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# TRANSMITTER INDUCED CALCIUM ENTRY ACROSS THE POST-SYNAPTIC MEMBRANE AT FROG END-PLATES MEASURED USING ARSENAZO III

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## SUMMARY

1. The $Ca^{2+}$ influx occurring across the post-synaptic membrane during transmitter action was studied at the frog neuromuscular junction, using the Ca sensitive dye arsenazo III to monitor the resulting changes in free myoplasmic $Ca^{2+}$ concentration.

2. Calibration experiments showed a linear relationship between the amount of $Ca^{2+}$ injected by ionophoresis into a muscle fibre, and the peak size of the arsenazo light absorbance record.

3. Ionophoretic application of acetylcholine (ACh) to voltage clamped end-plates gave rise to an arsenazo signal. The size of this response varied with the $Ca^{2+}$ concentration in the bathing solution.

4. The arsenazo light response increased in size steeply, and non-linearly, with hyperpolarization of the end-plate membrane, even when the end-plate current increased approximately linearly with hyperpolarization. The voltage dependence of the light response could be fitted well by an exponential with a voltage constant of 28 mV. Changes in $Ca^{2+}$ concentration of the bathing medium had little effect on this relationship.

5. At end-plates bathed in isotonic $CaCl_2$ solution the voltage dependence of both the arsenazo light response, and the end-plate current showed a closely similar, non-linear relationship.

6. Addition of 12 mM-$Co^{2+}$ to a bathing solution initially containing 12 mM-$Ca^{2+}$ substantially reduced the size of the arsenazo light response, and the voltage dependence of this response became more linear.

7. Arsenazo light responses were also recorded in response to transmitter release evoked by nerve stimulation. The size of the nerve evoked light response showed a non-linear voltage dependence, whilst the end-plate current was a linear function of membrane potential.

## INTRODUCTION

Several lines of evidence indicate that an influx of $Ca^{2+}$ ions accompanies transmitter activation of the post-synaptic membrane at the neuromuscular junction. These include studies using labelled $Ca^{2+}$ ions (Ahmad & Lewis, 1962; Jenkinson & Nicholls,

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1961; Evans, 1974), staining by $Ca^{2+}$ sensitive dyes (Csillick & Savay, 1963; Evans, 1974), and measurements of local contractures at end-plates of $K^+$ depolarized muscle fibres (Manthey, 1974). Electrophysiological experiments have also shown that $Ca^{2+}$ ions can carry a post-synaptic current, since miniature end-plate potentials, although of reduced size, have been recorded from muscles bathed in a medium in which $CaCl_2$ was substituted for NaCl (Takeuchi, 1963; Katz & Miledi, 1969*b*).

Transmitter induced post-synaptic influx of $Ca^{2+}$ is not confined to the neuromuscular junction: for instance it occurs also at the giant synapse of the squid (Katz & Miledi, 1969*a*). The influx of $Ca^{2+}$ ions across the post-synaptic membrane of the squid giant axon has been studied using the photoprotein aequorin as an intracellular $Ca^{2+}$ indicator. Influx was found to occur with activation resulting from either presynaptic nerve stimulation (Kusano, Miledi & Stinnakre, 1975*b*), or from ionophoretic application of glutamate (Miledi & Stinnakre, 1977). Depolarization of the membrane did not cause an appreciable $Ca^{2+}$ influx. The transmitter, or glutamate, activated influx was dependent upon the external $Ca^{2+}$ concentration, and varied linearly with the membrane potential of the post-axon.

We have examined the $Ca^{2+}$ influx at the frog neuromuscular junction, which occurs in response to nerve stimulation or application of acetylcholine, using the metallochromic indicator dye, arsenazo III (Miledi, Parker & Schalow, 1977*a*, *b*). Unlike the squid giant synapse, we find that the $Ca^{2+}$ influx varies as an exponential function of the membrane potential of the muscle fibre.

A preliminary account of this work has appeared (Miledi, Parker & Schalow, 1977*c*).

## METHODS

Experiments were made on the *cutaneous pectoris* muscle of *Rana temporaria*. The muscle was mounted in a glass bottomed chamber, and was stretched to a striation spacing of about 3·6 $\mu$m to reduce contraction artifacts. The basic composition of the bathing solution was (m. mole/l); NaCl, 120; KCl, 2; $CaCl_2$, 1·8; Na phosphate buffer 1; at pH about 7·2. In many experiments the $Ca^{2+}$ concentration was varied by substituting $CaCl_2$ for NaCl. Phosphate buffer was omitted in high $Ca^{2+}$ solutions, and the pH was adjusted to about 7 with KOH. Tetrodotoxin ($2 \times 10^{-6}$ g/ml.) was present in the solution in experiments where nerve stimulation was not used. The temperature was 6–9 °C.

End-plates were located visually under the microscope, and the end-plate region of a muscle fibre was penetrated with two micropipettes, separated by 100–200 $\mu$m, for intracellular recording and arsenazo III injection. The dye pipette was bevelled to a tip diameter of about 1 $\mu$m, and was filled with a solution of approximately 1 mM-arsenazo III (Sigma Chemical Co., Practical grade), together with 10 mM-$KHCO_3$, at pH 7. All experiments were performed with the end-plate voltage clamped, using the dye pipette as the current passing electrode. A third micropipette filled with 0·4 M-acetylcholine (ACh) chloride solution was used for ionophoretic application of ACh onto the end-plate. A backing current of 5–10 nA was applied to the pipette to prevent leakage of ACh. In some experiments this pipette was replaced by a KCl filled pipette, for local stimulation of the nerve terminal.

The procedures for injection of arsenazo III into muscle fibres, and optical recording of absorbance changes were as previously described (Miledi *et al.* 1977*b*; Parker, 1979). Briefly, a spot of light for measuring $Ca^{2+}$ dependent changes in absorption of the injected arsenazo III was focussed on the end-plate between recording and dye pipettes, and centred on the tip of the ACh pipette. Measurements of the light transmitted through the fibre were made at wavelengths of 532 and 602 nm, using two photomultipliers and interference filters. In the presence of $Ca^{2+}$, arsenazo III shows an increase in absorption at 602 nm, and a decrease at 532 nm. The difference signal of these two wavelengths is therefore sensitive to changes in $Ca^{2+}$ concentration,

but is little affected by movement artifacts during contraction of the fibre (Miledi *et al.* 1977*b*). Light responses are expressed as the fractional change in light transmitted through the fibre at the wave-length pair 532–602 nm.

