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CHANGES IN THRESHOLD FOR CALCIUM TRANSIENTS IN FROG SKELETAL MUSCLE FIBRES OWING TO CALCIUM DEPLETION IN THE T-TUBULES

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SUMMARY

1. Strength–duration curves were measured for voltage-clamp depolarizations required to elicit a just detectable rise in intracellular calcium, as monitored using arsenazo III, in frog twitch muscle fibres.

2. In normal Ringer solution, the threshold for a 5 sec duration depolarization was about 5 mV more negative than for a 200 msec duration pulse.

3. The shift in threshold comparing 200 msec and 5 sec pulses was almost abolished in bathing solutions including magnesium or nickel (4 mM), or where the free calcium concentration was buffered. The shift in threshold was little changed by substitution of barium for calcium.

4. These results can be explained by supposing that the 5 sec depolarization activates an inward calcium flux across the T-tubule membrane, which decreases the calcium concentration in the tubules, and hence alters the threshold for activation of excitation–contraction (e.–c.) coupling because of surface charge effects.

INTRODUCTION

We have recently described strength–duration curves for activation of calcium release from the sarcoplasmic reticulum (s.r.) of twitch muscle fibres (Miledi, Parker & Zhu, 1983*b*). Calcium release was measured using arsenazo III as an intracellular calcium monitor, and curves were derived by measuring the depolarization required to give a just detectable calcium signal for any given pulse duration. Data obtained in this way matched closely the strength–duration curves obtained using the threshold for mechanical contraction as an indicator of a response, a technique which has been used for many years (Adrian, Chandler & Hodgkin, 1969; Almers & Best, 1976; Kovács & Schneider, 1978). As the pulse duration was made longer, the depolarization required to elicit a threshold calcium signal decreased, and appeared to reach a minimum value (the rheobase potential) for durations longer than about 20 msec (at 10 °C).

However, in later experiments we found that the rheobase potential shifted to potentials about 5 mV more negative if a very long (5 sec) depolarizing pulse was used, as compared to a pulse of a few hundred milliseconds duration. This paper is concerned with an investigation of this phenomenon.

METHODS

Experiments were performed on the cutaneous pectoris muscle of *Rana temporaria*, at a temperature of 10 °C. Techniques for recording intracellular calcium transients using arsenazo III, and for voltage clamping fibres were as described previously (Miledi, Parker & Zhu, 1982, 1983*a*, *b*). Table 1 gives compositions of the bathing solutions used. When changing the bathing solution, the fluid in the chamber was washed several times, and the muscle allowed to equilibrate in the new solution for at least 15 min before readings were taken. All solutions included 5×10^{-7} g ml.$^{-1}$ tetrodotoxin (TTX) and 20 mM-tetraethylammonium bromide (TEA) to reduce the voltage-activated sodium and potassium currents, and hence improve clamp quality.

TABLE 1. Composition of solutions

Solution	NaCl	K^+	HEPES	EGTA	Ca^{2+}	Mg^{2+}	Ba^{2+}	Ni^{2+}	Sodium citrate
Normal Ringer	120	2	4	—	2	—	—	—	—
0 Ca^{2+} + 5 mM-Mg^{2+}	120	2	4	1	—	5	—	—	—
0 Ca^{2+} + 2 mM-Ba^{2+}	120	2	4	—	—	—	2	—	—
Ringer + 4 mM-Ni^{2+}	120	2	4	—	2	—	—	4	—
Ringer + 4 mM-Mg^{2+}	120	2	4	—	2	4	—	—	—
Ca^{2+} buffer Ringer	80	2	4	—	11	—	—	—	13
1 mM-Ca^{2+} Ringer	120	2	4	—	1	—	—	—	—

Concentrations are given in millimolar. All solutions were at pH 7·2, except the Ca^{2+}-buffered Ringer, which was at pH 7·1. All solutions included also 5×10^{-7} g ml.$^{-1}$ TTX and 20 mM-TEA.

