Elementary Components of Synaptic Transmission

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The analysis of chemical transmission at synapses, in particular at the neuromuscular junction, has proceeded from the cellular to the molecular level. Gradual refinements in electric recording technique have brought to light new features, first the quantal delivery of the transmitter substance, in multimolecular packets, from the nerve endings, and more recently the statistical elements of the postsynaptic potential change which arise from the molecular bombardment by acetylcholine and the transient opening of individual ion channels in the end-plate membrane

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The transmission of signals across many neuronal contacts ('synapses') and at neuro-effector junctions (in muscles and glands) is brought about by local secretion of a specific transmitter substance from the 'presynaptic' nerve endings and its reaction with the membrane of the 'postsynaptic' cell. The process has been particularly well studied at the vertebrate neuromuscular junction, where the transmitter was identified as acetylcholine by H.H. Dale and his colleagues over 40 years ago [1].

Transmission at the Neuromuscular Junction

When the impulse, that is an electric signal, arrives in the motor nerve endings, it causes them to release acetylcholine; this substance then diffuses rapidly across the small gap between nerve and muscle and there reacts with specific 'receptors', protein molecules anchored to the so-called end-plate membrane, i.e., the junctional surface area, of the muscle fibre. This reaction leads to the opening of channels or gates in the muscle membrane through which cations, particularly sodium ions, can pass – from the outside into the interior. This causes a local potential change, a depolarization which is known as the end-plate potential. Normally this is very large, and it acts as an electric stimulus starting up a propagating impulse in the muscle fibre which throws the whole fibre into action and causes it to contract.

In this essay, a brief account will be given of contributions which electrophysiological methods have made to the study of the mechanism of neuromuscular transmission and of its subcellular and molecular components. Progressive improvements of electric recording techniques over the years have enabled the research worker to examine more and more minute pieces of the synaptic process and, by using micromethods of high spatial and temporal resolution, to take the synaptic mechanism apart into its elementary components. The motor end plate is particularly well-suited for this type of investigation; it is easily accessible to the experimenter and presents him with a natural bio-electric 'preamplifier', or – more correctly – a sensitive chemo-electric transducer. It receives a chemical message in the form of acetylcholine from the nerve endings and normally translates it into a large muscle action potential. Long before the discovery of related cellular phenomena, of nerve and muscle impulses, acetylcholine and end-plate potentials, the biological production of powerful electric shocks had been known to occur in certain elasmobranch fish, and we realize today that the basic mechanism which underlies this massive discharge is precisely the same as that taking place at the neuromuscular junction, only it is amplified several thousand times by a tight packing of a large number of modified end plates into flat layers and by suitable stacking of several thousands of such layers in series, so that their electromotive forces add up and the currents produced by the cells can summate. Today, the research worker can attempt to measure the elementary

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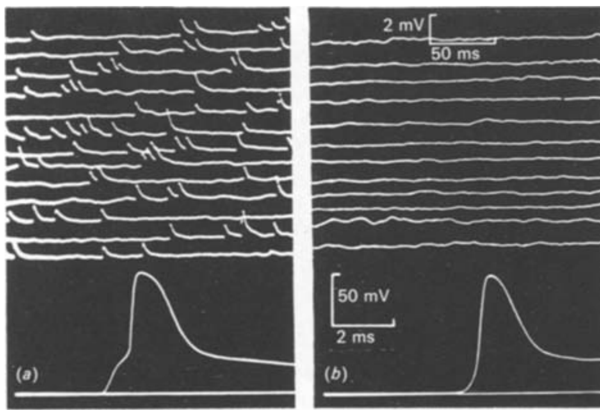

Fig. 1. Intracellular records from a muscle fibre of the frog [2], (a) from the end-plate region; (b) 2 mm away from the end plate. *Lower portions* were recorded at high oscillograph speed and low amplification. In these records, the electric response is shown to a motor nerve impulse initiated by a brief shock at the beginning of the trace. The response in (a) shows a step-wise end-plate potential leading to a propagating muscle impulse; in (b) the propagated impulse alone is seen after additional delay due to conduction along 2 mm of muscle fibre. *Upper portions* were recorded at low speed and high gain; they show the localized spontaneous 'miniature end-plate potentials' at the junctional region

voltages and currents which are generated when one or a few molecules of acetylcholine react with a single membrane receptor; these are quantities some nine orders of magnitude smaller than the discharge of an electric fish, and it is not difficult to appreciate that analytical progress and refinement of this order depended on the invention of sensitive recording methods and suitable microtechniques.

One of the important technical innovations made nearly 40 years ago was the introduction of the intracellular micro-electrode which enabled one to record the potential differences across the membrane of a single muscle fibre. Examples of such recordings are shown in Fig. 1.

