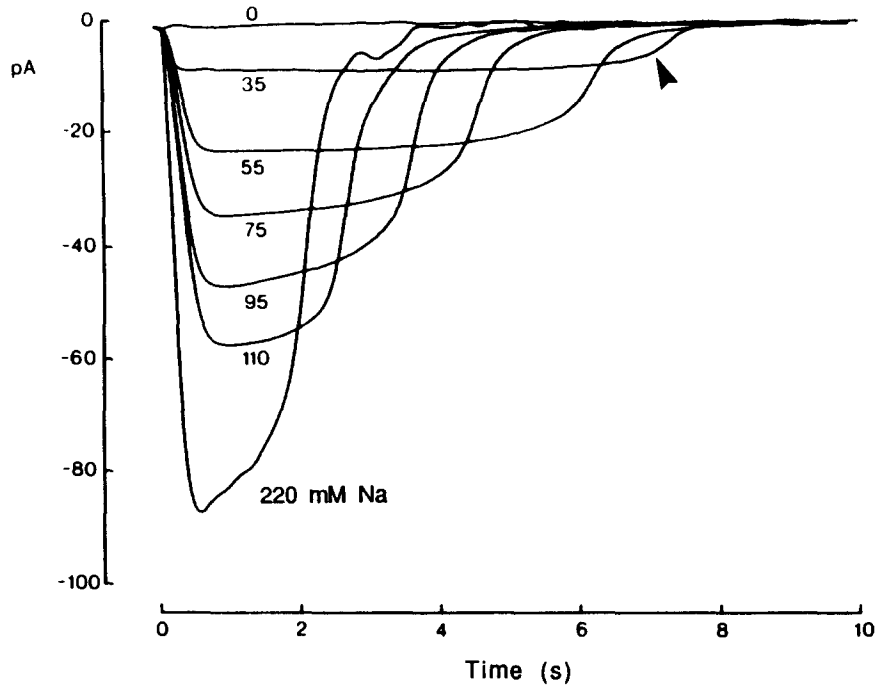

FIGURE 5. The effects of varying the external [Na^+] on the exchange currents at −14 mV. Exchange currents at the external [Na^+] indicated by each trace, recorded in the absence of external Ca^{2+}, Mg^{2+}, and K^+. All records are from the same outer segment. The record in 35 mM Na^+, indicated by the arrowhead, has been expanded by a factor of 0.5 to show the removal of the whole Ca load: the time-scale for this trace is therefore twice that of the others. Records smoothed by convolution with a Gaussian filter of SD = 100 ms. O BAPTA in patch solution.

The Net Charge Movement under Various Conditions

Fig. 4 *A* compares the exchange currents recorded at external Na^+ concentrations of 110 and 75 mM. The reduction in $[Na^+]_o$ reduces the saturated exchange current by 50% and slows the removal of the Ca^{2+} load (see also Hodgkin and Nunn, 1987). In

FIGURE 4 (*opposite*). The effects of lowered external [Na^+] and hyperpolarization on the net charge movement through the Na:Ca,K exchange. (*A*) Exchange currents in 110 mM Na^+, 0 Ca^{2+} (*bold trace*), and 75 mM Na^+, 0 Ca^{2+} (*thin trace*). Exposure to isotonic $CaCl_2$ and 0.5 mM IBMX was for 5 s (i.e., from 1 to 6 s). Light flash (2,600 photons μm^{-2}) and continuous light step (830 photons $\mu m^{-2}\ s^{-1}$) switched on at 4 s. V_m = −14 mV throughout. In calculating the value of q', Q_{exch} was corrected for the Ca^{2+} load present in darkness by subtracting the integral of the exchange current at the plateau of a saturating flash delivered at rest, which was 37.5 pC (see text). In 110 mM Na^+: Q_{exch} = 324.5 pC; Q_{load} = 610 pC; q' = 1.06; j_{sat} = 47 pA. In 75 mM Na^+: Q_{exch} = 317.5 pC; Q_{load} = 620 pC; q' = 1.02; j_{sat} = 23.3 pA. (*B*) Exchange currents at −14 mV (*bold trace*) and −64 mV (*thin trace*) in 110 mM Na^+ and 0 Ca^{2+}. The bold trace is that shown in *A*, and the same protocol was used except that the exchange current at −64 mV was recorded by stepping V_m (for 15 s; i.e., between t = 5 s and t = 20 s). At −64 mV: Q_{exch} = 287.5 pC; Q_{load} = 580 pC; q' = 0.99; j_{sat} = 67 pA. 0 BAPTA in patch solution.

this example, q' in 75 mM $[Na^+]_o$ was 1.02, and the mean value in this solution was 1.04 ± 0.05 (five experiments in four cells). An experiment using a wider range of $[Na^+]_o$ is shown in Fig. 5. In this experiment the exchange current was changed over a 7.2-fold range without a significant deviation from unity in the value of charge transported per calcium ion extruded. The reduction in the magnitude of the saturated exchange current in reduced $[Na^+]_o$ is therefore due to a reduction in the rate of operation of the carrier rather than to a change in the stoichiometry of the exchange.