The size of the light response elicited by a constant ACh pulse was found to increase approximately linearly with the amount of dye injected, up to a level giving a transmission decrease of about 0·2 at 532 nm. Injection at additional dye then caused a small decrease in response size. For example, in one fibre a maximum response was recorded when light transmission at 532 nm had decreased by 0·26, and after further injection of dye light transmission decreased by 0·42 while the response size was reduced by 22 %. Experiments were therefore performed by initially injecting fibres with arsenazo III to give a transmission decrease of about 0·25 at 532 nm, so that injection of any additional dye whilst clamping a fibre at hyperpolarized potentials would result in only a small change in response size.

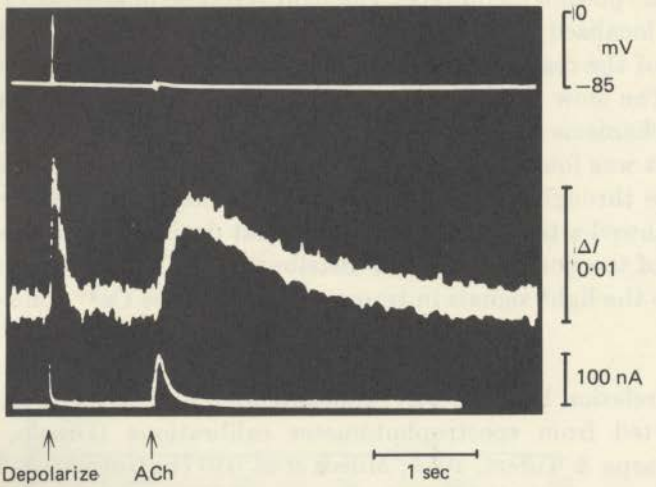


Fig. 1. Changes in light transmission at the wave-length pair 532–602 nm recorded from the end-plate region of a muscle fibre injected with arsenazo III. The fibre was voltage clamped. Top trace shows membrane potential, middle trace is light transmission, and lower trace is clamp current.

The first response was elicited by depolarizing the fibre to 0 mV for 10 msec. The second response resulted from application of a 20 msec ionophoretic pulse of ACh to the end-plate. Bathing solution contained 12 mM-$Ca^{2+}$. The optical trace was recorded with a time constant of 3·3 msec. Temperature 6 °C.

## RESULTS

### *Light responses to ionophoretic ACh pulses*

The change in light absorption at the wave-length pair 532–602 nm which accompanies activation of an arsenazo III loaded end-plate by an ionophoretic pulse of ACh is illustrated in Fig. 1. Control records obtained *before* injecting dye showed no appreciable change after ACh application. The light response induced by ACh does not arise as a consequence of change in membrane potential. In Fig. 1 the voltage clamp error during ACh action was less than 2 mV, and depolarizing pulses of up to 30 mV did not produce any light response.

The time course of the light response is much slower than the time course of the end-plate current. In Fig. 1, the light response begins to rise about 50 msec after the beginning of the end-plate current, and reaches a peak after about 400 msec. At this

time the end-plate current has returned virtually to zero. The subsequent decline in the light response lasts about 3 sec. This contrasts with the time course of the response to a strong, brief depolarizing pulse (Fig. 1), where the peak occurs in about 20 msec, and the falling phase has a time constant of about 50 msec (see also Miledi *et al.* 1977*b*).

The slow time course of the ACh light response probably results from slow diffusion of $Ca^{2+}$ within the muscle fibre. In the case of a depolarizing pulse, $Ca^{2+}$ ions are released from the sarcoplasmic reticulum, at sites distributed throughout the volume of the muscle fibre at spacings, in our experiments, of *ca.* 3·6 $\mu$m. Homogenous mixing of $Ca^{2+}$ with arsenazo III will therefore occur rapidly. Ionophoretic application of ACh on the other hand will produce a very localized region of raised $Ca^{2+}$ at the inside surface of the plasma membrane. The light response of arsenazo III will be non-linear for a very localized region of elevated $Ca^{2+}$ concentration, and an increase in the amplitude of the response will be expected as $Ca^{2+}$ ions diffuse out into the bulk of the fibre. The slow decline of the response may be due to saturation of the $Ca^{2+}$ uptake mechanisms of the fibre at the localized region. In agreement with this interpretation, it was found that when ionophoretic pulses of $Ca^{2+}$ were injected into the muscle fibre through an intracellular micropipette filled with 0·4 M-$CaCl_2$, the light response showed a time course similar to that during ACh activation (Fig. 2*A*).

In view of the non-homogenous distribution of $Ca^{2+}$ ions, we have not attempted to calibrate the light signals in terms of absolute free $Ca^{2+}$ concentration changes.

### *Linearity of the light response*

A linear relation between $Ca^{2+}$ concentration and arsenazo III light response has been reported from spectrophotometer calibrations (Dipolo, Requena, Brinley, Mullins, Scarpa & Tiffert, 1976; Miledi *et al.* 1977*b*; Gorman & Thomas, 1978). This relationship appears to hold also in the muscle fibre, using ionophoretic pulses of $Ca^{2+}$ to simulate the localized entry of $Ca^{2+}$ which is induced by ACh.

Results of one experiment are shown in Fig. 2*B*. The tip of the $Ca^{2+}$ pipette was positioned in the centre of the measuring light spot, which in this case was not at an end-plate region.

There was a good linear relationship between the total charge passed through the $Ca^{2+}$ pipette, and the peak size of the light response. This relationship was independent of ionophoretic pulse length for durations up to about 1 sec, and the pulses used covered most of the range of light response sizes seen with ACh activation.