RESULTS

Strength–duration curves for calcium signal

Strength–duration curves were obtained by adjusting the amplitude of a test depolarizing pulse, so that for any given pulse duration a just detectable arsenazo signal was obtained (Fig. 1 and see Miledi *et al.* 1983*b*). For pulse durations shorter than about 20 msec, the pulse amplitude required to elicit a calcium signal increased steeply with decreasing duration, as we have described previously (Miledi *et al.* 1983*b*). At longer pulse durations the relationship flattened, but a change in threshold of about 5 mV to more negative values was seen when the duration was increased from 100 msec to 5 sec. These effects are illustrated in Fig. 2, where it can be seen that the strength–duration curve appears to comprise two distinct components. The first is the steep relationship at short pulse durations, which becomes almost flat for durations longer than about 50 msec (Fig. 2*A*). However, on a slower time scale (Fig. 2*B*) the threshold potential continues to fall with increasing pulse duration, and reaches a minimum value only at durations longer than about 2 sec.

Complete measurements of strength–duration curves at long pulse durations were difficult to obtain, and instead we quantified the extent of the second (slow) component by comparing thresholds for pulse durations of 200 msec and 5 sec. The protocol generally used was to measure the threshold first for the 200 msec pulse, then for the 5 sec pulse, and finally to repeat the measurement with the 200 msec pulse. An interval of at least 1 min was allowed after each test with the 5 sec pulse. The two sets of measurements with the 200 msec pulse gave consistent values for the

threshold; for example, of eleven fibres examined in 1 or 2 mM-calcium Ringer solution, nine fibres showed no detectable difference when measured before and after the 5 sec pulses, and two fibres showed differences of about 1 mV.

A mean difference in threshold of 5·1 mV (S.E. of mean 0·4 mV) was found from measurements on nine fibres (four muscles) bathed in normal Ringer solution, comparing pulse durations of 200 msec and 5 sec.

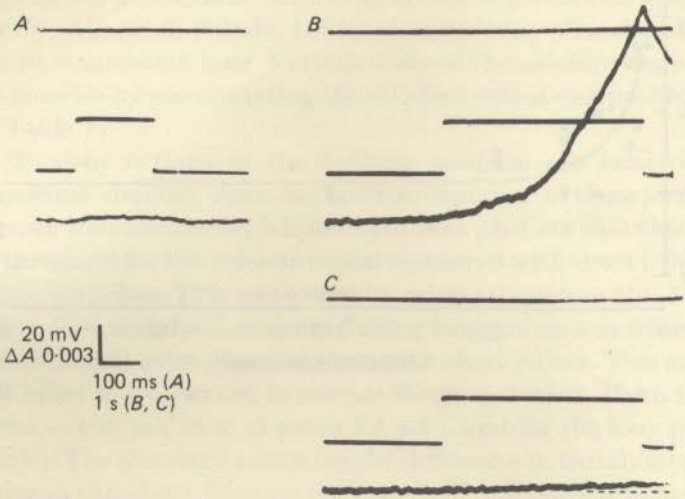

Fig. 1. Arsenazo signals elicited by voltage-clamp depolarizing pulses close to threshold. In each frame the top trace is the 0 mV reference line, the middle trace membrane potential, and the lower the arsenazo light absorbance signal recorded at 650–700 nm. *A*, the pulse duration was 200 msec, and the pulse amplitude was adjusted to elicit a just detectable arsenazo signal. *B*, the pulse amplitude was the same as in *A*, but with a duration of 5 sec. A large arsenazo signal was elicited. *C*, the same as in *B*, except that the pulse amplitude was decreased by 5 mV. The arsenazo signal was then just detectable (dashed line indicates base line). Temperature 10 °C.

Calcium-depletion hypothesis

At first sight, it may not appear surprising that a more negative threshold is seen with long depolarizing pulses. The threshold calcium signal presumably represents a balance between calcium release and re-uptake by the s.r., and this might be closer to equilibrium during long pulses, thus giving a higher cytoplasmic free calcium concentration. However, the decline of the arsenazo signal following an action potential follows a roughly exponential time course, with a time constant of about 80 msec (Miledi *et al.* 1982), so that a pulse duration of 200 msec would be expected to give a calcium level close to equilibrium. Also, a strong argument against this explanation is provided by the observation that changes in divalent ion composition of the bathing solution have a considerable effect on the change in threshold seen with long pulses. For example, addition of 4 mM-magnesium to the Ringer solution almost abolishes the shift in threshold comparing 200 msec and 5 sec duration pulses (see later).