TABLE II

Net Inward Charge Movement through the Na:Ca,K Exchange during the Extrusion of a Single Ca^{2+} Ion

$[Na^+]_o$	V_m	No. of observations	No. of outer segments	Net charge, q' (mean ± SEM)
mM	*mV*			
35	−14	4	4	0.93 ± 0.05
	−64	1	1	0.96
55	−14	7	5	0.95 ± 0.03
	−64	2	2	0.96 ± 0.09
75	−14	9	7	0.95 ± 0.03
	−44	1	1	0.99
	−64	4	2	1.09 ± 0.05
	+16	1	1	0.96
95	−14	9	7	1.01 ± 0.03
	−64	2	1	0.99 ± 0.03
110	−14	28	13	1.02 ± 0.02
	−44	1	1	0.99
	−64	4	3	1.00 ± 0.05
220	−14	2	2	0.94 ± 0.09
Normal Ringer				
	−14	8	8	0.98 ± 0.03
	−44	1	1	1.10
Collected results		84	16	1.004 ± 0.011

All results, except for those in normal Ringer, are in solutions containing nominally 0 Ca^{2+}, with either 1 mM EGTA or 1 mM EDTA (for 0 $[Mg^{2+}]$ solutions) added; the contaminating $[Ca^{2+}]$ was measured to be 6 μM by atomic absorption spectrophotometry, so free $[Ca^{2+}]_o$ was $\sim 2 \times 10^{-10}$ M. Results in the presence and absence of external K^+ (2.5 mM) and Mg^{2+} (1.6 mM) are collected together as these ions did not appear to alter the stoichiometry of the exchange. The third column gives the number of observations in each condition, and the fourth column gives the number of different outer segments in which these measurements were made.

The thin trace in Fig. 4 *B* shows an experiment in which the effect of membrane potential on the exchange stoichiometry was examined. A 50-mV hyperpolarization increased the saturated exchange current by 47% and accelerated the removal of the Ca^{2+} load, but the value of q' at −64 mV was unchanged (0.99 in this experiment) and the mean value for this hyperpolarization was 1.00 ± 0.05 (see Table II).

The results from these experiments are shown in Fig. 6. The net charge movement per calcium ion transported is given as a function of $[Na^+]_o$ at a membrane potential of −14 mV (filled circles), and at V_m = −64 mV (filled squares). Varying $[Na^+]_o$

between 35 and 220 mM does not cause the value of q' to differ significantly from unity. Over the range of conditions investigated here, therefore, there is no effect either of the electrochemical gradient for Na^+ or of the membrane potential on the stoichiometry of the exchange. The exchange current in these experiments varied over a 40-fold range, from 2 pA in 35 mM Na^+, 1.6 mM Mg^{2+}, and 2.5 mM K^+, to >80 pA in 220 mM Na^+, 0 Mg^{2+}, and 0 K^+.

The values of q' measured under a wider variety of conditions are shown in Table II. The stoichiometry is independent of the external [Na^+] (220–35 mM), of