### *Effect of changing outside $Ca^{2+}$ concentration*

We did not examine the effects of changing the $Ca^{2+}$ concentration in the bathing fluid whilst recording from a single fibre, because movements during changing of solutions frequently dislodged the micropipettes. Instead, Fig. 3 shows data obtained from thirteen fibres (eight muscles) in which solutions with $Ca^{2+}$ concentrations between 0·18 and 18 mM were used. In the 0·18 mM-$Ca^{2+}$ Ringer, 5 mM-$MgCl_2$ was added to stabilize the muscle membrane. To normalize for the different sizes of ionophoretic ACh pulses used in these experiments, the peak size of the light response has been scaled by the peak end-plate current, and is expressed as the light transmission change ($\Delta I$) per nA of ACh clamp current. The values shown were obtained with the

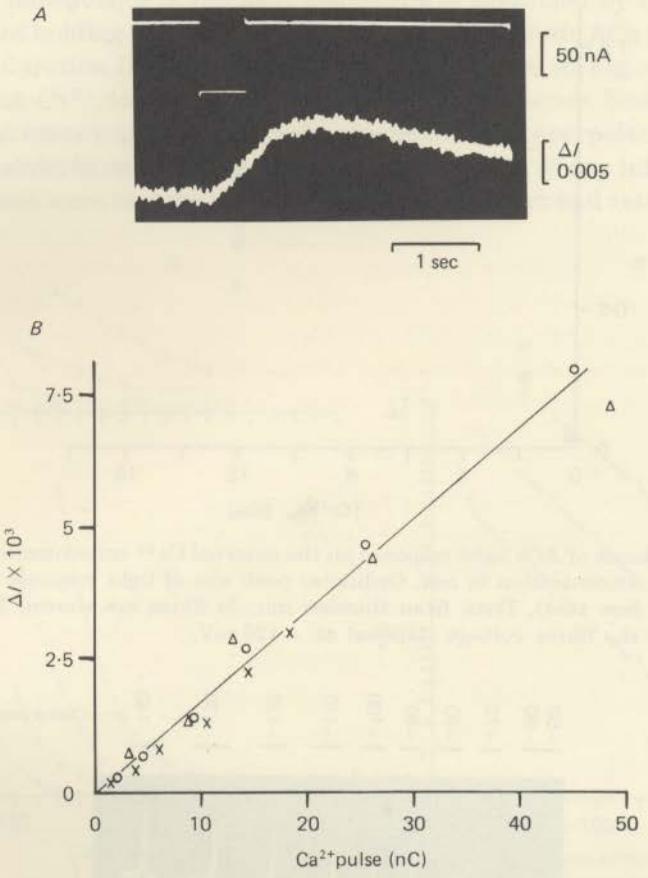


Fig. 2. *A*, light transmission records at 532–602 nm showing the response to ionophoretic injection of $Ca^{2+}$ into a muscle fibre previously loaded with arsenazo III. Upper trace is ionophoretic current through the $Ca^{2+}$ pipette, and lower trace is light transmission. The tip of the $Ca^{2+}$ pipette was positioned in the centre of the measuring light spot. The fibre was depolarized from $-90$ to $-55$ mV by the ionophoretic pulse; the same depolarization applied through the arsenazo III pipette gave no response. A backing current of 5 nA was applied to the $Ca^{2+}$ pipette. Optical recording time constant was 10 msec. Temperature 8 °C. *B*, relationship between light response and amount of $Ca^{2+}$ injected. Abscissa: charge injected through $Ca^{2+}$ pipette in nC. Ordinate: peak change in light transmission. Ionophoretic currents of 17 nA (×), 41 nA (○), and 77 nA (△) were used, with pulse durations varying between 50 and 1100 msec. A backing current of 9 nA was applied to the $Ca^{2+}$ pipette, and the values on the abscissa have been corrected for this. Data from same fibre as *A*.

fibres clamped at $-120$ mV, to give responses sufficiently above the noise level in low $Ca^{2+}$ solution (see below).

The size of the normalized light response depended strongly upon the external $Ca^{2+}$ concentration. In the 0·18 mM-$Ca^{2+}$ solution responses were barely detectable, even with large ACh pulses giving end-plate currents of 250 nA. The data of Fig. 3 do not lie exactly on a straight line, but the deviation from linearity may not be significant, considering the errors introduced by pooling results from different fibres.

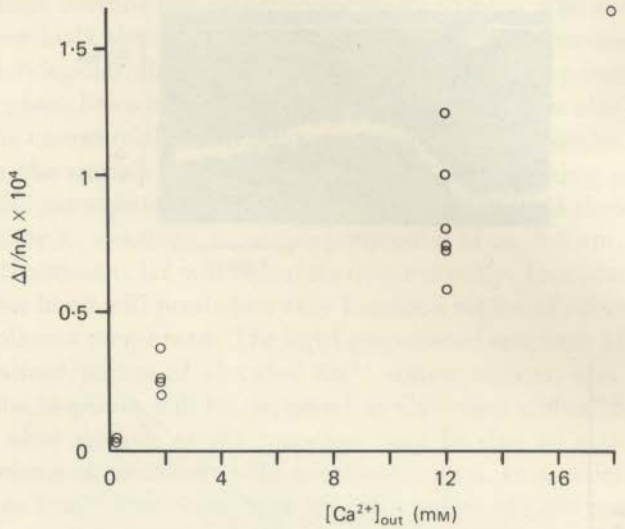


Fig. 3. Dependence of ACh light response on the external $Ca^{2+}$ concentration. Abscissa: external $Ca^{2+}$ concentration in mM. Ordinate: peak size of light response per nA end-plate current (see text). Data from thirteen muscle fibres are shown. Results were obtained with the fibres voltage clamped at $-120$ mV.

