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EFFECTS OF MANGANESE AND OTHER AGENTS ON THE CALCIUM UPTAKE THAT FOLLOWS DEPOLARIZATION OF SQUID AXONS

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SUMMARY

1. The Ca-sensitive photoprotein aequorin was injected into squid axons and the light response to stimulation or depolarizing voltage clamp pulses recorded.

2. The effects of Mn^{2+}, Co^{2+}, Ni^{2+}, La^{3+} and of the organic Ca antagonists D-600 and iproveratril on the early tetrodotoxin-sensitive and late tetrodotoxin-insensitive components of the light response were studied.

3. The late tetrodotoxin-insensitive component can be blocked, reversibly, by concentrations of Mn, Co and Ni that reduce but do not block the tetrodotoxin-sensitive component. The late component can also be blocked by La^{3+} and the organic Ca antagonists D-600 and iproveratril.

4. Mn^{2+}, Co^{2+}, Ni^{2+} and the drug D-600 all reduce the Na currents, but have little effect on either outward or inward K currents. Tetraethylammonium blocks the outward K current but has no appreciable effect on the tetrodotoxin-insensitive entry of Ca.

5. Concentrations of Mn between 5 and 50 mM substantially reduce the light output during a train of action potentials; they also slightly reduce the rate of rise of the action potential.

6. On pharmacological grounds it is concluded that the tetrodotoxin-insensitive component of Ca entry does not represent Ca ions passing through the K permeability channels. There must exist a potential-dependent late Ca channel that is distinct from the well known Na and K channels of the action potential. A possible function for this late Ca channel in the coupling of excitation to secretion is discussed.

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INTRODUCTION

Depolarization of squid axons leads to an uptake of Ca (Hodgkin & Keynes, 1957). The time course of this uptake has recently been examined by Baker, Hodgkin & Ridgway (1971) using the Ca-sensitive protein aequorin. Ca entry occurs in two phases: an early phase that is blocked by tetrodotoxin and seems to reflect Ca entering through the Na permeability channels, and a late phase that is insensitive to external tetrodotoxin or internal tetraethylammonium ions.

The experiments described in this paper examine more closely the possibility that the late entry of Ca occurs through the K channel of the action potential. Although the time course of the late Ca entry is similar to that of the K conductance, it is possible to find substances that block selectively either the K conductance or the late Ca entry. The tetrodotoxin-insensitive Ca entry can be blocked by Mn^{2+}, Co^{2+}, Ni^{2+} and La^{3+} ions at concentrations that do not substantially affect the K inward or outward currents. The transition metals and La are known to block Ca entry in various preparations, e.g. in barnacle muscle fibres (Hagiwara & Takahashi, 1967) and also to abolish the 'Ca response' of the presynaptic nerve terminal of the stellate ganglion of the squid (Katz & Miledi, 1969*a*; Miledi, 1971). Essentially similar results were obtained with the organic Ca antagonists iproveratril and its methoxy-derivative D-600 both of which selectively diminish Ca influx and contractile activity in mammalian heart muscle (Fleckenstein, Tritthart, Fleckenstein, Herbst & Grün, 1969; Kohlhardt, Bauer, Krause & Fleckenstein, 1972). Our tentative conclusion is that the tetrodotoxin-insensitive entry of Ca does not take place through the K channel of the action potential.

Part of this work has already been described briefly (Baker, Meves & Ridgway, 1971).

METHODS

Material. Giant axons with diameters between 600 and 1000 μm were isolated from mantles of *Loligo forbesi*. Living squid were used occasionally but as a rule axons were dissected from refrigerated mantles.

Procedures. These were essentially the same as described in detail by Baker, Hodgkin & Ridgway (1971). After the axon had been cleaned 0·2–0·35 μl. of the aequorin solution was injected over a length of 1–2 cm. The light emission was measured with a photomultiplier and was usually recorded with a pen-recorder (Unicam AR 25 Linear Recorder). The response of the pen-recorder was 100 % complete in 0·5 sec. For voltage clamp experiments an internal double spiral electrode was used with the voltage wire exposed for about 7 mm and the current wire for 15–20 mm. In some experiments the K outward currents were blocked in the injected region by overlapping the aequorin patch with an injection of 2·5 M tetraethylammonium chloride to give a final concentration of about 70 mM.

Solutions. The basic solution was high-Ca sea-water containing 112 mM-$CaCl_2$,

400 mM-NaCl, 10 mM-KCl and 2·5 mM-$NaHCO_3$. $MnCl_2$, $CoSO_4$ (cobaltous sulphate) and $NiSO_4$ (nickelous sulphate) were added as concentrated solutions (0·5–2 M) or sometimes as solids just before use. It should be stressed that solutions of $MnCl_2$ must be made fresh for each experiment. La was used in the absence of bicarbonate in Tris-buffered sea-water. Chemicals were Analar where available.

The drug iproveratril and its methoxy derivative D-600 were obtained from Knoll Pharmaceuticals. Iproveratril (0·1 mg/ml.) was dissolved directly in sea-water. A strong solution of D-600 was made in ethanol (usually 100 mg/ml.) and diluted 1:1000 with the experimental solution. The final ethanol concentration never exceeded 0·1 % (v/v).