When one records from the end-plate region, the response to a nerve impulse is a two-step electric signal, the first component of which represents the large normal end-plate potential leading to the second step, the propagating muscle impulse. Recording 2 mm away in the same fibre, one only sees a simple wave, the propagated impulse which arrives after an additional delay, due to the time taken in travelling 2 mm away from the junctional region. The two components of the electric response have very different properties, in their reactions to various chemical agents and to electric currents. The initial, end-plate step can be blocked by substances like curare which specifically antagonize the effect of acetylcholine on the end-plate membrane, and it is potentiated by a variety of cholinesterase inhibitors which prevent the rapid destruction of acetylcholine due to the presence of a potent hydrolytic enzyme at the end plate. Neither of these drugs has any appreciable effect on the propagated action potential, which can be initiated by an electric current of suitable strength passed directly across the muscle-fibre surface. This electrical muscle impulse, however, can be blocked by the highly specific pufferfish poison, tetrodotoxin, which – in contrast to curare – leaves the depolarizing, electro-motive action of acetylcholine on the end plate quite unaffected.

The Quantal Release of the Transmitter Substance

In 1950, Paul Fatt and I, using the intracellular recording technique, came across an interesting and quite unexpected observation. We found that even in the absence of all impulse activity, when the muscle appears to be 'silent' and completely at rest, there is an intermittent, 'spontaneous' electric activity going on at the end plate all the time, in the form of randomly recurring very small 'miniature end-plate potentials', brief blips of depolarization, of the order of 0.5 mV in magnitude, which rise within 1–2 ms and decay within about 20 ms (frog muscle, 20 °C, see Fig. 1), and have an average frequency of about 1 Hz. Their amplitude is about one hundredth of the full-size end-plate potential evoked by a nerve impulse – far below the threshold of muscle excitation; but these 'mini-potentials' have the same time course and the same local origin at the junctional region, and they react to curare or cholinesterase inhibitors in the same way as the normal end-plate potential or the response to an ionophoretically applied pulse of acetylcholine. Clearly, the chemo-sensitive mechanism of the end plate enables us to register the impact of minute subthreshold amounts of acetylcholine. The end plate serves as a kind of 'micro-droplet counter' for acetylcholine which is secreted by the nerve endings, even in their 'resting', unexcited condition. I cannot recount here the experiments which have led to the conclusion that the miniature potentials originate by spontaneous secretion of fair-sized multi-molecular packets of acetylcholine from the nerve terminals. The spontaneous potentials are certainly much too large to be attributed to a random escape and local impact of only a few molecules. There is good evidence [3] indicating that the transmitter substance is pre-packaged inside the nerve terminal in parcels which contain several thousand molecules, that these are secreted in an all-or-none manner, the discharge of one quantal packet producing one miniature end-plate potential. There is moreover a great deal of circumstantial evidence [4] suggesting that this quantal, packet-wise delivery has a structural basis in the synaptic vesicles revealed by the electron microscope

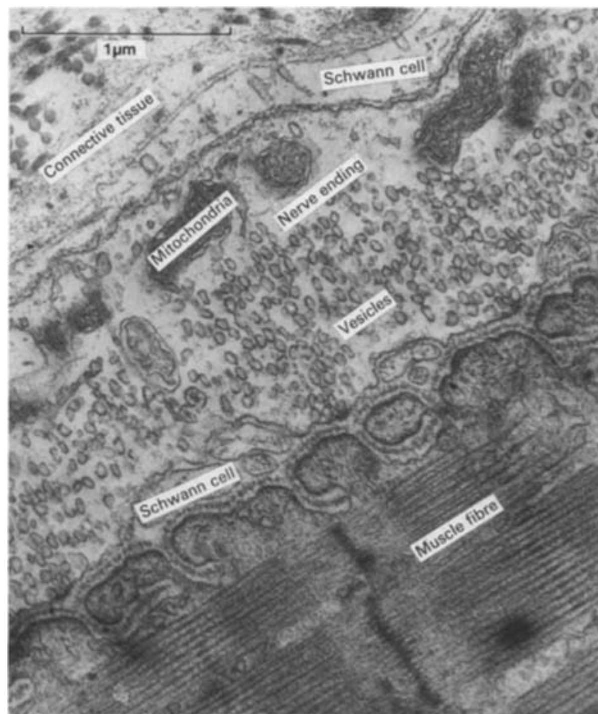

Fig. 2. Electron micrograph of a small portion of a neuromuscular junction from a frog muscle [5]. Longitudinal section of the muscle fibre