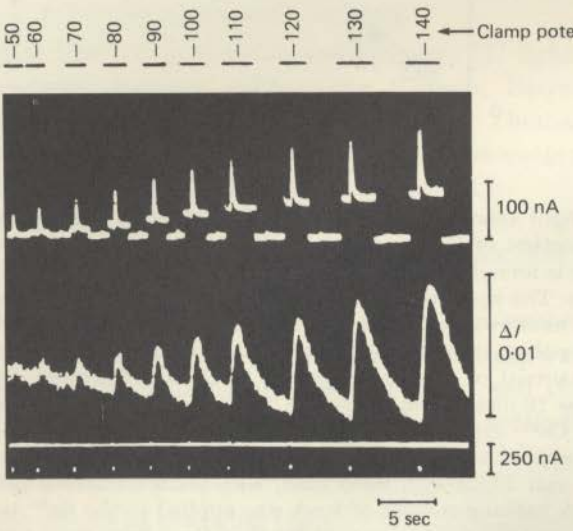


Fig. 4. Effect of changes in membrane potential on the end-plate current, and light response produced by ionophoretic application of ACh. Upper trace is clamp current, middle trace is light transmission at 532–602 nm, and lower trace is ACh ionophoretic current. The fibre was voltage clamped at $-50$ mV and hyperpolarized to the potentials indicated for the duration of the bars. There is a slow drift in the optical recording, which was probably due in part to injection of additional arsenazo III into the fibre during periods of hyperpolarization. Ringer solution contained 12 mM-$Ca^{2+}$. Optical recording time constant, 33 msec. Temperature 7 °C.

The voltage dependence of the light response was examined by clamping the end-plate at different holding potentials, and applying ionophoretic ACh pulses of constant intensity and duration (Fig. 4). In the experiment shown in Fig. 4 the muscle was bathed in 12 mM-$Ca^{2+}$, to increase the size of the light response. Since the dye pipette was used as the current passing electrode, the periods of hyperpolarization were kept as short as possible, in order to minimize further injection of dye into the fibre. However, test voltage were maintained until the clamp current had returned to the base

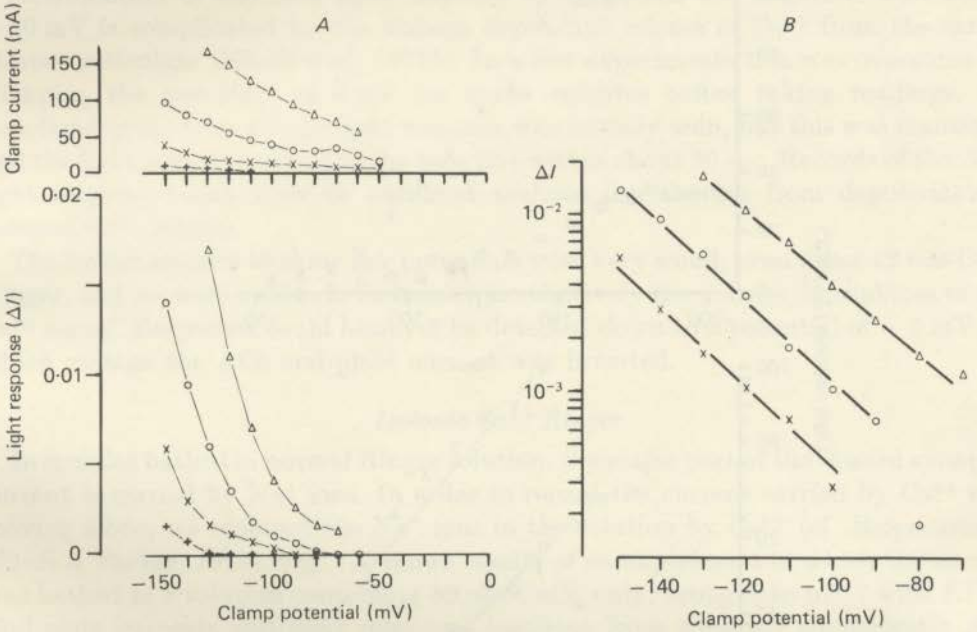


Fig. 5. *A*, relationship between membrane potential, and end-plate current (upper graph), or light response (lower graph), elicited by constant ionophoretic ACh pulses. Data were obtained from records similar to Fig. 3. The four curves shown are for ionophoretic pulses of 0·35 (+), 0·7 (×), 1·4 (○), and 3·5 (△) nC, (pulse duration 0·5, 1, 2 and 5 msec respectively). The muscle was bathed in 12 mM-$Ca^{2+}$ Ringer solution. Temperature 6·5 °C. *B*, the data of the light responses from *A*, replotted on semilogarithmic co-ordinates. The responses from the lowest intensity ACh pulse have been omitted. Lines were fitted by eye.

line after ACh application. Changes in response size resulting from an increase in dye concentration were small, since dye was injected initially to a level where further increases would cause only a slight change in response (see Methods). This was checked in six fibres by repeating runs similar to Fig. 4, using a constant ACh dose. At a given voltage, the light response in the second run was 107 % (± 13·9 % S.D.) of the size in the first run.

The relationship between the peak light response and the membrane potential measured at one end-plate, with four different ACh doses, is shown in Fig. 5*A*. The peak ACh-currents induced by the respective ACh pulses are also plotted.

The relationship between membrane potential and end-plate ACh-current over the range investigated (− 50 to − 150 mV) was approximately linear, although showing a

small upward curvature with hyperpolarization (cf. Dionne & Stevens, 1974). In contrast the light response showed a highly non-linear relationship, increasing steeply with increasing hyperpolarization.

Fig. 5*B* shows the light response data of Fig. 5*A* plotted on semilogarithmic co-ordinates. The results are well fitted by straight lines, demonstrating that the light response to the ionophoretic pulses of ACh is an exponential function of membrane