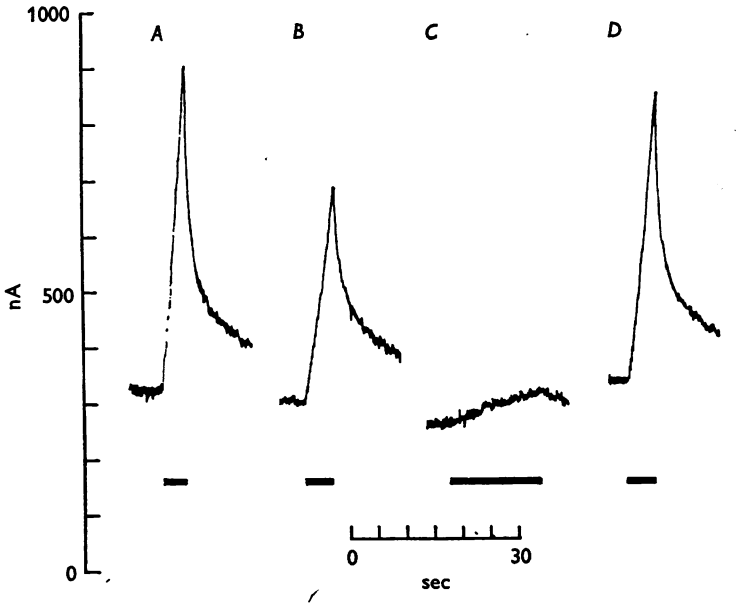

Fig. 1. Increase in rate of light emission in response to repetitive stimulation at 100 impulses/sec. Ordinate: light (nA); abscissa: time (sec). The interval between records varied between 10 and 30 min. The external solution was 112 mM-Ca-Na ASW. *A*, control; *B*, after addition of 5 mM-$MnCl_2$; *C*, after addition of 50 mM-$MnCl_2$; *D*, after removal of $MnCl_2$. The horizontal bars represent the periods of stimulation. Axon diameter 800 μm. Temp. 20° C. The initial rate of rise of light intensity increased as the square of the action potential frequency.

RESULTS

Effects of Mn^{2+} and other agents on Ca entering during the action potential.

Repetitive stimulation reversibly increases the light output from aequorin-injected axons (Fig. 1*A*). The addition of $MnCl_2$ (50 mM) to the sea-water markedly reduces the light output during repetitive stimulation (Fig. 1*C*). At the same time, although not shown, the overshoot of the membrane action potential is decreased by about 4 mV and the slope of its rising

phase is diminished slightly, but the duration of the action potential is unchanged. The effects of Mn on the light production and on the action potential are fully reversible (Fig. 1*D*). In some axons a reduction in light output is seen with Mn concentrations as low as 5 mM (Fig. 1*B*). Co^{2+} (5–25 mM) and Ni^{2+} (25 mM) have similar actions to those of Mn^{2+}. La^{3+} (4 mM) and the drugs iproveratril (0·1 mg/ml.) and D-600 (0·1 mg/ml.) also reduce the light output. La prolongs the action potential whereas iproveratril reduces the overshoot by about 10 mV and D-600 eventually blocks conduction of the action potential. These drugs do not significantly alter the resting potential.

Experiments under voltage clamp conditions

The voltage clamp technique was used to characterize more fully the mode of action of those agents that effect an apparent reduction in Ca entry during the action potential. The phrase 'apparent reduction in Ca entry' is used because Ca entry is measured in terms of light produced by aequorin that has been injected into the fibre, and the possibility must always be considered that a reduction in light output results not from a smaller entry of Ca, but from inhibition of the aequorin reaction so that less light is produced for a given change in internal Ca. This is discussed in more detail in the Discussion (see p. 525).

It is convenient to describe the results of the voltage clamp experiments under two headings: (1) the effects of Mn^{2+} and other agents on the apparent entry of Ca as measured by aequorin, and (2) the effect of these same agents on the Na and K conductances.

Effects of Mn^{2+} and other agents on the apparent entry of Ca

On the basis of experiments in which axons were depolarized by pulses of different duration, Baker, Hodgkin & Ridgway (1971) divided the Ca entry into two components: an early entry that is blocked by tetrodotoxin and a late component that is insensitive to tetrodotoxin (see also Fig. 2*A*, *B*). The relative sizes of these two components varied widely. Fig. 2*C* shows the effects of including 50 mM-$MnCl_2$ in the external solution (112 Ca-ASW) on the light output in response to brief trains of depolarizing pulses. Mn reduces, but does not eliminate, the light output especially in response to long pulses. The effects of Mn are reversible. Lower concentrations of Mn (10 mM) reduce the light output but not to the same extent as 50 mM-Mn. Higher concentrations of Mn were not tested.

The action of Mn is quite different from that of tetrodotoxin. Tetrodotoxin blocks Ca entry in response to pulses of short duration (up to about 200 μsec for depolarizations of 100 mV at 18° C) and has less effect on the Ca entry associated with longer pulses. Mn has the opposite action: blocking

Ca entry in response to long pulses and leaving an appreciable fraction of the entry that is normally associated with pulses of short duration. The experiments with Mn suggest that it blocks the late component of Ca entry. If this is true, the Ca entry that persists in the presence of 50 mM-Mn should be blocked by tetrodotoxin. Fig. 3 shows this to be the case. In the presence of both Mn and tetrodotoxin, the initial rate of light production in response to 80 mV depolarizing pulses of 825 μsec duration was less than 3 % of that in the absence of these agents.

