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DEPENDENCE OF ACETYLCHOLINE DESENSITIZATION ON THE MEMBRANE POTENTIAL OF FROG MUSCLE FIBRE AND ON THE IONIC CHANGES IN THE MEDIUM

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SUMMARY

1. The rate of desensitization of the post-synaptic membrane to prolonged action of acetylcholine was investigated during (*a*) potassium depolarization of the frog muscle fibre, (*b*) artificial changes of the membrane potential and (*c*) in the presence of some multivalent cations and of caffeine.

2. Depolarization of the muscle fibre by 15 mM-K^+ led to a slowing down in the development of desensitization by 80 %. This effect on desensitization produced by membrane potential changes could account for the previously described effect of increased K^+ concentration.

3. Electrotonically produced depolarization of the muscle fibre membrane resulted in a decrease, hyperpolarization in an increase of the rate of desensitization.

4. Several multivalent cations can be, according to their ability to increase rate of desensitization, arranged in the following series:

$$Mg^{2+} \ll Ca^{2+} \leqslant Ba^{2+} < Sr^{2+} \ll Al^{3+} < La^{3+}$$

5. Caffeine in a concentration of 1·5 mM does not affect the rate of desensitization.

6. A hypothesis is presented suggesting that desensitization is not only restricted to the receptor level but that it also occurs, at least partly, at the terminal stages of the activation system.

INTRODUCTION

According to existing views, desensitization, i.e. the decrease in sensitivity of the post-synaptic membrane to the action of acetylcholine (ACh) and related compounds, is explained by the transition of ACh receptors from an active to an inactive state during the prolonged contact of ACh with the membrane. It is assumed that the rate of the receptor inactivation depends on the concentration of ACh applied to the chemosensitive surface of the muscle fibre. The higher the concentration of ACh, the more rapidly the inactivation occurs (Katz & Thesleff, 1957).

All factors affecting the desensitization rate have so far been considered from the point of view of their action on the ratio between active and inactive ACh-receptors (Manthey, 1966; Nastuk, 1967). In this paper we wish to present some facts which cannot be unambiguously explained by the theory based on the receptor level of desensitization. A study was made of how the desensitization is affected by (1) high concentration of potassium just below the threshold of contracture, (2) artificial changes of the membrane potential of the muscle fibre and (3) some multivalent cations.

METHODS

Experiments were performed on the isolated m. sartorius of the frog *Rana temporaria*. The muscles were slightly stretched and fixed on the convex bottom of a Perspex chamber (5 ml.) which was connected with a device for the quick exchange of solutions. The initial Ringer solution had the following composition (mM): Na^+ 117; K^+ 2·5; Ca^{2+} 1·8; Cl^- 120·6; HCO_3^- 2·4; pH = 7·4 at room temperature (18–21° C). When $[K^+]_o$ (concentration in the solution) was increased, the $[Na^+]_o$ was simultaneously lowered so as to keep the ionic strength constant. In the solutions in which a higher level of multivalent cations was present, Tris-buffer was used (4 mM Tris + HCl in concentrations necessary for pH 7·4). Standard glass micro-electrodes filled with 2·75 M-KCl (resistance 7–30 MΩ) were used both for intracellular recording and for the passage of polarizing current. The diameter of the joint tip of double-barrel glass micropipettes containing 2 M ACh chloride (Merck), which served for iontophoretic application, was approximately 1 μ and the resistance of each barrel was 20–50 MΩ. These micropipettes were selected, and those with a resistive connexion between the channels or with uneven tips were discarded. Double-barrel micropipettes filled with ACh were placed on the sensitive area of the fibre and the 'braking' negative voltage (preventing the diffusion of ACh from the channels) was carefully adjusted.

When rectangular positive pulses (duration 5–30 msec) were applied through one of the channels at a frequency of 0·5–0·3/sec to liberate small amounts of ACh, short-lasting depolarizations reaching about 5–10 mV were recorded. The experiments were discontinued when the amplitude of the test responses did not remain at a constant level for a sufficiently long period (5–20 min).