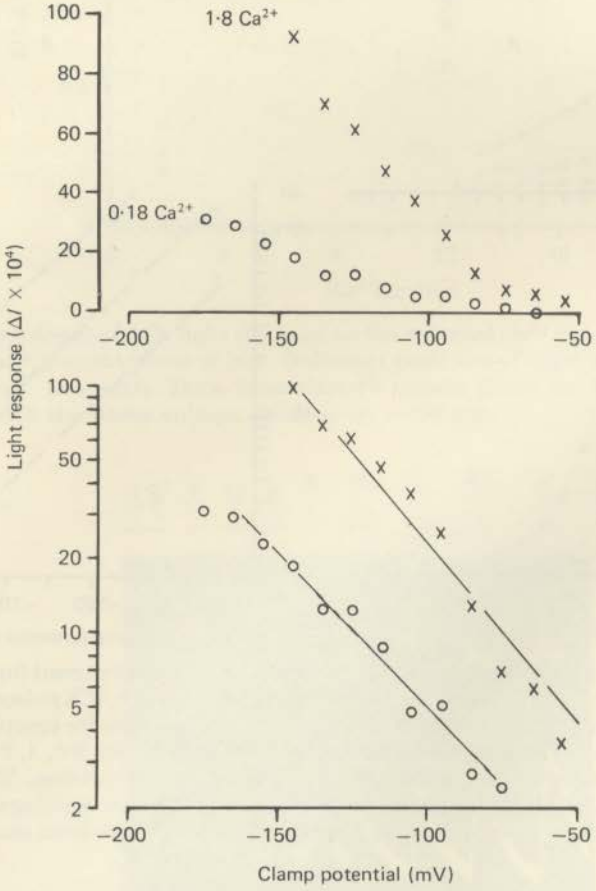


Fig. 6. Membrane potential dependence of the ACh light response measured from two fibres bathed in Ringers solution containing 1·8 mM (×), and 0·18 mM (○) $Ca^{2+}$. Abscissa, clamp potential. Ordinate, peak change in light transmission at 532–602 nm. The results are shown plotted on linear (upper graph), and semilogarithmic (lower graph) co-ordinates. The 0·18 mM-$Ca^{2+}$ Ringer contained additionally 5 mM-$MgCl_2$. Temperatures: 7 °C (×), and 5 °C (○).

hyperpolarization. The slopes of the lines for the three ACh doses are closely similar. An e-fold change in response was produced by a change in potential of 28 mV for the largest pulse, and 25·5 mV for the two smaller pulses. From a total of eight experiments on muscles bathed in 12 mM-$Ca^{2+}$ ringer, a mean value of $27{\cdot}9 \pm 3{\cdot}5$ mV ($\pm 1$ S.D.) was obtained for the voltage change required to give an e-fold change in light response.

A similar voltage dependence was found when the bathing solutions contained normal (1·8 mM) or low (0·18 mM) $Ca^{2+}$ concentrations, although the size of the responses was reduced. Fig. 6 shows results from two fibres, where solutions containing 1·8 and 0·18 mM-$Ca^{2+}$ were used. The light response in both solutions varied as an exponential function of membrane potential. In the experiments shown, an e-fold change in response resulted from a potential change of 30 mV in 1·8 mM-$Ca^{2+}$, and 34 mV in 0·18 mM-$Ca^{2+}$.

Examination of the ACh light response at potentials less negative than about $-50$ mV is complicated by the voltage dependent release of $Ca^{2+}$ from the sarcoplasmic reticulum (Miledi *et al.* 1977*b*). In a few experiments this was overcome by clamping the end-plate at 0 mV for a few minutes before taking readings. On depolarizing the fibre a large light response was initially seen, but this was transient, and the light record returned to the base line within about 30 sec. Records of the ACh light response could then be obtained without interference from depolarization induced $Ca^{2+}$ release.

The light responses at these low potentials were very small, even using 12 mM-$Ca^{2+}$ Ringer, and we were unable to examine quantitatively the voltage dependence of the $Ca^{2+}$ signal. Responses could however be detected down to a potential of $-5$ mV, at which voltage the ACh end-plate current was inverted.

### *Isotonic $Ca^{2+}$ Ringer*

In muscles bathed in normal Ringer solution, the major part of the inward synaptic current is carried by $Na^+$ ions. In order to record the current carried by $Ca^{2+}$ ions moving alone, we replaced the $Na^+$ ions in the solution by $Ca^{2+}$ (cf. Bregestovski, Miledi & Parker, 1979). Fig. 7*A* shows results of an experiment in which the muscle was bathed in a solution containing 80 mM-$CaCl_2$ only, brought to pH 7 with KOH. End-plate currents and light responses resulting from constant ionophoretic ACh pulses were measured as before.

In isotonic $Ca^{2+}$ solution the light response displayed the usual exponential voltage sensitivity, and in this experiment this behaviour was also mirrored by the end-plate current. In other experiments in isotonic $Ca^{2+}$ (P. Bregestovski, R. Miledi & I. Parker, unpublished data), it has been found that the voltage dependence of the ACh current has an exponential form if the tip of the ACh pipette is not close to the end-plate, but that with close application the voltage relationship could be fitted well by two straight line segments. All the arsenzo experiments reported here were performed with non-focal positioning of the ACh pipette. Fig. 7*B* shows the data of Fig. 7*A* replotted on semilogarithmic coordinates. Both end-plate currents, and light responses, lie well on straight lines with similar slopes. Potential changes of 28 and 29 mV gave e-fold changes in respectively the light response and the end-plate current. Plotting the light response against peak endplate current on linear co-ordinates gave a good fit to a straight line, with a slope of $3{\cdot}12 \times 10^{-4}$ $\triangle I$ $nA^{-1}$. A total of five runs on two other fibres, using ACh pulses varying over a tenfold amplitude range, gave similar results. The voltage dependence of both the peak current and the light response were fitted well by exponentials, e-fold changes in current and light occurring with voltage changes of $34{\cdot}2 \pm 2{\cdot}5$ and $33{\cdot}8 \pm 3$ mV (mean $\pm$ 1 S.D.) respectively.

The size of the light response produced by a given charge movement of $Ca^{2+}$ ions

across the end-plate membrane is in reasonable agreement with that predicted from the experiment with ionophoretic $Ca^{2+}$ injection into the muscle fibre. For example, in Fig. 7*A* a light response of $\triangle I$ 0·02 was recorded at −190 mV, and the total charge movement during the endplate current was 14 nC. From Fig. 2, the same size of light response would be expected from an ionophoretic $Ca^{2+}$ pulse of 116 nC. Assuming a transport number of 0·1 for the $Ca^{2+}$ pipette (Kusano *et al.* 1975*a*), this gives the total charge carried by $Ca^{2+}$ ions as 11·6 nC.