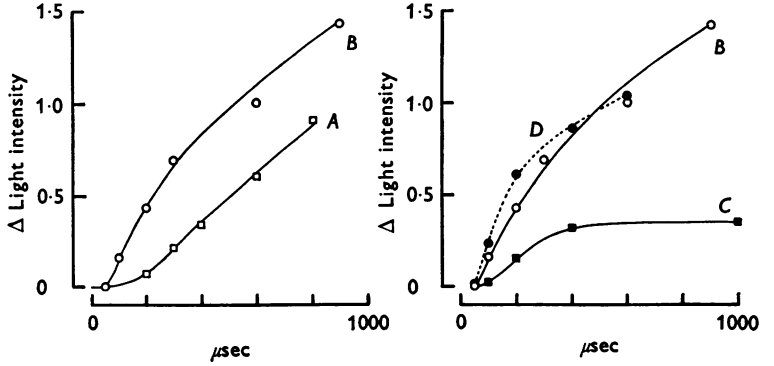

Fig. 2. Relation between pulse duration (abscissa) and the increment in light intensity per pulse. *A*, in the presence of 1·4 μM tetrodotoxin; *B*, after removal of tetrodotoxin; *C*, in the presence of external $MnCl_2$ (50 mM); *D*, after removal of $MnCl_2$. The records were obtained in the order *A*, *B*, *C*, *D*. The ordinate is the initial rate of rise of light intensity at 100 pulses/sec ÷ the initial rate of rise of light intensity at 100 action potentials/sec. Pulse amplitude 100 mV. External solution Mg-free sea-water containing 112 mM-Ca. The initial rate of rise of light intensity increased linearly with pulse or action potential frequency. Temp. 18° C. Axon diameter 750 μm.

Mn is not a completely selective inhibitor of the late component of Ca entry: it also substantially reduces the early, tetrodotoxin-sensitive entry. Fig. 2*C* shows that Mn reduced the response to 200 μsec pulses to about 30 % of its normal value (see also Fig. 3). This reduction of the early light is accompanied by a reduction in the Na inward current (see p. 521).

The actions of Co^{2+} and Ni^{2+} are essentially similar to those of Mn^{2+}. The results are collected in Table 1. The last column gives the relative sizes of the late component in the presence and absence of each inhibitor. These figures for the late component were obtained either by subtracting the early component on the assumption that all the light at 200 μsec is due to the early component or by eliminating the early component by tetrodotoxin. In spite of considerable scatter, it seems clear that 50 mM $MnCl_2$, 25 mM-$CoSO_4$, 25 mM-$NiSO_4$ and 5 mM-$LaCl_3$ were about equally effective in suppressing the late light. 10 mM-$MnCl_2$ and 5 mM-$CoSO_4$ had a much

smaller effect. Both 50 mM-$MnCl_2$ and 25 mM-$CoSO_4$ also reduced the light response to short pulses (early component) to 30–64 % of its normal size. Finally, in most axons a decrease in the resting light was seen. In addition, the Table shows that a late component of the usual size was present in axons that had been injected with tetraethylammonium chloride (TEA), so that they had very little or no outward K current. The insensitivity of the late component to internal TEA is in agreement with the observations of Baker, Hodgkin & Ridgway (1971).

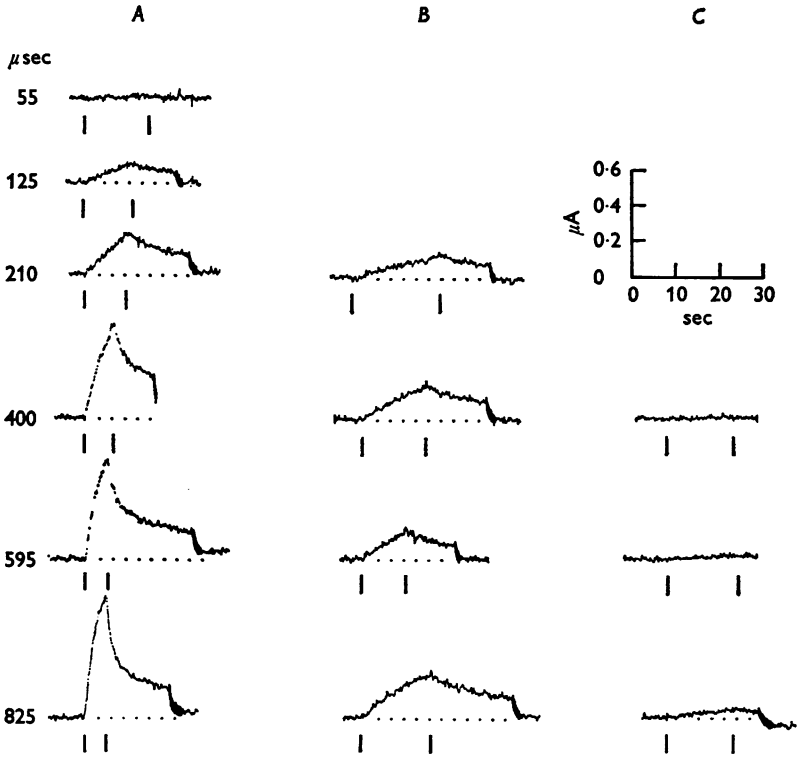

Fig. 3. Inkwriter records of aequorin responses to trains of pulses of different duration. *A*, 112 mM-Ca ASW; *B*, 112 mM-Ca ASW with 50 mM-$MnCl_2$; *C*, 112 mM-Ca ASW with 50 mM-$MnCl_2$ and 1·6 μM tetrodotoxin. The duration of the pulse, in μsec, is shown at the left. The amplitude was 80 mV and the frequency 200/sec. The duration of the train varied between 5 and 20 sec and is indicated by the vertical bars. The recorder was slowed by a factor of 12 during part of the recovery phase. The resting glow was 1020 nA at the beginning of the experiment; it began to drop after application of 50 mM-$MnCl_2$ and was 720 nA at the end of the experiment. The axon was kept 20 hr at 4° C before injection of aequorin. The experiment was started 3 hr after injecting 0·35 μl. aequorin over 2 cm and lasted 50 min. Axon diameter 950 μm. Temp. 20° C.