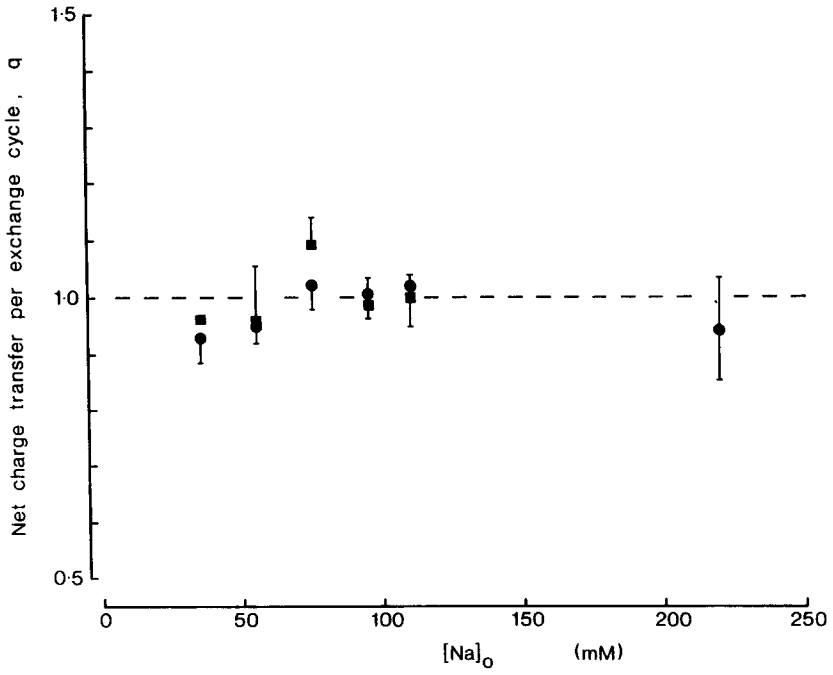

FIGURE 6. The net charge movement through the Na:Ca,K exchange under a variety of conditions. Each point is the value of q' at the external [Na^+] indicated, either at −14 mV (●) or −64 mV (■). All the results shown were obtained in solutions containing 0 Ca^{2+}, but the results in the presence and absence of external K^+ (2.5 mM) and Mg^{2+} (1.6 mM) are collected together as these ions did not appear to affect the value of q'. Bars show ±SEM. Over the range of conditions tested the value of q' did not differ significantly from 1.

membrane potential (−64 to +16 mV), and of the presence or absence of external Ca^{2+} (1 mM), K^+ (2.5 mM), or Mg^{2+} (1.6 mM). From the collected results, the net charge movement through the exchange during the extrusion of a single Ca^{2+} ion was calculated to be $q' = 1.004 \pm 0.011$ (84 measurements on 16 cells).

DISCUSSION

The main conclusion from these experiments is that the Na:Ca,K exchange in rods transports one net positive charge into the cell during the extrusion of a single Ca^{2+}

ion, and that the charge transfer is independent of the electrochemical gradients for Na^+, Ca^{2+}, and K^+ over a wide range. The charge transfer is also independent of whether the exchange is operating in the forward or the reversed mode (Cervetto et al., 1989). These properties simplify the interpretation of kinetic studies in which the exchange current is measured at different membrane potentials and/or ionic conditions (Lagnado et al., 1988); the size of the exchange current can be taken as a direct measure of the rate of Ca^{2+} transfer through the carrier.

The charge moved by the Na:Ca,K exchange in exchange for each Ca^{2+} extruded has been found using the suction pipette method to be 0.94 in the absence of $[Ca^{2+}]_o$ by Yau and Nakatani (1984) and 0.74 in normal Ringer by Hodgkin et al. (1987). The deviations from a value of unity are probably related to the disadvantages inherent in the suction pipette method, many of which are eliminated by the more direct technique used in this paper. First, the membrane potential is not under experimental control in the suction pipette method. Second, the suction pipette typically collects only ~50% of the outer segment membrane current, and the possibility remains open that this fraction might change in the course of an experimental manipulation. Third, the seal resistance of the whole-cell pipette (typically 10–20 GΩ) is three orders of magnitude higher than that of the suction pipette (5–10 MΩ). The currents caused by junction potentials between solutions of different ionic composition, which must be removed from suction pipette records by subtraction, are therefore typically too small in the whole-cell records to require subtraction. Finally, the internal ionic environment is under experimental control when using the whole-cell technique, an advantage which has been used in the present study to remove internal Na^+.

The simplest stoichiometry consistent with the influx of a single charge during the efflux of a single Ca^{2+} ion is one in which $3Na^+$ ions exchange with $1Ca^{2+}$, as was found in recent experiments investigating the reversal potential of the exchange in cardiac sarcolemmal vesicles (Reeves and Hale, 1984) and intact ventricular myocytes (Ehara, Matsuoka, and Noma, 1989). In rod outer segments, though, Cervetto et al. (1989) have demonstrated that the reversed exchange, mediating Ca^{2+} influx, depends on the presence of external K^+, and by varying $[Na^+]_o$, $[Ca^{2+}]_o$, $[K^+]_o$, and membrane potential, it was found that the equilibrium condition for the exchange was best described by the relation expected for the transport of $4Na^+$ ions in exchange for $1K^+$ and $1Ca^{2+}$. Evidence for Na:Ca,K cotransport in photoreceptors is also provided by the results of flux measurements in bovine rod outer segments, where it has been found that external K stimulates Ca uptake via the reversed exchange, and internal K stimulates the component of Ca efflux which is dependent on external Na (Schnetkamp, Szerencsei, and Basu, 1989).