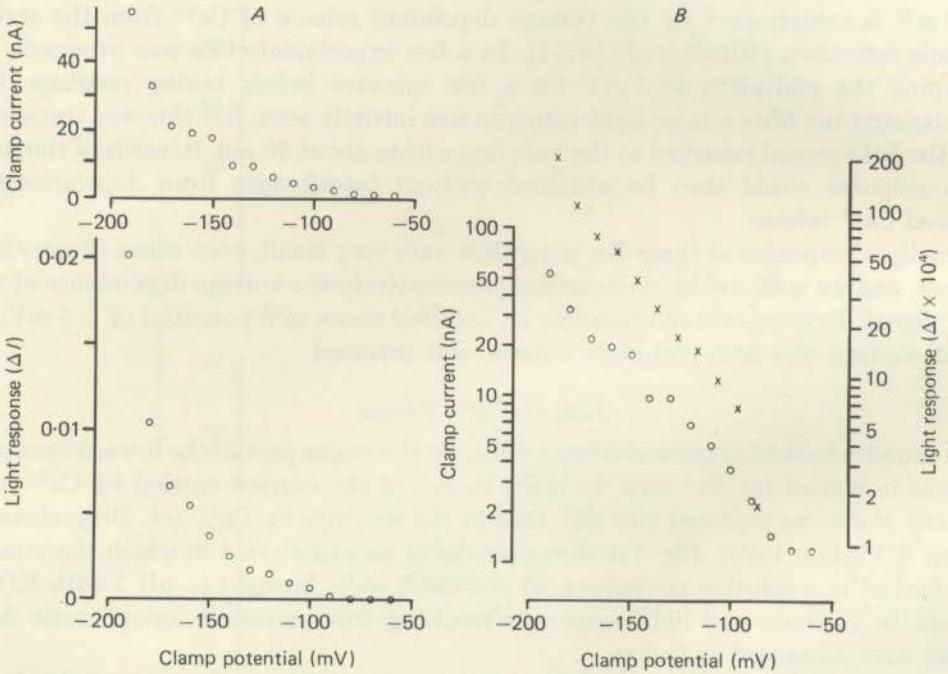


Fig. 7. *A*, voltage dependence of the ACh end-plate current (upper graph), and light response (lower graph), recorded from a fibre bathed in 80 mM-$CaCl_2$ solution. The ionophoretic ACh pulse was 800 nA, and 5 msec duration. Temperature 5·5 °C. *B*, the data of *A* plotted on semilogarithmic co-ordinates, showing the peak end-plate currents (○), and light responses (×), as a function of membrane potential.

### *Action of $Co^{2+}$ on the light response*

$Co^{2+}$ ions are known to block the late phase of depolarization induced $Ca^{2+}$ entry into squid giant axons (Baker & Glitsch, 1975). The effects of $Co^{2+}$ on the ACh induced light response were examined by obtaining control records from a muscle bathed in 12 mM-$Ca^{2+}$ Ringer, and then repeating these observations about 15 min after $CoCl_2$ had been added to the bath to a final concentration of 12 mM.

Fig. 8*A* shows one experiment where the voltage dependence of the light response was examined before and after addition of $Co^{2+}$. The position of the ACh pipette may have shifted slightly between the two sets of readings, and so the small decrease in size of the end-plate current is of doubtful significance. The light response was however considerably reduced in size after addition of $Co^{2+}$, and the steeply curved voltage

dependence became more linear. Fig 8*B* illustrates results from three other fibres, which had been bathed in a solution containing 12 mM-$Co^{2+}$ and 12 mM-$Ca^{2+}$ for about 1 hr before recording. In all cases the voltage dependence of the light response is approximately linear, and the size of the response considerably reduced. For example at $-120$ mV the mean light response per nano-ampere synaptic current was $1{\cdot}5 \times 10^{-5}$ $\triangle$I $nA^{-1}$, compared with $8{\cdot}5 \times 10^{-5}$ $\triangle I$ $nA^{-1}$ in 12 mM-$Ca^{2+}$ Ringer (Fig. 3).

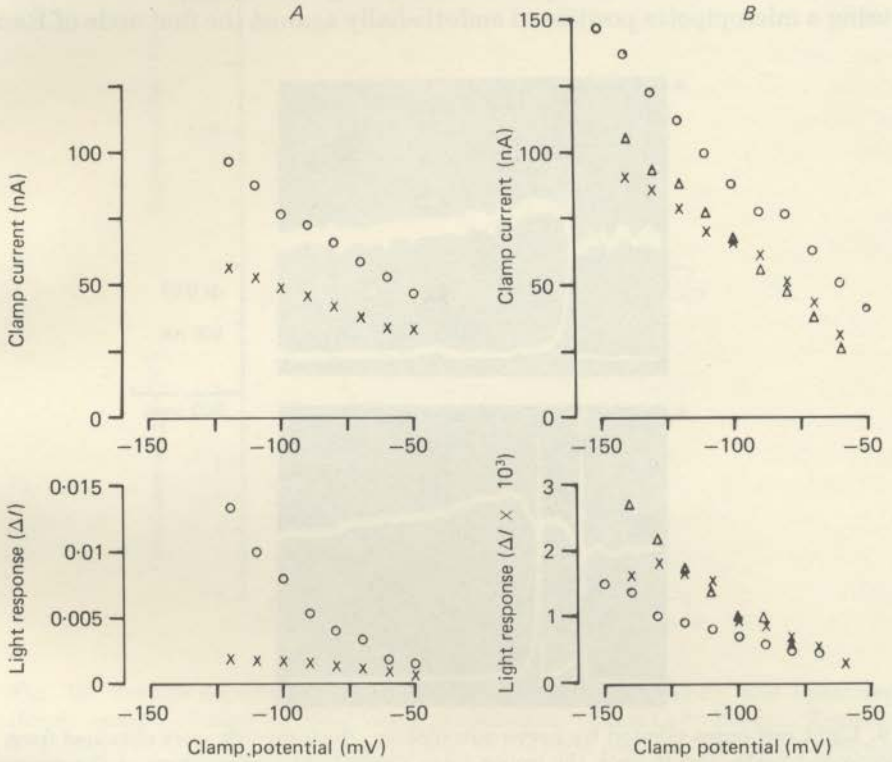


Fig. 8. *A*, effect of Co on the voltage sensitivity of the end-plate current, and light response, produced by constant ionophoretic ACh pulses. The records were obtained from a single fibre; using a Ringer solution containing 12 mM-$Ca^{2+}$ (○), and 10 min after adding 12 mM-$CoCl_2$ to the solution (×). Temperature 6·5 °C. *B*, data from three fibres which had been bathed in Ringer solution containing 12 mM-$Co^{2+}$ and 12 mM-$Ca^{2+}$ for about 1 hr. Co-ordinates as in *A*. Temperature 6–7 °C.