TABLE 1. Effect of Mn, Co, Ni, La and D-600 on aequorin responses

Test	Axon	Resting light (nA) Normal	Resting light (nA) Test	Pulse amplitude (mV)	Pulse frequency (sec^{-1})	Response to short pulses: Pulse duration (msec)	Response to short pulses: Initial rate of rise (nA/sec) Normal	Response to short pulses: Initial rate of rise (nA/sec) Test	Response to long pulses: Pulse duration (msec)	Response to long pulses: Initial rate of rise (nA/sec) Normal	Response to long pulses: Initial rate of rise (nA/sec) Test	Late comp. test / Late comp. normal
50 mM-Mn	1	5610	4840	80	200	0·30	77	49·5	0·80	286	122	0·35
	2	1125	1020	80	200	0·21	30	9	0·82	171	22·5	0·10
	6 (TTX)	2640	3190	90	200	—	—	—	1·40	20	0	0
	9	185	60	80	200	0·20	5·5	1·9	0·82	19	2·7	0·06
	10 (TTX, TEA)	220	150	80	100	—	—	—	7·70	440	5·5	0·01
10 mM-Mn	11 (TTX, TEA)	930	290	80	100	—	—	—	8·10	34·5	18	0·52
		2090	715	80	100	—	—	—	6·05	247·5	134	0·54
25 mM-Co	44	800	750	80	200	0·19	6·5	2	1·00	18	5	0·26
	45	985	940	80	200	—	—	—	1·03	50·3	20·3	0·40
	41 (TTX)	600	720	80	Single	—	—	—	33	9000	0	0
5 mM-Co	41 (TTX)	600	640	80	Single	—	—	—	33	9000	6600	0·73
25 mM-Ni	41 (TTX)	960	960	80	Single	—	—	—	33	10500	0	0
5 mM-La	1	4620	4070	80	200	—	—	—	1	132	0	0
D-600	3	1485	1375	80	200	—	20	8	0·80	1331		0·32
(10^{-4} g/ml)	12 (TTX, TEA)	1095	1020	80	100	—	—	—	8·65	63	0	0
	39	335	265	70	100	—	—	—	1·95	12·5	2·5	0·2

External solution Mg-free, NaCl-ASW containing 112 mM-Ca. Temperature 17·5–21·5° C. Three of the axons were injected with TEA to give a final internal concentration of about 70 mM; in five experiments the sea-water contained tetrodotoxin (0·8–1·6 μM). Axon 41 was kept at 4° C for 20 hr before injecting aequorin; the measurements were made 8 hr after injection. The other axons were injected shortly after the dissection and the measurements were done 2–7 hr later; the second measurements on axon 11 were made 11·5 hr after injection.

The response to short pulses was subtracted from the response to long pulses to obtain the late component. The entry in the last column gives the ratio late component in the test solution (112 Ca-sea-water containing Mn, Co, Ni, La or D-600) to late component in normal sea-water.

The organic Ca antagonist D-600 also decreases both the early and the late components of Ca entry. The effect of D-600 on the tetrodotoxin-insensitive component is illustrated in Fig. 4. Quantitative results from three experiments are collected in Table 1. It can be seen that the drug substantially reduces or completely abolishes the late component of Ca entry as measured with long pulses. The resting light is also diminished. The effect of D-600 on the early component of Ca entry was tested in one experiment. The initial rate of rise of the light response to short pulses

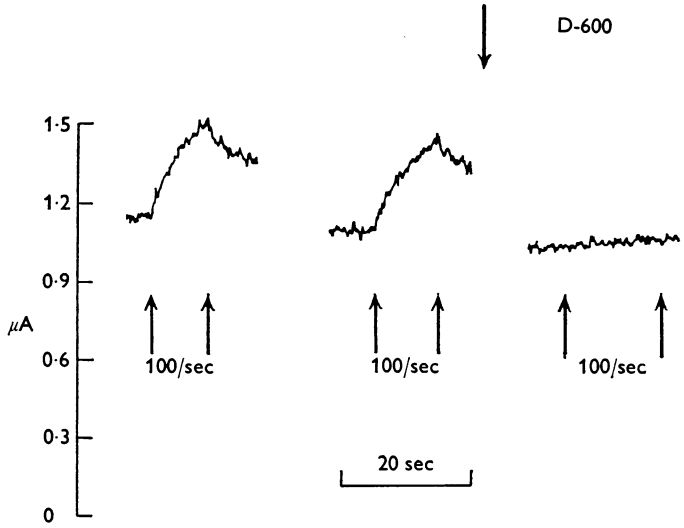

Fig. 4. Effect of D-600 on the late component of the aequorin response. Inkwriter records of light responses to trains of pulses. Ordinate: light output (μA); abscissa: time (sec). Pulse amplitude 80 mV, pulse duration 8·65 msec, repetition rate 100/sec, duration of trains indicated by arrows. External solution: 112 mM-Ca ASW with 0·8 μM tetrodotoxin for first two records, same with 0·8 μM tetrodotoxin and D-600 (10^{-4} g/ml.) for last record. 12 min between first and second record; third record 7 min after adding D-600. The axon was injected with aequorin and 2·5 M-TEA and the records were taken 7 hr after injection. Axon diameter 925 μm. Temp. 21° C.

decreased from 20 to 8 nA/sec in the presence of D-600. Simultaneously the Na inward current was markedly reduced (see p. 521). Similar, but less complete results were obtained with iproveratril.