An alternative hypothesis is that the change in threshold with long pulses results

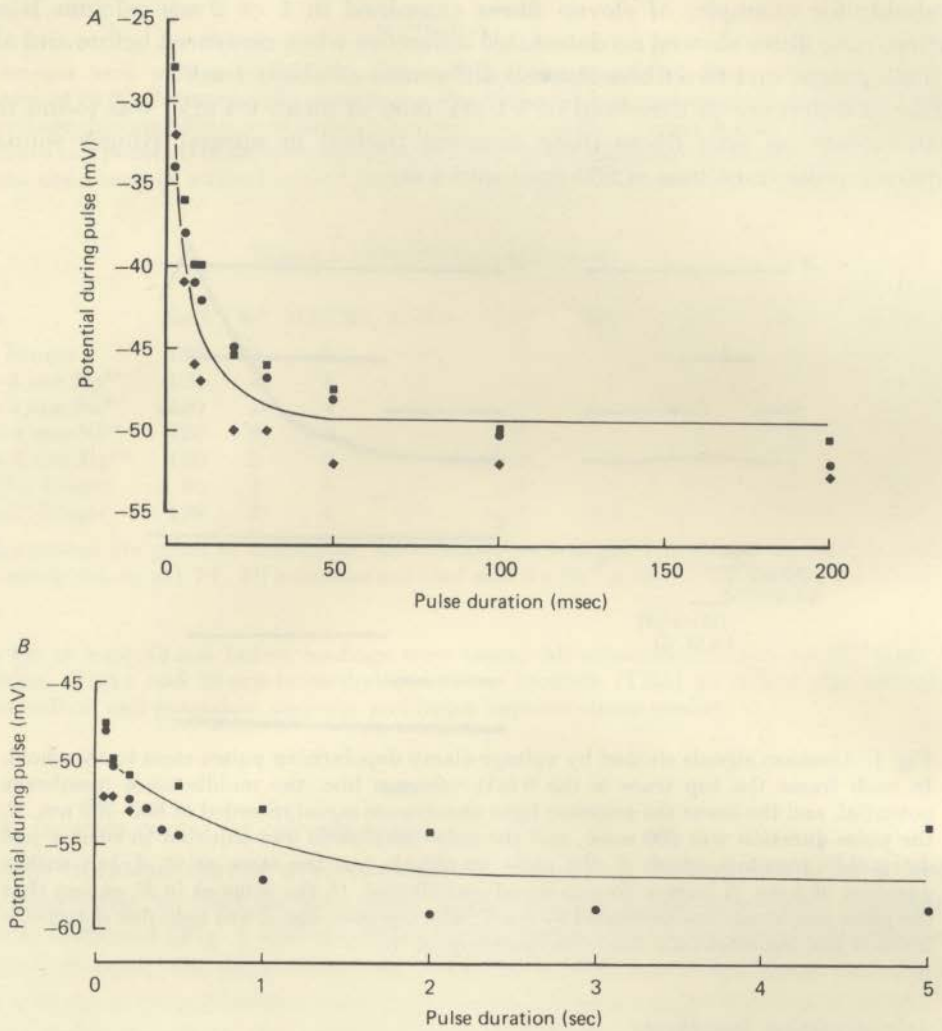

Fig. 2. Strength–duration curves showing the potential required to elicit a threshold arsenazo signal for any given pulse duration. Data in *A* and *B* from the same three fibres (indicated by different symbols).

from a depletion of calcium ions in the lumen of the T-tubules, which leads to a change in surface potential of the T-tubule membrane. It is known that depolarization of a muscle fibre leads to an influx of calcium ions from the bathing medium (Bianchi & Shanes, 1959; Beaty & Stefani, 1976; Stanfield, 1977; Sanchez & Stefani, 1978; Almers & Palade, 1981), and that under certain conditions this influx is sufficient to deplete appreciably the calcium level in the lumen of the T-tubules (Almers, Fink & Palade, 1981). If the calcium concentration in the T-tubule lumen falls during long depolarizations close to threshold for activation of e.–c. coupling, this would be expected to change the surface potential of the tubular membrane because there would be less screening of the negative surface charges on the membrane (Hille, Woodhull & Shapiro, 1975; Costantin, 1968). This change in surface potential would

be in the correct direction to shift the threshold for activation of calcium release from the s.r. to more negative values.