The action of $Co^{2+}$ could be reversed by washing the muscle with normal Ringer solution. We were unable to obtain records from the same fibre before and after washing, but results from several fibres which had been washed for about 50 min following immersion in Ringer solution containing 12 mM-$Co^{2+}$ all showed the normal exponential relationship between membrane potential and light response.

*Nerve stimulation*

It was difficult to study the light response produced by nerve evoked transmitter release. Initially, (+)-tubocurarine chloride was used to avoid muscle contraction, but then the end-plate currents were relatively small, and no light response was visible under these conditions. Experiments were therefore performed in the absence of curare by locally stimulating only the axon leading to the end-plate under investigation, using a micropipette positioned endothelially against the first node of Ranvier

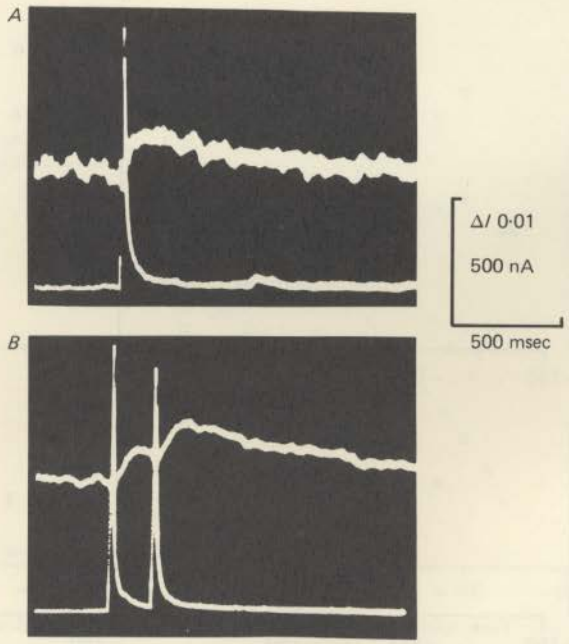


Fig. 9. Light responses elicited by nerve stimulation. Both records were obtained from the same end-plate, and in each the upper trace shows light transmission at the wavelength pair 532–602 nm, and the lower trace clamp current. The end-plate was voltage clamped to a potential of −170 mV.

A single nerve stimulus was given in *A*, and two stimuli at an interval of 200 msec in B. Optical recording time constant was 3·3 msec for *A*, and 33 msec for *B*. Temperature 7 °C.

from the nerve terminal (cf. Katz & Miledi 1977). Pipette positive current pulses of 1 msec duration were used for stimulation, and the current was adjusted to be above that required to give a maximal endplate current. The fibre was maintained under voltage clamp to prevent the generation of action potentials. Twitches did occur in a few other fibres in the muscle, due to impulses propagating back along the axon onto other fibres in the same motor unit, but this did not produce any serious artifact.

The light response resulting from a single nerve stimulus is shown in Fig. 9*A*. The responses were very small, and to obtain the record shown the fibre was bathed in 12 mM-$Ca^{2+}$ ringer, and was hyperpolarized to −170 mV. Paired stimuli gave rise to light responses which appeared to summate linearly (Fig. 9*B*). Various stimulus

intervals between 25 and 200 msec were examined in one fibre, and we found no evidence for facilitation of the light response to the second stimulus, as has been reported for the squid giant synapse (Kusano *et al.* 1975*b*).

The voltage dependence of the light response to single nerve stimuli was investigated in the same way as for the ACh response. Fig. 10 shows results from one fibre,

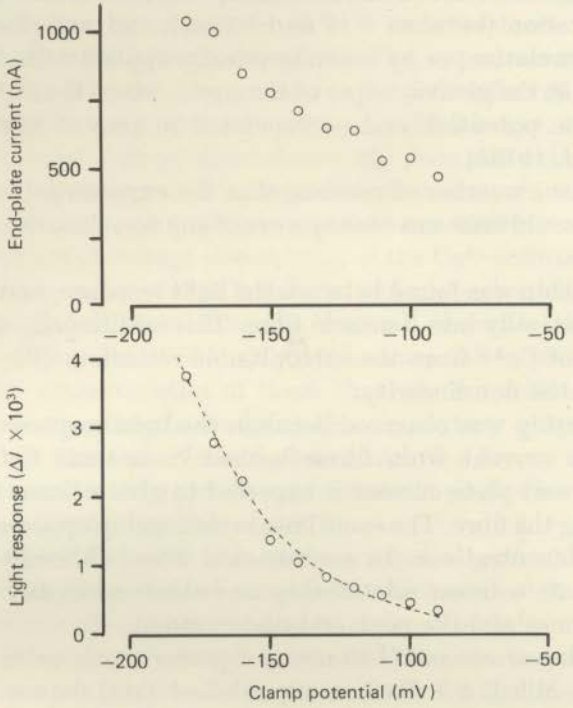


Fig. 10. Voltage dependence of end-plate current (upper graph), and light response (lower graph), elicited by local nerve stimulation. 12 mM-$Ca^{2+}$ Ringer. Temperature 7 °C. Same fibre as Fig. 9. The curve fitted to the light response data is an exponential, with a voltage constant of 34 mV.

which was bathed in 12 mM-$Ca^{2+}$ Ringer solution. The end-plate current varied linearly with membrane potential, whilst the light response showed an exponential relationship, with an e-fold change in response produced by a potential change of 34 mV. A second experiment, on a different muscle, gave a similar voltage dependence for the light response, with a voltage constant of 35 mV.

## DISCUSSION

A number of observations strongly suggest that the changes in light transmission recorded from arsenazo III loaded end-plates, in response to transmitter action, result from an influx of $Ca^{2+}$ ions into the muscle fibre from the bathing solution. These include the dependence of the light response upon the external $Ca^{2+}$ concentration, the antagonistic action of externally applied $Co^{2+}$ ions, and the observation that the response is present in fibres where $Ca^{2+}$ release from the sarcoplasmic reticulum

has been inactivated by prolonged depolarization. The influx of $Ca^{2+}$ ions is expected to be a linear function of the external $Ca^{2+}$ concentration (Meves, 1968), and the size of the response was found to vary approximately linearly with $[Ca]_{out}$.