It should be pointed out, however, that the net charge movement per Ca^{2+} ion transported is an average measurement over a large population of exchange molecules over many seconds. If two conditions are met the macroscopic charge flow can be equated to the charge transported per exchange cycle. First, there must be a homogenous population of exchange molecules in the outer segment membrane, and second, each cycle extruding a Ca^{2+} ion must be identical. We do not think it likely that there is more than one type of Na:Ca,K exchange, but one might imagine, for example, that half of the exchange cycles operate with a stoichiometry of

$4Na^+:1Ca^{2+}$ (net charge +2) and that the other half operate with a stoichiometry of $4Na^+:1Ca^{2+},2K^+$ (net charge 0), leading to an average net movement of one positive charge per Ca^+ ion. These two cycles would have to be tightly coupled since we find that the exchange is strictly dependent on K^+. Such possibilities raise the issue of the ways in which the microscopic behavior of the exchange might be studied. Clearly there is no equivalent to single channel recording at present, but if the unitary conductance of the exchange is large enough, another possibility may be the use of noise analysis.

An investigation of the net charge movement through the cardiac Na:Ca exchange has recently been carried out along similar lines by Bridge et al. (1990) using isolated ventricular myocytes under whole-cell voltage clamp. These authors used caffeine to prevent Ca^{2+} uptake and release from intracellular stores, and the Ca^{2+} channel blocker nifedipine to isolate the Ca^{2+} current. They found the charge entering the cell through the Ca^{2+} channels to be about twice that moved by the exchange in expelling the Ca^{2+} load, consistent with a stoichiometry of $3Na^+:1Ca^{2+}$ for the exchange in this tissue. The electrochemical gradient for Na^+ was not changed in these experiments, but the net charge transport was fixed over a range of Ca loads.

There is evidence to suggest that the electrogenic Na:K pump also operates with a fixed stoichiometry (Thomas, 1969). Using dialyzed squid giant axons under voltage clamp, Rakowski, Gadsby, and De Weer (1989) simultaneously identified ^{22}Na fluxes and membrane current through the pump by specifically blocking its activity with a cardiotonic steroid. They found that the ratio of the change in ^{22}Na efflux to the change in the membrane current on applying the steroid to be independent of membrane potential (between 0 and −60 mV) and external $[Na^+]$ (between 0 and 390 mM). In all conditions the results were not significantly different from those expected for a stoichiometry of $3Na^+:2K^+$, showing that changes in the Na:K pump current simply reflect changes in the rate at which the carrier operates.

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REFERENCES

Barbour, B., H. Brew, and D. Attwell. 1988. Electrogenic glutamate uptake in glial cells is activated by intracellular potassium. *Nature.* 335:433–435.

Baylor, D. A., and T. D. Lamb. 1982. Local effects of bleaching in retinal rods of the toad. *Journal of Physiology.* 328:49–71.

Baylor, D. A., and B. J. Nunn. 1986. Electrical properties of the light-sensitive conductance of rods of the salamander Ambystoma tigrinum. *Journal of Physiology.* 371:115–145.

Bridge, J. H. B., J. R. Smolley, and K. W. Spitzer. 1990. The relationship between charge movements associated with I_{Ca} and $I_{Na\text{-}Ca}$ in cardiac myocytes. *Science.* 248:376–378.

Capovilla, M., L. Cervetto, and V. Torre. 1983. Ionic movements through light-sensitive channels of toad rods. *Journal of Physiology.* 343:277–294.

Cervetto, L., L. Lagnado, R. J. Perry, D. W. Robinson, and P. A. McNaughton. 1989. Extrusion of calcium from rod outer segments is driven by both sodium and potassium gradients. *Nature.* 317:740–743.

Corey, D. P., and C. F. Stevens. 1983. Science and technology of patch recording electrodes. *In* Single Channel Recording. B. Sakmann and E. Neher, editors. Plenum Publishing Corp., New York. 53–68.

Ehara, T., S. Matsuoka, and A. Noma. 1989. Measurement of reversal potential of Na^+-Ca^{2+} exchange current in single guinea-pig ventricular cells. *Journal of Physiology.* 410:227–250.

Fesenko, E. E., S. S. Kolesnikov, and A. L. Lyubarski. 1985. Induction by cyclic-GMP of cationic conductances in plasma membrane of retinal rod outer segment. *Nature.* 313:310–313.