The experiments described in the following sections were designed to test this calcium-depletion hypothesis.

Changes in divalent cation composition

The voltage-activated calcium channel in muscle is permeable also to barium ions, but is relatively (Almers & Palade, 1981), or completely (Sanchez & Stefani, 1978), impermeable to magnesium ions. Various tests of the calcium-depletion hypothesis are therefore possible by manipulating the divalent cation composition of the bathing solution (cf. Table 1).

(i) If the divalent cations in the bathing medium are relatively impermeant through the calcium channel, there can be little depletion of these ions in the T-tubule lumen, and hence the calcium-depletion hypothesis predicts that there would be little difference in threshold for the calcium signal measured with short (200 msec) and long (5 sec) depolarizing pulses. This was tested by using a 0 calcium plus 5 mM-magnesium Ringer solution. The threshold measured using long pulses was 0·8 mV (S.E. of mean 0·26 mV; twelve fibres) more negative than with short pulses. This may be compared with the shift of 5·1 mV observed in normal Ringer solution. Mean threshold for the short pulse was −49·2 mV (S.E. of mean 1·4 mV), and for the long pulse −50·0 (S.E. of mean 1·5 mV). The standard errors for the difference in threshold are smaller than those for the mean threshold, because the threshold difference is obtained from paired measurements on the same fibre, thus cancelling out variability between fibres and errors due to electrode potentials.

(ii) Replacement of calcium by another permeant divalent cation would be expected to give a difference in threshold, as measured with short and long pulses, similar to that seen in normal Ringer solution. This was tested using 0 calcium plus 2 mM-barium solution. The threshold for the long pulse was 4·6 mV (S.E. of mean 0·18 mV) more negative than for the short pulse (five fibres from three muscles). Mean thresholds were −54·6 mV (S.E. of mean 2 mV) for the short pulse and −59·3 mV (S.E. of mean 2 mV) for the long pulse.

(iii) Addition of 4 mM-magnesium to the normal Ringer solution would be expected to reduce the threshold difference, because even if the calcium concentration in the lumen of the T-tubules fell to zero, this would reduce the total divalent cation concentration only from 6 to 4 mM. Any changes in surface potential would thus be much smaller than in normal Ringer solution. The threshold in normal Ringer solution plus 4 mM-magnesium for the long pulse was 1·2 mV (S.E. of mean 0·49 mV) more negative than for the short pulse (three fibres, two muscles). Mean thresholds were −45·1 mV for the short pulse and −46·4 mV for the long pulse.

A similar experiment was also performed using normal Ringer solution plus 4 mM-nickel. Since nickel ions are reported to be impermeant (Almers & Palade 1981), the calcium-depletion hypothesis predicts a reduction in threshold difference similar to that observed with 4 mM-magnesium. The observed threshold for the long pulse was 1·6 mV (S.E. of mean 0·41 mV) more negative than for the short pulse.

Calcium-buffered solutions

Depletion of calcium ions in the T-tubule lumen can be circumvented by using a high concentration of a calcium-buffering system in the bathing fluid, such that the free calcium concentration in the tubules remains fairly constant, even though there is an influx of calcium into the fibre (Almers *et al.* 1981). For our purposes citrate provided a convenient buffer, since it has an affinity constant giving good buffering power at a free-calcium level of around 1 mM. The composition of the calcium-buffered Ringer solution (Table 1) was chosen to give a free-calcium concentration of 1 mM, with a total calcium concentration of 11 mM, taking a value for the apparent affinity constant of citrate for calcium of $10^{3.53}$ M^{-1} at 20 °C, pH 7·1 and ionic strength 0·1 M (Campi, Ostacoli, Meirone & Saini, 1964; Simons, 1976). A small error in estimation of free calcium concentration may have resulted because our experiments were at 10 °C and at a slightly higher ionic strength. The sodium chloride concentration in the calcium-buffered solution was reduced to 80 mM in order to maintain isotonicity.

Experiments were made on one muscle, examining five fibres whilst bathing in 1 mM-calcium Ringer solution, and then another five fibres after changing to calcium-buffered Ringer. In the 1 mM-calcium Ringer the threshold for long pulses was 4·5 mV (S.E. of mean 0·54 mV) more negative than for short pulses, whilst the corresponding shift in calcium-buffered Ringer was 1·3 mV (S.E. of mean 0·3 mV) in the same direction. Mean thresholds for the short pulse were −51·9 mV in 1 mM-calcium solution, and −54·4 mV in calcium-buffered Ringer.