The most interesting feature of the $Ca^{2+}$ influx is its exponential dependence upon membrane potential. An e-fold change in size of the light response was produced by a change in potential of about 28 mV. The relationship was little affected by changes in external $Ca^{2+}$ concentration (between 0·18 and 80 mM), and was closely similar for activation by nerve stimulation, or by ionophoretically applied ACh. This behaviour differs from that found at the giant synapse of the squid, where the $Ca^{2+}$ influx varied linearly with membrane potential, and extrapolated to zero at between +150 to +300 mV (Kusano *et al.* 1975*b*).

It is most unlikely for a number of reasons, that the exponential voltage dependence which we observe could arise as a consequence of any non-linearity of the arsenazo III recording technique.

(i) A linear relationship was found between the light response, and the amount of $Ca^{2+}$ injected ionophoretically into a muscle fibre. This additionally suggests that a $Ca^{2+}$ triggered release of $Ca^{2+}$ from the sarcoplasmic reticulum (Ford & Podolsky, 1972) is not a cause of the non-linearity.

(ii) A linear relationship was observed between the light response, and the peak ACh induced endplate current from fibres bathed in isotonic $CaCl_2$ solution; a condition in which the end-plate current is expected to give a linear measure of the amount of $Ca^{2+}$ entering the fibre. The same linear relationship appears to hold also at lower external $Ca^{2+}$ concentrations. In experiments where different doses of ACh were applied (e.g. Fig. 5), a linear relationship was observed at any *fixed* potential between the light response and the peak end plate current.

(iii) Experiments using arsenazo III to monitor postsynaptic entry of $Ca^{2+}$ at the squid giant synapse (R. Miledi & I. Parker, unpublished data) show a linear relationship between membrane potential and $Ca^{2+}$ entry, similar to that observed with aequorin by Kusano *et al.* (1975*b*).

(iv) Experiments using aequorin at the frog neuromuscular junction (R. Miledi, J. Stinnakre & T. Takahashi, unpublished data) are in agreement with our results in showing a steeply non-linear voltage dependence of the $Ca^{2+}$ light response.

At strongly hyperpolarized potentials the $Ca^{2+}$ influx may begin to contribute significantly to the total end-plate current, even with normal (1·8 mM-$Ca^{2+}$) Ringer solution. For example, the experiment of Fig. 7. using isotonic $Ca^{2+}$ bathing solution, gave a light response of $3{\cdot}12 \times 10^{-4}$ $\triangle I$ per nA of $Ca^{2+}$ current across the end-plate membrane. In the experiment of Fig. 6, where the muscle was bathed in 1·8 mM-$Ca^{2+}$ Ringer, a light response of about $6 \times 10^{-3}$ $\triangle I$ was observed at a potential of $-125$ mV, with an end-plate current of 210 nA. Of this current, about 20 nA would have to have been carried by $Ca^{2+}$, in order to account for the size of the light response. This calculation is approximate, and does not take into account factors such as differences in fibre diameter and amounts of dye injected, in the two experiments. It does however suggest that the contribution of the $Ca^{2+}$ current is not negligible at hyperpolarized potentials, and may be an additional factor in producing a non-linear relationship between end-plate current and membrane potential (see also Dionne & Stevens, 1974).

On the basis of the Goldman–Hodgkin–Katz model of ion permeation (Goldman, 1943; Hodgkin & Katz, 1949; Meves, 1968) the $Ca^{2+}$ influx across the end-plate should approximate a linear function of membrane potential for values hyperpolarized from about $-30$ mV (extrapolating to zero at zero mV), but curving off at potentials depolarized from $-30$ mV, to reach zero at approximately $+130$ mV. The observed voltage dependence of the $Ca^{2+}$ influx therefore suggests that the permeability of the endplate membrane to $Ca^{2+}$ is not constant, but shows a voltage dependent increase with hyperpolarization. The experiments with cobalt further suggest that there may be two components to the $Ca^{2+}$ influx; (i) a small Co insensitive influx, which varies linearly with potential, and (ii) a larger, cobalt sensitive influx, which has a non-linear voltage dependence. Mg does not exhibit a similar blocking action to Co, since an exponential voltage dependence was found in fibres bathed in a Ringer solution containing 5 mM-$Mg^{2+}$ and 0·18 mM-$Ca^{2+}$.

The steep exponential voltage dependence of the $Ca^{2+}$ influx might be explained in two ways: (i) by the existence of ACh induced end-plate channels specific for $Ca^{2+}$ ions, and having properties different from the 'normal' channels which regulate $Na^+$ and $K^+$ permeability (Takeuchi, 1963*b*). (ii) $Ca^{2+}$ ions are able to pass through the $K^+/Na^+$ channels, but the characteristics of these channels are different for the passage of $Ca^{2+}$ ions. Recent experiments studying the elementary properties of the channels through which $Ca^{2+}$ ions cross the end-plate membrane favour the second hypothesis, and have also demonstrated a non-linear increase in single channel $Ca^{2+}$ current with hyperpolarization (Bregestovski *et al.* 1979).

The function of the $Ca^{2+}$ influx is unclear, especially considering that it becomes prominent only at hyperpolarized potentials, which are not encountered in life. The interior of the muscle fibre is subject also to changes in $Ca^{2+}$ concentration occurring during contractile activation, but it is possible that the transmitter activated $Ca^{2+}$ influx may give rise to a high local concentration at the inside surface of the end-plate membrane, which could exceed the levels resulting from $Ca^{2+}$ release from the sarcoplasmic reticulum. It has been postulated that the local intracellular $Ca^{2+}$ concentration plays a role in the mechanisms of desensitization of the end-plate (Devore & Nastuk, 1977; Scubon-Mullieri & Parsons, 1977; Manthey, 1974; Nastuk & Parsons, 1970), and it is interesting that the rate of desensitization is a strong function of membrane potential (Magazanik & Vyskočil, 1970).

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