Hodgkin, A. L., P. A. McNaughton, and B. J. Nunn. 1985. The ionic selectivity and calcium dependence of the light-sensitive pathway in toad rods. *Journal of Physiology.* 358:447–468.

Hodgkin, A. L., P. A. McNaughton, and B. J. Nunn. 1987. Measurement of sodium-calcium exchange in salamander rods. *Journal of Physiology.* 391:347–370.

Hodgkin, A. L., P. A. McNaughton, B. J. Nunn, and K.-W. Yau. 1984. Effects of ions on retinal rods from Bufo marinus. *Journal of Physiology.* 350:649–680.

Hodgkin, A. L., and B. J. Nunn. 1987. The effects of ions on sodium-calcium exchange in salamander rods. *Journal of Physiology.* 391:371–398.

Kimura, J., S. Miyamae, and A. Noma. 1987. Identification of Na-Ca exchange current in single ventricular cells of guinea-pig. *Journal of Physiology.* 384:199–222.

Koch, K.-W., and L. Stryer. 1988. Highly cooperative feedback control of retinal rod guanylate cyclase by calcium ions. *Nature.* 334:64–66.

Lagnado, L., L. Cervetto, and P. A. McNaughton. 1988. Ion transport by the Na-Ca exchange in isolated rod outer segments. *Proceedings of the National Academy of Sciences, USA.* 85:4548–4552.

Lagnado, L., and P. A. McNaughton. 1987. Light responses and Na:Ca exchange in isolated salamander rod outer segments. *Journal of Physiology.* 390:11P. (Abstr.)

Lagnado, L., and P. A. McNaughton. 1988. The stoichiometry of Na:Ca exchange in isolated salamander rod outer segments. *Journal of Physiology.* 407:82P. (Abstr.)

Lagnado, L., and P. A. McNaughton. 1989. The sodium:calcium exchange in photoreceptors. *In* Sodium-Calcium Exchange. T. J. A. Allen, D. Noble, and H. Reuter, editors. Oxford University Press, Oxford, UK. 261–297.

Lagnado, L., and P. A. McNaughton. 1990. Electrogenic properties of the Na:Ca exchange. *Journal of Membrane Biology.* 113:177–191.

Lipp, P., and L. Pott. 1988. Transient inward current in guinea-pig atrial myocytes reflects a change of sodium-calcium exchange current. *Journal of Physiology.* 397:601–630.

Lolley, R. N., and E. Racz. 1982. Calcium modulation of cGMP synthesis in rat visual cells. *Vision Research.* 22:1481–1486.

McNaughton, P. A., L. Cervetto, and B. J. Nunn. 1986. Measurement of the intracellular free calcium concentration in salamander rods. *Nature.* 322:261–263.

Menini, A., G. Rispoli, and V. Torre. 1988. The ionic selectivity of the light-sensitive current in isolated rods of the tiger salamander. *Journal of Physiology.* 402:279–300.

Rakowski, R. F., D. C. Gadsby, and P. De Weer. 1989. Stoichiometry and voltage-dependence of the sodium pump in voltage-clamped, internally dialyzed squid giant axon. *Journal of General Physiology.* 93:903–941.

Reeves, J. P., and C. C. Hale. 1984. The stoichiometry of the cardiac sodium-calcium exchange system. *Journal of Biological Chemistry.* 259:7733–7739.

Sather, W. A., and P. B. Detwiler. 1987. Intracellular biochemical manipulation of phototransduction in detached rod outer segments. *Proceedings of the National Academy of Sciences, USA.* 84:9920–9294.

Schnetkamp, P. P. M. 1986. Sodium-calcium exchange in the outer segments of bovine rod outer segments. *Journal of Physiology.* 373:25–45.

Schnetkamp, P. P. M., R. T. Szerencsei, and D. K. Basu. 1989. Na^+-Ca^{2+} exchange in bovine rod outer segments requires and transports K^+. *American Journal of Physiology.* 257:C153–157.

Thomas, R. C. 1969. Membrane current and intracellular sodium changes in a snail neurone during extrusion of injected sodium. *Journal of Physiology.* 201:495–514.

Yau, K.-W., and K. Nakatani. 1984. Electrogenic Na-Ca exchange in retinal rod outer segment. *Nature.* 311:661–663.

Zimmerman, A. L., and D. A. Baylor. 1986. Cyclic GMP sensitive conductance of retinal rods consists of aqueous pores. *Nature.* 321:70–72.