A positive rectangular pulse of low amplitude (duration 20–25 sec) was then applied through the second channel of the micropipette to produce a wave of ACh depolarization, i.e. the conditioning response. In the course of such a long depolarization wave the amplitude of the test responses diminished. The rate of this decrease

of test responses served as a measure of the diminishing sensitivity to ACh. Switching off the conditioning pulse was in most cases followed by a gradual increase of the test responses to the initial value. In some cases the recovery of the test response was not complete and the amplitudes returned to only 70–90 % of their original value. The rate of desensitization was measured by plotting the test responses as the percentage of the initial amplitude against the duration of the conditioning pulse.

RESULTS

Effect of increased potassium concentration in the perfusion solution on the rate of desensitization

In most experiments in which normal Ringer solution was used, the conditioning ACh pulse, causing a depolarization of approximately 5–6 mV, produced a 50 % decrease of the amplitude of test responses in 15–20 sec. The rate of this decrease depended only on the amplitude of the conditioning response, and was not influenced by the amplitude and frequency of the rest responses (the intervals between the test responses were 10–50 times longer than their duration). When the normal solution surrounding the muscle was exchanged for a solution containing 15 mM-K^+ instead of 2·5 mM, the membrane potential was lowered to about 50 mV. Simultaneously, the amplitude of ACh response diminished (Fig. 1). According to the equations presented by Takeuchi (1963*a*) the acetylcholine equilibrium potential (E_r) of a muscle fibre, placed in a solution containing 15 mM-K^+ and 104 mM-Na^+, equals $-2{\cdot}5$ mV. The theoretical relation between the amplitude of the ACh response and the level of membrane potential is expressed by the continuous line in Fig. 2. The points represent our experimental values of the amplitude of ACh responses obtained in a solution with increased K^+ concentration; thus the lowering of the response amplitude is evidently caused by the decrease in potential difference across the membrane. Although there is no reason to believe that either the number of interacting ACh-receptors or the amount of the applied ACh on the membrane was changed, the rate of desensitization markedly decreased (Fig. 1). The half-time of desensitization was prolonged to 181 ± 16 % of the control (nine experiments). The rate of desensitization returned to its original value when the solution with high K^+ concentration was replaced by the Ringer with normal K^+ concentration. However, it was possible to reach the control rate of desensitization even in a high $[K^+]_o$ solution when the amount of ACh applied was increased to such an extent that the ACh responses, at a still lower membrane potential, had the same amplitude as at the normal one.

The influence of artificial changes of membrane potential on the rate of desensitization

On the basis of a series of experiments in which a solution with increased $[K^+]_o$ was applied, it might be assumed that the mechanism of the decreased desensitization rate is closely related to the potential difference across the membrane. If this is so, then the artificial shift of the membrane potential to the initial level would restore the initial rate of desensitization even in