One criticism of the experiments described so far is that the agents that reduce Ca entry may not be blocking the Ca channels but modifying them such that they can only be opened by larger depolarizations. This was examined by observing the effects of Mn, Co, Ni and D-600 over a wide range of voltages. Baker, Hodgkin & Ridgway (1971) showed that the

voltage-dependence of the late component of Ca entry follows a roughly bell-shaped curve with a maximum at depolarizations of 70–90 mV from the resting potential. Fig. 5 shows measurements obtained at different voltages with repetitive clamp pulses of 7·7 msec duration given at a repetition rate of 100/sec. The fibre was treated with external tetrodotoxin and internal tetraethylammonium. In 112 mM-Ca-ASW without Mn the light response increased steeply at depolarizations between 60 and 80 mV, reached a maximum at 90–100 mV, and decreased again for larger depolarizations. The curve resembles the voltage–response curve obtained by Baker, Hodgkin & Ridgway (1971). 50 mM-$MnCl_2$ reversibly reduced the light response to zero over the whole potential range.

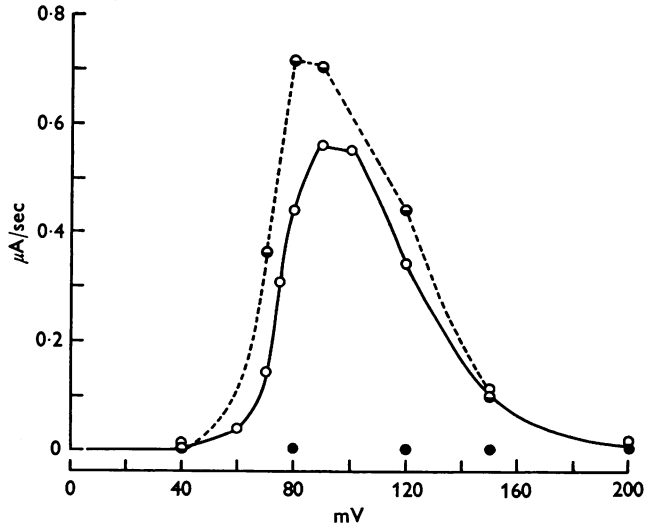

Fig. 5. Effect of pulse amplitude on the aequorin response to repetitive pulses (100/sec) of 7·7 msec duration. Abscissa: pulse amplitude; ordinate: initial rate of rise of light response, recorded on inkwriter. ○, 112 mM-Ca ASW with 1·6 μM tetrodotoxin; ●, 112 mM-Ca ASW with 1·6 μM tetrodotoxin and 50 mM-$MnCl_2$; ◒, 7–18 min after returning to 112 mM-Ca ASW with 1·6 μM tetrodotoxin without $MnCl_2$. The resting light was 5·8 μA at the beginning of the experiment, dropped continuously during the first measurements in Mn-free ASW and stayed constant at 220–330 nA for the rest of the experiment. 2·5 M-TEA was injected over 2 cm so as to overlap the patch of aequorin. Measurements were started 2 hr after injection. Tests at the start of the experiment showed that the initial rate of rise of light intensity was linearly proportional to the frequency of stimulation. Axon diameter 775 μM. Temp. 18° C.

The experiment shown in Fig. 5 makes it unlikely that the nearly complete blockage of the late light by 50 mM-Mn is due to a shift of the voltage–response curve along the voltage axis. This conclusion is further supported

by the experiment illustrated in Fig. 6. The experiment was done on an old axon with large light responses (Baker, Hodgkin & Ridgway, 1971) so it was possible to use single voltage clamp pulses of 33 msec duration. Mn, Co and Ni reduced the light response at all the voltages tested. The position of the peak was not shifted significantly by 5 mM-$CoSO_4$, but its height was reduced. In a separate experiment D-600 at a concentration of 100 μg/ml. also reduced the light response at all the voltages tested.

It is difficult to be quantitative about the extent of the reduction by 5 mM-Co. Assuming a linear relation between light and Ca entry, the reduction was about 50 %, but in this experiment the square root of the light response may be a better measure of the ionized Ca in which case the maximum response was reduced to 67 % of its value in the absence of Co. The use of the square root is justified by the observation that this axon, unlike the axon of Fig. 5, showed a non-linear (square) relation between the light response and frequency of stimulation (see also Baker, Hodgkin & Ridgway, 1971).