DISCUSSION

Calcium-depletion hypothesis

The threshold depolarization of a muscle fibre bathed in normal Ringer solution which is required to elicit a just detectable rise in intracellular calcium is shifted by about 5 mV more negative when the pulse duration is increased from 200 msec to 5 sec. Our explanation for the major part of this shift is that depolarization opens calcium channels in the membrane of the T-tubules (Sanchez & Stefani 1978; Almers & Palade 1981; Almers *et al.* 1981), and that the resulting influx of calcium decreases the calcium level in the tubules, thus causing a negative shift in the threshold for activation of e.–c. coupling owing to changes in surface potential of the tubular membrane (Hille *et al.* 1975). Evidence supporting this conclusion is: (i) the shift in threshold is almost abolished when calcium in the bathing solution is replaced by magnesium, an ion which is relatively impermeant through the tubular calcium channel; (ii) addition of 4 mM-magnesium or nickel to normal Ringer solution greatly reduced the shift in threshold, (iii) a bathing solution in which calcium was replaced by another permeant ion, barium, gave a similar shift to that seen in normal Ringer, and (iv) the shift in threshold was greatly reduced in a solution where the free calcium concentration was buffered at 1 mM, in order to reduce any fall in tubular free calcium level.

Depletion of calcium in the tubules resulting from influx across the tubular membrane has been directly demonstrated in cut-fibre preparations (Almers *et al.* 1981), and there is also evidence from intact fibres (Nicola Siri, Sanchez & Stefani, 1980; Stefani & Chiarandini, 1982).

Alternative possibilities to account for the shift in threshold with long pulses

include the following: (i) potassium ions may accumulate in the tubules, and depolarize the tubular membrane, (ii) changes in divalent ion concentration in the tubules might occur because of electrophoretic movement caused by a voltage gradient along the tubular lumen. Both of these mechanisms can be ruled out as being responsible for more than a small part of the observed shift in threshold, since neither is expected to show differences with different species of divalent cations. For the case of potassium accumulation, note that all bathing solutions included 20 mM-TEA, which would reduce the delayed rectifier potassium current to about 20 % of normal (Stanfield, 1970).

The calcium-depletion hypothesis assumes that the calcium signal detected with arsenazo arises entirely from calcium ions released from the s.r., and that the calcium influx across the tubular membrane is too small to be directly detectable. Almost certainly this is the case. For example, we can consider the extreme situation where all of the calcium in the T-tubule system enters the muscle cytoplasm. The volume of the T-system is about 0·4 % of the fibre volume (Peachey, 1965; Almers *et al.* 1981), so the cytoplasmic total calcium concentration would be raised by about 8 μM (assuming 2 mM-calcium present initially in the T-system). This increase in total cytoplasmic calcium is only about 4 % of the change occurring during a single twitch (Miledi *et al.* 1982), and even if it occurred instantaneously would give an arsenazo signal close to the limits of resolution. In fact, the calcium influx would occur over a period of a few seconds, and would be taken up rapidly by the s.r., without appreciably raising the free calcium level.

In cases where depletion of divalent cations in the T-system is expected to be negligible (e.g. in bathing solutions containing magnesium, nickel, or calcium buffer), a small negative shift of around 1 mV in threshold was consistently observed when the pulse duration was lengthened from 200 msec to 5 sec. Most probably this is simply due to the cytoplasmic calcium level approaching closer to an equilibrium between release and re-uptake during the longer pulse, although the mechanisms of potassium accumulation and electrophoretic movement described above might also contribute. In normal Ringer solution, the corresponding shift was about 5 mV, so that depletion of tubular calcium would have accounted for a shift in threshold of about 4 mV.

Does sufficient calcium current flow to cause tubular depletion?

In order to produce a shift in surface potential of 4 mV, the calcium concentration in the tubules would have to fall from 2 mM to between 1 and 0·5 mM (Costantin, 1968; Hille *et al.* 1975). At first sight it may appear unlikely that the calcium conductance in the tubules would be activated sufficiently at potentials of between −55 and −45 mV to cause this depletion. However, some rough calculations based on data from calcium current measurements suggest that the expected influx is sufficiently large.