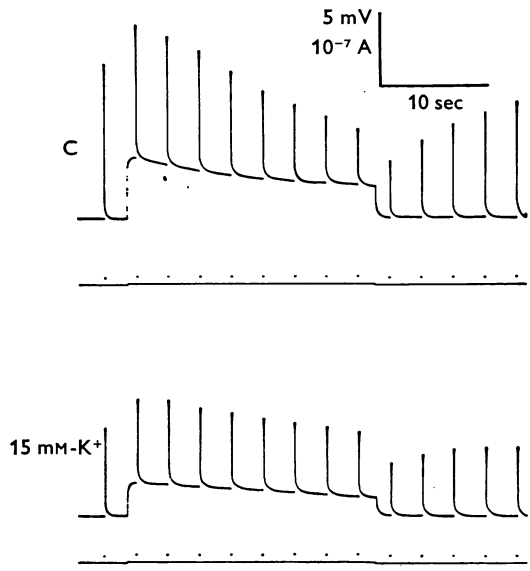

Fig. 1. Intracellular recordings of desensitization evoked in the end-plate zone of muscle fibre. Small amounts of ACh from one barrel of a double-barrel micropipette were ejected repeatedly causing quick depolarizations (test responses). From the second barrel, ACh was applied for 23 sec (monitored on the lower line in each recording); during application of this conditioning pulse the amplitude of the test responses decreased. C, control (membrane potential = 88 mV); the lower recording, 20 min after application of 15 mM-K^+ (membrane potential = 51 mV). The rate of test responses diminution (rate of desensitization) is noticeably decreased. No anti-cholinesterase present, temperature 20° C (slightly retouched).

solution in high $[K^+]_o$. This assumption was tested directly in solutions with high $[K^+]_o$ as well as in normal Ringer by altering the level of the membrane potential artificially. In the high $[K^+]_o$ solution the membrane potential was shifted from -52 to -90 mV, i.e. normal value, by a current passing through a second micro-electrode inserted into the muscle fibre 50–100 μ from the recording electrode. It has been found in four experiments that this artificial shift of membrane potential caused an increase in

the rate of desensitization approaching the control rate observed in the normal solution (Fig. 3).

A series of five experiments was performed in normal Ringer solution in which the membrane potential was artificially shifted by an appropriate amount of current. In all the experiments the hyperpolarization resulted in an increase, and depolarization in a decrease, of the rate of the desensitization (Fig. 4).

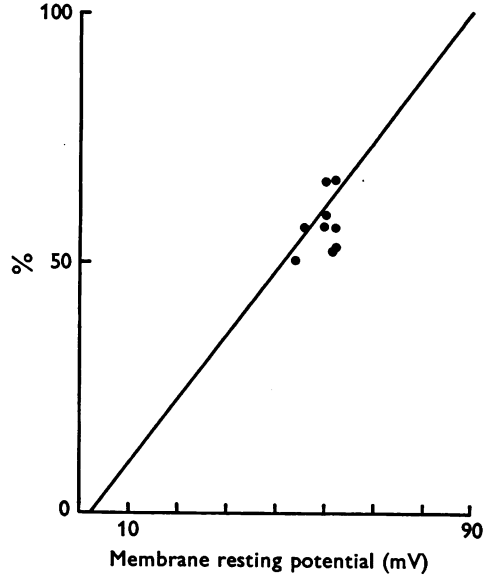

Fig. 2. Theoretical course of the correlation between the amplitude of the ACh responses and the membrane potential level, calculated according to eqns. (1) and (2) of Takeuchi (1963*a*). E_r is 2·5 mV at 15 mM-K^+ and 104 mM-Na^+. Ordinate, percentage of response decrease (100 % ACh response at membrane potential = 90 mV), abscissa, resting membrane potential in mV. Circles indicate values, measured in nine experiments at 15 mM-K^+.

Effect of multivalent cations on the rate of desensitization

Manthey (1966), Magazanik (1968, 1969) and Magazanik & Shekhirev (1970) showed that the rate of desensitization depends on the concentration of Ca^{2+}; lowering the external Ca^{2+} causes a decrease of the rate of the desensitization and the increase in the Ca^{2+} concentration has opposite effect. Manthey (1966) considers the interference of Ca^{2+} with the interaction between ACh and the receptor to be the most plausible explanation of this phenomenon. A less specific effect of Ca^{2+}, involving changes of the membrane permeability caused by the interaction of Ca^{2+} with membrane phospholipids (Abood, Koyama & Kimizuka, 1963; Blaustein, 1967) could also explain the influence of the ion on desensitization. Therefore it was

of interest to learn how other multivalent cations, which differ from Ca^{2+} in their interaction with phospholipids, influence the rate of desensitization. Experiments were carried out using Mg^{2+}, Ca^{2+}, Ba^{2+}, Sr^{2+}, Al^{3+} and La^{3+}. The cations were either added in small amounts to the normal solution, or Ca^{2+} in the solution was replaced by another cation.