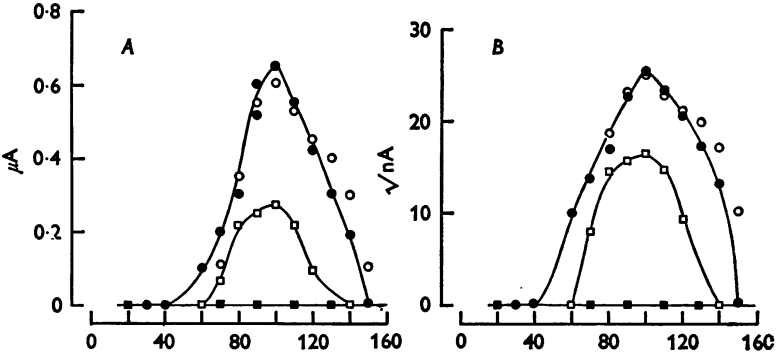

Fig. 6. Effect of pulse amplitude on the aequorin response to single pulses of 33 msec duration. Abscissa: pulse amplitude (mV); ordinate: the peak light response plotted linearly in *A* and as the square root in *B*. ○, 112 mM-Ca ASW; □, 112 mM-Ca ASW with 5 mM-$CoSO_4$; ■ 112 mM-Ca ASW with 25 mM-$CoSO_4$; ●, 112 mM-Ca ASW after removal of cobalt. Inclusion of 25 mM-$NiSO_4$ or 50 mM-$MnCl_2$ also resulted in no detectable light response to depolarizations of 0–200 mV. All solutions contained 1·6 μM tetrodotoxin. The resting glow was 600 nA at the beginning and increased continuously to 960 nA at the end of the experiment. The axon was kept 20 hr at 4° C before injection of aequorin. The measurements were started 8 hr after injecting 0·2 μl. aequorin over 1 cm and lasted 18 min. Tests at the start of the experiment showed that the initial rate of light production was proportional to the square of the frequency of stimulation. Axon diameter 675 μm. Temp. 20·5° C.

The experiment shown in Fig. 6 was done in the presence of tetrodotoxin but without internal TEA. It therefore rules out the rather remote possibility that the bell-shaped voltage–response curve found in the previous experiments was caused by the presence of TEA in the axon. In the experi-

ment of Fig. 6 voltage–response curves were also measured with single longer pulses (120 msec duration) and with repetitive shorter pulses (1 msec duration, repetition rate 50/sec). The curves were similar to the one illustrated except that the decline of the light response at large depolarizations was more pronounced with long pulses than with short ones. This may be because Ca entering after the pulse is an appreciable fraction of the Ca that enters in response to short pulses.

Effect of Mn, Co and D-600 on the Na and K currents

The results can be summarized by saying that any decrease in the early light was accompanied by a decrease of the Na inward current; the decrease or blockage of the late component by Mn, Co or D-600, however, took place without large changes in the potassium outward or inward currents.

In three experiments (axons 1, 2 and 9 in Table 1) the Na inward current measured with 80 mV depolarizing pulses decreased to 43, 38 and 60 % after changing from 112 mM-Ca-ASW to 112 mM-Ca-ASW with 50 mM-$MnCl_2$. 25 mM-$CoSO_4$ and 5 mM-$LaCl_3$ reduced I_{Na} to 59 and 31 % respectively. These changes were partially reversible. The drug D-600 (0·1 mg/ml.) had an even stronger effect on the Na inward current. It completely blocked I_{Na} in two experiments and markedly reduced it in a third (see Fig. 8). The Na current could not be restored by hyperpolarizing prepulses and its blockage was not accompanied by any significant changes in resting potential. It was partially reversible on return to D-600-free sea-water.

To investigate the reduction of I_{Na} more closely the Na currents were measured during depolarizing pulses of 0 to 90 mV in 112 mM-Ca-ASW with and without 25 mM-$CoSO_4$ and the Na conductance g_{Na} was plotted against voltage. The curve for 25 mM-$CoSO_4$ differed from the normal curve in two respects: (*a*) it was slightly shifted towards more positive potentials, the potential for half maximum g_{Na} being 4·5 mV more positive, and (*b*) the maximum g_{Na} was reduced to 59 % of its normal value. This is in agreement with observations of Takata, Pickard, Lettvin & Moore (1967) and Blaustein & Goldman (1968) on the lobster axon; they found a marked reduction of the maximum g_{Na} when La, Co, or Ni were substituted for Ca in the external solution. Since the shift of the conductance curve is so small it seems to be the decrease of the maximum g_{Na} that is responsible for the reduction of I_{Na} measured with 80 mV depolarizing pulses. The reduction in I_{Na} was of the same order of magnitude as the decrease of the early light (see Table 1).

The marked reduction of I_{Na} by D-600 (0·1 mg/ml.) was not due to the ethanol in which the drug was dissolved; this was shown by a control experiment.

The effects of Mn, Co, Ni and D-600 on the K outward current were much smaller and difficult to distinguish from a run-down of the fibre. The small and irregular effects on I_K are in contrast to the nearly complete suppression of the late light. The only agent that produced a clear effect on I_K was La which in a single experiment reduced I_K to 10 % of its normal value.

In axon 41 (see Table 1) the maximum I_K (reached during a 70 or 110 mV clamp pulse of 33 msec duration) was reversibly reduced to 93, 91 and 84·5 % by 5 mM-$CoSO_4$, 25 mM-$CoSO_4$ and 25 mM-$NiSO_4$ respectively, whereas another experiment (axon 44) with 0 to 90 mV clamp pulses of 3 msec duration showed no effect of 25 mM-$CoSO_4$ at all. In axon 9, a reversible decrease of I_K (measured with 80 mV pulses of 1 msec duration) to 92 % of its normal value occurred in 50 mM-$MnCl_2$. Larger reductions to 77–88 % were found in three other experiments with 50 mM-$MnCl_2$ (axons 1, 2 and 6) where I_K was measured with 60–120 mV pulses of 0·8–1·4 msec duration; these effects, however, were not reversible.

As illustrated in Fig. 8, D-600 affected the K outward current only slightly. The final value of the outward current in *b* is 89 % of that in the control record *a*. In two other experiments D-600 reduced I_K (measured with 50–90 mV pulses of 0·8 and 2 msec duration) to 80 and 88 % respectively. The effect, although small, seems genuine and not due to a run-down of the fibre because the K current was stable before application of the drug.