We can first estimate the calcium current which would be needed to deplete the tubules. The mean diameter of fibres in our experiments was around 60 μm, so considering a 100 μm length of fibre, the volume of the T-system would be about $1{\cdot}5 \times 10^{-12}$ l. (assuming T-tubule volume as 0·4 % of fibre volume). Thus, to reduce the tubular calcium concentration from 2 to 1 mM, a calcium flux equivalent to a charge of 3×10^{-10} C would be required. If this movement of charge took place over a 2 sec period, the mean current would be 0·15 nA for a 100 μm length of fibre, or about 0·075 μA cm^{-2} membrane area (assuming tubular membrane area to be ten times the surface membrane area).

By comparison, Almers & Palade (1981) report that the peak calcium current at 0 mV in a solution containing 10 mM-calcium varied between 28 and 143 μA cm^{-2} in different fibres. This range encompasses also the values observed by Sanchez & Stefani (1978) and Stanfield (1977), after taking into account differences in free calcium concentration in the bathing fluids. Assuming that the calcium influx varies linearly with external free calcium concentration, then activation of between 0·26 and 1·3 % of this peak calcium conductance would be sufficient to cause the required degree of depletion in our Ringer solution containing 2 mM-calcium. Stefani, Sanchez & Nicola Siri (1980) have proposed that steady state activation of the calcium channel may be described by an m^3h formulation, where the steady-state value of m is given by $(1+\exp((V_m-E_m)/K_m))^{-1}$: see Stephani *et al.* (1980) for definition of terms. From their values ($E_m = 38{\cdot}8$ mV, $K_m = 9{\cdot}7$ mV), activation of 2 % of the channels would be expected at a potential of about −48 mV. However, this applies to 10 mM-external calcium, so that the corresponding value in 2 mM-calcium would be around −58 mV, after taking into account the change in surface membrane potential.

The above calculation can only be approximate, and an important factor which is not taken into consideration is the replenishment of tubular calcium during the depolarizing pulse by diffusion from the external solution. This effect is difficult to estimate quantitatively, but it seems likely that the expected calcium currents, even at a potential of −55 mV, may be sufficient to cause appreciable depletion in at least the deeper parts of the tubular system.

A related problem is the extent to which the calcium channels become inactivated during the 5 sec depolarizations, since it is thought that in intact fibres the majority of the decline in the calcium current during depolarization results from inactivation, rather than calcium depletion (Stefani & Chiarandini, 1982). If this is the case, then our results suggest that sufficient calcium channels must still remain open during the 5 sec depolarization to cause appreciable depletion.

Possible physiological importance of tubular calcium depletion

The shifts in threshold potential which we attribute to calcium depletion in our experiments were small (4 mV), but this was with depolarizations to potentials which would activate only slightly the tubular calcium conductance (see above). The question therefore arises as to whether much larger effects may occur following tetanic stimulation of muscle. We attempted to investigate this by using voltage-clamp pulse trains to simulate a train of action potentials, and preliminary results suggest that the shift in threshold may be only 5–10 mV following prolonged stimulation. However, these experiments are very difficult, owing to technical problems associated with the large calcium transient and accompanying contraction, so the conclusion is uncertain. Other evidence (Almers *et al.* 1981) indicates that calcium in the central part of the tubular system may be depleted to as low as 2 % of the external concentration during long tetanic stimulation. In the absence of any other divalent cations, the resulting change in surface potential could shift the threshold for contractile activation to potentials as negative as −75 mV (Hille *et al.* 1975). Thus, the contractile threshold may approach quite close to the resting potential, and in situations where the potential was depressed, or where the external calcium concentration was low, could perhaps give rise to a maintained contracture following stimulation. A regenerative element might also be present in this case, since the threshold for activation of the tubular calcium conductance is probably shifted in a similar manner to more negative potentials, thus leading to an increased calcium influx and greater calcium depletion in the tubules.

Shifts in threshold potential for contractile activation may present a complication in experiments involving potassium contractures; for example, calcium depletion in the tubules might account for the long latency of contraction seen in slow muscle fibres depolarized by a potassium concentration just sufficient to elicit a response (Lannergren, 1967).

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