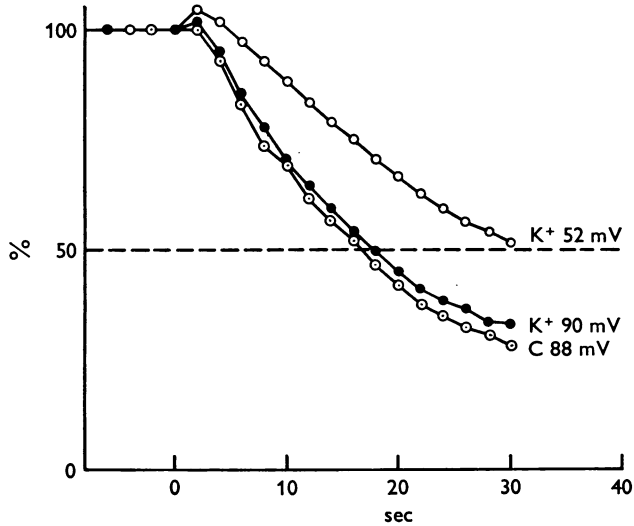

Fig. 3. Effect of 15 mM-K^+ on the rate of desensitization. The rate of desensitization is expressed as a percentage of the decrease of the test response (100 % = amplitude before desensitization in time = 0). ⊙, rate of desensitization in normal Ringer solution. C, control (membrane potential 88 mV). ○, after 14 min in a solution with 15 mM-K^+ (membrane potential 52 mV). The rate is lowered and the half-time of desensitization is about 150 % of the control. ●, membrane potential was artificially increased to initial level (90 mV) by passing current through a second intracellular micro-electrode (7 MΩ). The rate of desensitization recovered approximately to the initial value.

The exchange of Ca^{2+} for an equivalent amount of Mg^{2+} decreased the rate of desensitization and prolonged the half-time of desensitization to 170 ± 20 % of the control (five experiments). The addition of various amounts of Mg^{2+} (up to 10 mM) to the normal solution did not appreciably influence the rate of desensitization. Mg^{2+} evidently cannot be a substitute for Ca^{2+} in its effect concerning the rate of desensitization.

It was not possible to examine the action of Ba^{2+} in Ca^{2+} free solutions as spontaneous activity of the muscle fibres prevented the stable intracellular recordings. Ba^{2+} was therefore added to a solution containing the standard amount of Ca^{2+} (1·8 mM). The addition of 5 mM-Ba^{2+} reversibly increased the rate of desensitization so that half-time was 70 ± 5 % of the control (four experiments).

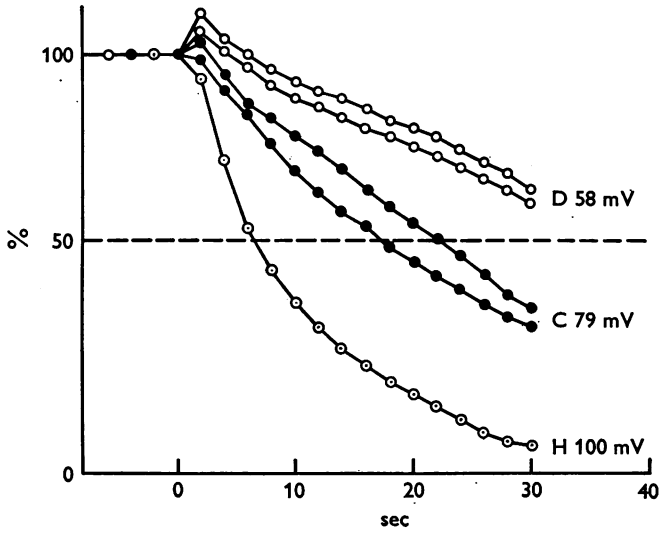

Fig. 4. The relationship between the rate of desensitization and membrane potential level. Desensitization was first registered at a normal membrane potential = 79 mV (C, control), then after artificial depolarization (D) to 58 mV (○) and hyperpolarization (H) to 100 mV (⊙) and finally again at 79 mV. A second intracellular electrode was used for polarizing the muscle fibre.