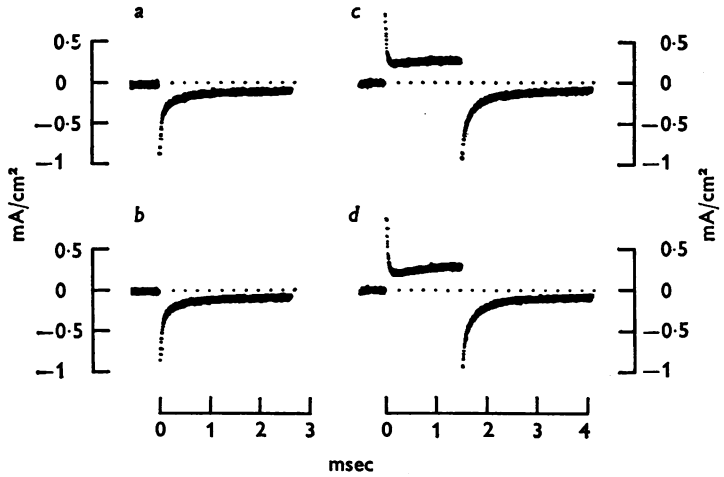

Fig. 7. Voltage clamp currents in K-rich sea-water. *a* and *c*: 210 mM-KCl, 200 mM-NaCl, 112 mM-$CaCl_2$, 2·5 mM-$NaHCO_3$; *b* and *d*: same with 25 mM-$CoSO_4$. Hyperpolarizing pulse of 70 mV amplitude, in *c* and *d* preceded by depolarizing pulse of 50 mV amplitude and 1·5 msec duration. No holding current. The axon was injected with 0·2 μl aequorin over 1 cm and the records were taken in the sequence *a*, *c*, *b*, *d*. Axon diameter 755 μm. Temp. 19·5° C.

Since the aequorin responses are thought to be due to Ca inward movements it seemed especially important to study the effects of Mn, Co and D-600 on the K inward currents. These were obtained by applying hyperpolarizing clamp pulses in sea-water containing 210 mM-KCl (Figs. 7*a–d* and 8*c*, *d*). At the beginning of the hyperpolarizing pulse there was a large surge of capacity current followed by a tail of inward current that declined to a small steady-state value (Fig. 7*a*). A depolarizing prepulse slightly increased the tail of inward current but did not alter the steady-state value

(Fig. 7*c*). Inward currents such as these (see similar records in Fig. 4*a* of Armstrong & Binstock, 1965) are expected if the driving force for K ions is suddenly increased; the decline of the current is explained by the shutting-off of the K conductance at more negative internal potentials. As seen in Fig. 7*b* and *d*, 25 mM-$CoSO_4$ had no effect on the K inward currents. A semi-logarithmic plot did not reveal any significant change of the time constant or the extrapolated instantaneous current. The only noticeable effect of 25 mM-$CoSO_4$ was a slight delay in the rise of the outward current during the depolarizing prepulse (compare records *c* and

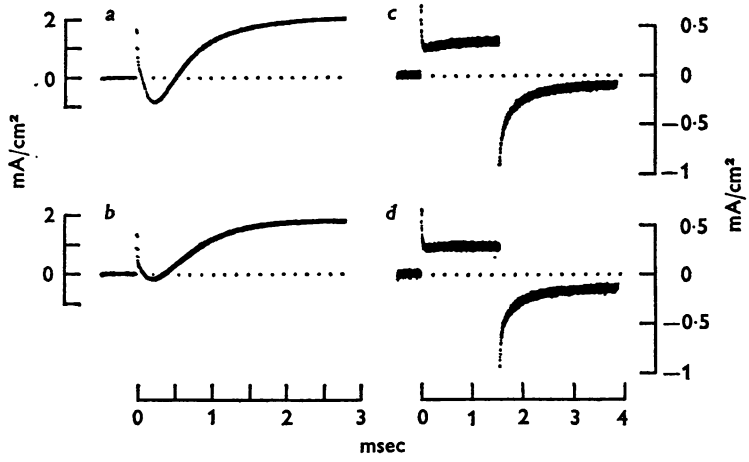

Fig. 8. Effect of D-600 on voltage clamp currents. *a*: 112 mM-Ca ASW, depolarizing pulse of 60 mV; *b*: same with 10^{-4} g/ml. D-600; *c*: 210 mM-KCl, 200 mM-NaCl, 112 mM-$CaCl_2$, 2·5 mM-$NaHCO_3$; depolarizing pulse of 50 mV amplitude and 1·5 msec duration, followed by hyperpolarizing pulse of 70 mV; *d*: same with 10^{-4} g/ml. D-600. No holding current. The axon was injected with 0·2 μl. aequorin over 1 cm and the records were taken in the sequence *a*, *c*, *d*, *b*. Axon diameter 910 μm. Temp. 21° C.

d in Fig. 7). The current–voltage curve, obtained by varying the clamp pulse between −70 and +70 mV and measuring the current at 2·5 msec, was identical for 210 mM-KCl ASW with and without 25 mM-$CoSO_4$. Two further experiments showed that 50 mM-$MnCl_2$ also does not affect the K inward currents measured in sea-water with 210 mM-KCl.

The same method was used to study the effect of D-600 on the K inward currents. Again, inward currents were measured in sea-water with 210 mM-KCl using a hyperpolarizing pulse, preceded by a depolarizing prepulse. Record *c* in Fig. 8 which is similar to Fig. 7*c* was taken before adding D-600. The drug did not alter markedly the K outward or inward current (Fig. 8*d*). Closer inspection reveals that the final value of the outward current in *d* is 79% of that in *c*; semi-logarithmic extrapolation of the

inward current to its initial value shows a slight reduction of the initial value.