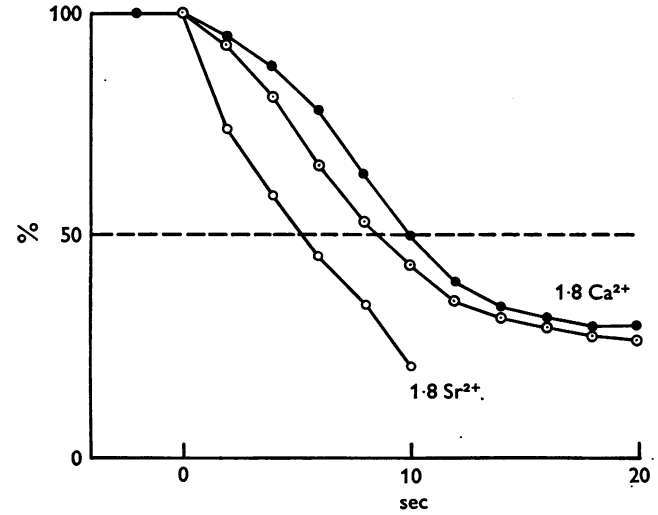

Fig. 5. Rate of desensitization in Ringer solution with 1·8 mM-Ca^{2+} (●), 15 min after replacing the Ca^{2+} with 1·8 Sr^{2+} (○) and after 25 min washing with normal Ringer solution (⊙).

When Ca^{2+} was substituted by Sr^{2+} the amplitude of ACh potentials was decreased to 65 % of the control and time course elongated mainly in the decaying phase. The rate of desensitization was at the same time markedly increased (Fig. 5).

When Ca^{2+} was replaced by an equal molar concentration of Al^{3+} or La^{3+}, the ACh responses were profoundly diminished and prolonged. In a

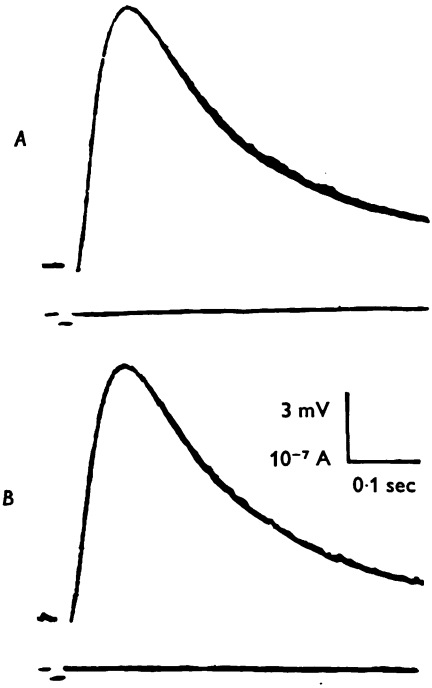

Fig. 6. The effect of Al^{+3} on the amplitude and shape of ACh response in the presence of 2×10^{-3} prostigmine. *A*, control; *B*, 15 min after addition of 0·3 mN-$AlCl_3$. The same amplitude and shape indicates that there are no changes in the ACh effectiveness on the receptors under a given dose of Al^{-3}.

solution containing 1·8 mM-La^{3+} they decreased gradually until they disappeared completely. This effect, however, was irreversible. The addition of 0·3 mM-Al^{3+} or La^{3+} to the normal solution did not affect the amplitude and shape of ACh responses both in the presence and absence of antiocholinesterase (Fig. 6), but markedly increased the rate of desensitization (2 and 3 times respectively, four experiments). The increase of the rate produced by La^{3+} is comparable with the effect of 18 mM-Ca^{2+} (Magazanik, 1968).

The influence of caffeine on the rate of desensitization

It has been demonstrated (e.g. Bianchi, 1961; Novotný & Vyskočil, 1966) that caffeine affects the fluxes of calcium across the membrane of the muscle and enhances the amount of exchangeable Ca ions in sarcoplasm. Caffeine, when added to the normal Ringer solution in a concentration below the threshold for evoking contracture ($1{\cdot}5 \times 10^{-3}$ M), influenced neither the amplitude of ACh potentials, nor the rate of desensitization (Fig. 7).

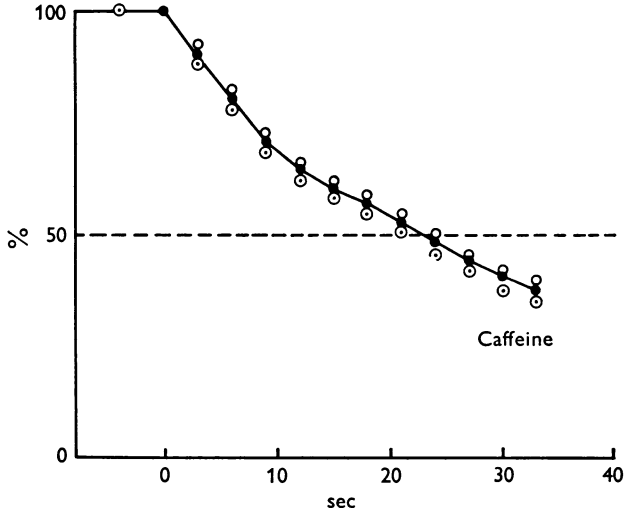

Fig. 7. The rate of desensitization in the absence of caffeine (●), 12 min after the application of 1·5 mM caffeine into the muscle bath (○), and after 20 min of washing with the initial solution (⊙).

DISCUSSION

Our knowledge of the mechanism of the activation of the post-synaptic membrane is limited to the interaction of the transmitter (ACh) with the receptors and the flow of ions across the membrane. In their hypothesis on the desensitization of the post-synaptic membrane Katz & Thesleff (1957) suggested that this phenomenon occurs at the input of the activation system.

The following scheme was presented:

$$\begin{array}{ccc} & \overset{a}{} & \\ S + A & \rightleftharpoons & SA \\ \Big\| k_2 \quad \Big\| k_4 & \text{(fast)} & \Big\| k_3 \quad \Big\| k_1 \text{ (slow)} \\ S + B & \rightleftharpoons & SB \\ & \underset{b}{} & \\ & \text{(fast)} & \end{array} \quad \text{(slow)}$$

where S is the conditioning ACh concentration, SA is the 'effective' ACh-receptor compound, and SB is the 'refractory' compound.

According to this hypothesis the receptor can exist in two states: active (A) and inactive (B), but only in A it is able to trigger the depolarization. The necessary conditions for this hypothesis are (Katz & Thesleff, 1957): that the affinity constant of the inactive receptor (b) must be many times higher than the affinity constant of the active receptor (a); the rate constant of the transition of SA to SB (k_1), the inactivation constant, must be higher than the constant of the opposite process (k_3) and $a/b > k_1/k_2$. The rate of desensitization under standard conditions is determined by the concentration of ACh in the solution adjacent to the post-synaptic membrane.

This hypothesis explains very well the relationship between the rate of desensitization and the restitution of the initial sensitivity, as well as the dependence of the rate of desensitization on the amount of ACh applied.