The time constant τ_n of the K conductance variable n, determined by plotting log $(\sqrt[4]{I_K} - \sqrt[4]{I_{K\infty}})$ against time, was 0·93 msec in Fig. 7*a* and 0·76 msec in Fig. 7*c*; this is somewhat smaller than τ_n = 1·22 msec calculated from the equations of Hodgkin & Huxley (1952) for the same temperature and a membrane potential of −90 mV (assuming a resting potential of −20 mV in the experiment of Fig. 7 and a resting potential of −62 mV in the experiments of Hodgkin & Huxley). In Fig. 8*c* the time constant τ_n was 0·82 msec as compared with a Hodgkin & Huxley value of 1·03 msec. The difference between our time constants and those calculated from the data of Hodgkin & Huxley could be partly due to the high Ca concentration of our sea-water.

DISCUSSION

The results described in this paper confirm and extend the earlier work of Baker, Hodgkin & Ridgway (1971). They show that the tetrodotoxin-insensitive Ca entry can be completely blocked by externally applied Mn^{2+} and Co^{2+} ions at concentrations that reduce but do not block the early component. In the presence of blocking concentrations of both Mn^{2+} and tetrodotoxin the total depolarization-dependent Ca entry is reduced to less than 3 % of its normal value. Furthermore, our experiments show that the organic Ca antagonist D-600 markedly reduces both the tetrodotoxin-sensitive and the tetrodotoxin-insensitive Ca entry.

A major aim of this work was to determine whether or not the tetrodotoxin-insensitive component of Ca entry represents Ca entry through the K-selective permeability channels. The insensitivity of this component to tetraethylammonium chloride (TEA) is not sufficient proof for two distinct channels because in squid axons K inward current through the delayed channel is also not affected by TEA (Armstrong & Binstock, 1965). The evidence summarized in Table 2, however, indicates that the two channels are quite distinct pharmacologically. The decrease or blockage of the tetrodotoxin-insensitive Ca entry by Mn, Co or D-600 took place without large changes in the K outward or inward currents. On pharmacological grounds there seems little doubt that the depolarization-induced entry of Ca which is insensitive to tetrodotoxin is quite distinct from the well-known Na and K permeability systems that underlie the action potential. The results suggest that there must be a third potential-dependent permeability system in the squid axon membrane that is selective for Ca ions. We shall call this 'the late Ca channel'.

Entry of Ca through the late Ca channel is insensitive to tetrodotoxin and TEA but can be reduced or blocked reversibly by external Mg^{2+}, Mn^{2+} and Co^{2+} ions and by the organic calcium antagonist D-600. The mode of action of these agents has not been investigated further. There is some evidence that the onset of inhibition by Mn^{2+} is very fast (Baker, Meves &

Ridgway, 1973) suggesting that Mn^{2+} acts at a very superficial site. But we have not examined whether the blocking effect of Mn^{2+} or D-600 can be overcome by an increase of the Ca concentration as described by Katz & Miledi (1969*b*) and Kohlhardt *et al.* (1972).

TABLE 2. Comparison of the pharmacological and other properties of the tetrodotoxin-insensitive Ca permeability channels and the K permeability channels in squid axons. Data refer to experiments at 18–22° C

Treatment	Tetrodotoxin-insensitive Ca entry	K channel
Tetrodotoxin	No effect	No effect
TEA	No effect	Blocks K outward currents
Mg*	Blocks	Little effect
Mn	Blocks	Little effect
Co	Blocks	Little effect
La	Blocks	Blocks
D-600	Blocks	Little effect
Recovery from inactivation†	Slow (τ = 3·5–5 min)	Faster

* See Baker, Hodgkin & Ridgway (1971).
† See Baker, Meves & Ridgway (1973).

The substances which reduce or block Ca entry through the late Ca channel have little effect on the potassium currents. However, they reduce substantially the Na inward current, the rising phase of the action potential and the tetrodotoxin-sensitive Ca entry. The effect of Mn or Co on the Na current is in accord with observations of Blaustein & Goldman (1968) who found that the sodium inward current of the lobster axon is decreased by Co, Ni or high concentrations of Ca. Kohlhardt *et al.* (1972) describe only a 7 % decrease of the Na inward current in cardiac muscle fibres treated with D-600 in a concentration of 0·5 mg/l. The marked effect of D-600 on I_{Na} in our experiments may be due to the 200 times higher concentration we used (0·1 mg/ml.).

It should be stressed that the evidence for the late Ca channel and its sensitivity to agents such as Mn rests entirely on experiments with aequorin and it is possible that the action of Mn^{2+}, for instance, might result more from interference with the aequorin reaction than with Ca entry. This seems unlikely because (*a*) a number of different agents reduce Ca entry through the late Ca channel without completely abolishing Ca entry through the early tetrodotoxin-sensitive channel, (*b*) the rate of onset of inhibition is fast suggesting an external site of action, and (*c*) recovery is rapid. Nevertheless, it seems essential to verify these results using other techniques.

The existence of a late Ca channel may have particular relevance to excitation-secretion coupling. As already pointed out by Baker, Hodgkin

& Ridgway (1971) and Baker (1972) there is a striking parallel between the properties of the late Ca channel and the Ca-dependent transmitter release mechanism and this is further supported by the observation that Mn, Co, La and D-600 which block the late Ca channel can also block secretion (Katz & Miledi, 1969*b*; Miledi, 1971; Dreifuss, Grau & Nordmann, 1973).

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