In our experiments, we have found that changes of the membrane potential produced either by increasing $[K^+]_o$ or by passing current through the membrane, markedly alter the rate of desensitization. As the concentration of ACh remained constant under these experimental conditions, it would have to be assumed on the above-mentioned hypothesis, that hyperpolarization increases and depolarization decreases the value of the receptor inactivation rate constant. In fact, the stability of the receptor–ACh complex has been suggested to change during the hyperpolarization or depolarization (see Kordaš, 1969). This was put forth when it was found that hyperpolarization prolongs and depolarization shortens the end-plate current and potential (Takeuchi & Takeuchi, 1959; Kordaš, 1969; Magazanik & Vyskočil, 1969). But on the other hand, no substantial changes of the shape of the ACh potentials were observed during the depolarization by K^+, or strong depolarization by current (Magazanik & Vyskočil, 1969). Therefore it is possible that changes in the stability of the receptor–ACh complex are not the main reason for the changes of the rate of desensitization, caused by polarization of the membrane.

Another additional assumption would have to be introduced to the Thesleff's & Katz's hypothesis in order to explain the facts (a) that many cholinomimetics which differ in their affinity for the receptor, when applied in an amount causing identical initial responses, can desensitize the post-synaptic membrane almost to the same extent, and (b) that there is no effect of curare (Magazanik, 1968) and atropine (L. G. Magazanik & F. Vyskočil, unpublished) on the rate of desensitization. The ratio a/b of the affinity constants of the active (A) and inactive (B) forms of receptor for all cholinomimetics and cholinolytics would have to be very similar. Assuming A and B represent different steric forms of the cholinergic

receptor, it is rather difficult to imagine that the a/b ratio has an identical value for all the individual compounds, which differ markedly in their chemical structure.

An hypothesis that factors influencing the rate of desensitization act not only at the receptor level but that at least partly at the intermedial or terminal stages of the activation system would fit our experimental data.

The following tentative scheme of this mechanism can be presented. During the prolonged action of ACh on the post-synaptic membrane receptors, free Ca^{2+} ions accumulate in the membrane and can be bound to the phosphate groups of phospholipids, forming complexes which control the pathways for ion permeability. This would alter the normal function of ionic channels and account for the diminution of the electrical responses (depolarizations) which serve as our only measure of the sensitivity to the ACh. Under conditions when increased binding of multivalent cations in the membrane can be expected, i.e. raising Ca^{2+} concentration or hyperpolarization of the cell (Frankenhauser & Hodgkin, 1957; Khodorov & Peganov, 1969) or replacement of Ca^{2+} by cations with a higher affinity to phospholipids, the rate of desensitization is higher. On the other hand, the absence of Ca^{2+} in the medium and the lowering the potential difference across the membrane slowed down the time course of desensitization. There are several other observations which are in line with this hypothesis. When the ability of multivalent cations to increase the rate of desensitization was studied, we found that they can be arranged in the following series:

$$Mg^{2+} \ll Ca^{2+} \leqslant Ba^{2+} < Sr^{2+} \ll Al^{3+} < La^{3+}.$$

A similar sequence of potency was demonstrated when studies were made of the effect of these cations on membrane resistance at the Ranvier node (Khodorov & Peganov, 1969), on the increase of permeability of the lobster axon membrane (Blaustein & Goldman, 1968), as well as on their binding to the phospholipids of the cell membrane fraction and phosphatidylserine (Blaustein, 1967).

Although the changes of permeability for ions during the activation of the axon membrane and the post-synaptic membrane are different, the mechanism controlling these changes seems to have common stages. It is well known that an increase of $[Ca^{2+}]_o$ which accelerates the desensitization decreases the Na permeability not only of the axon membrane (Frankenhaeuser & Hodgkin, 1957) but also of the muscle post-synaptic membrane (Takeuchi, 1963*b*).

The assumption that desensitization does not occur only at the receptor level enables us to comprehend the influence of factors of a different nature, such as ionic changes in the medium or the effect of depolarization of the post-synaptic membrane, on this phenomenon.

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