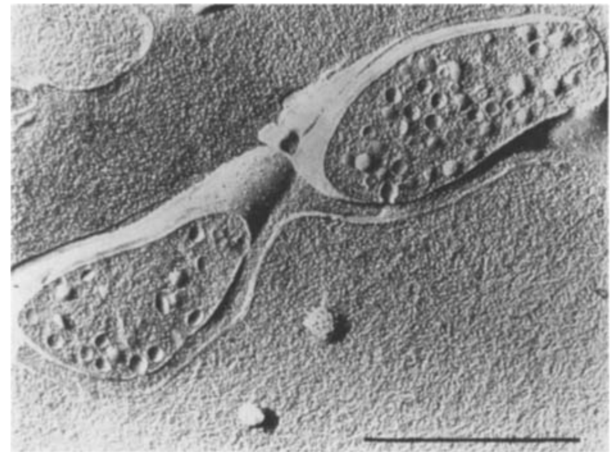

Fig. 3. Freeze-etch electronmicrograph of an electroplaque (modified end plate) from the electric fish, *Torpedo* [6]. The picture shows synaptic vesicles in the nerve endings; some of the vesicles have fused with the plasma membrane and opened to the 'synaptic cleft'. Scale 1 µm

(Figs. 2, 3). Calcium is instrumental in this process. Apparently, calcium ions enable the colliding membranes of a vesicle and the nerve therminal to fuse; this initiates a reaction which causes the fused membrane to break and so allows the whole diffusible content of the vesicle to impinge on the postsynaptic membrane.

The discovery of the spontaneous miniature end-plate potentials was followed by the even more important realization that they represent the basic coin, the quantum in the normal process of transmission [7]. The nerve impulse, in fact, does not initiate the secretion of acetylcholine *de novo*, but it vastly accelerates a process that goes on all the time, in other words it increases the statistical probability, or the frequency, at which the same quantal packets are released. In the resting state, the mean frequency of the spontaneous miniature potentials is of the order of one per second; after arrival of the impulse, about 300 quantal packets are secreted almost synchronously within less than one millisecond, a transient increase in secretory rate by a factor of several hundred thousand. This vast increase in the statistical probability of an elementary event is probably mediated by the transient surge of calcium ions which enter the nerve terminal when a wave of depolarization reaches it [8].

The Molecular Components of the End-Plate Potential

R. Miledi and I wanted to find out what happens when single molecules of acetylcholine react with a receptor in the end plate. The size and time course of the molecular action of acetylcholine, and also of other clinically applied neuromuscular stimulants and blocking agents, was clearly of great theoretical and practical interest, but until less than 10 years ago did not seem amenable to effective experimental approach.

The combined progress of investigations of synaptic action, from the cellular all-or-none response, to the subcellular miniature end-plate potential, to the molecular response, has depended decisively on progress in increasing the sensitivity and stability of the recording technique. 30 years ago, the discovery of miniature end-plate potentials, that is the quantal response to a single packet of acetylcholine, containing thousands of molecules, may have been regarded as a technical achievement, which in a certain sense it was, by comparison with the registration of the much larger full-fledged impulse or end-plate potential. In 1970, we wanted to go further and determine the amplitude and lifetime of the molecular effect of the transmitter substance. It was clear, of course, that acetylcholine – one molecule, or maybe two or three in cooperation – acts on a membrane protein so as to open a single ion channel, allowing sodium ions to enter and produce a minute depolarizing current pulse. The question was: can one demonstrate this as a discrete molecular event and measure its size and time course? Ten years ago, we had little hope of recording it directly. We knew that if one gives progressively small-

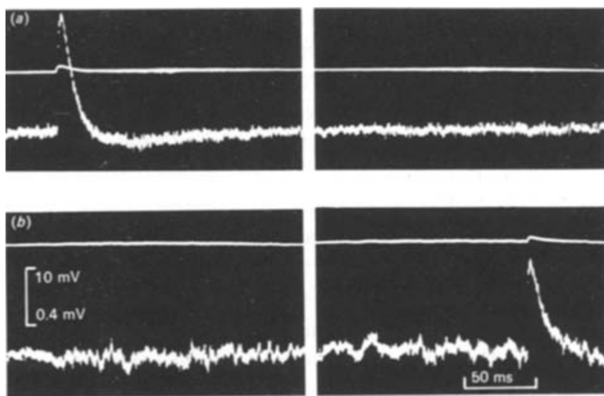

Fig. 4. 'Acetylcholine noise' (fluctuations in membrane potential due to application of acetylcholine), recorded from a frog muscle end plate [9]. In each block, the upper trace was recorded on a low-gain direct-coupled channel (scale 10 mV); the lower trace was simultaneously recorded on a high-gain, condenser-coupled channel (scale 0.4 mV). (a) Controls (no acetylcholine); (b) membrane fluctuations during a period of steady acetylcholine dosage, by diffusion from a drug-filled micropipette. In (b) the increased distance between the low- and high-gain traces is due to upward displacement of the direct-coupled trace because of acetylcholine-induced depolarization. Two sponaneous miniature end-plate potentials are also seen

er doses of acetylcholine to an end plate by discharging it from a nearby micropipette, the resulting electrical membrane response seemed to be continuously graded and certainly failed to show any discrete molecular components. Their size was clearly too small to be resolved. So we thought of the next best thing and began to look for statistical fluctuations in a steady average response produced by a constant dose. We decided, in fact, to search for the statistical play that might be produced by the random molecular bombardment of the end plate involving fluctuations of hundreds of molecular effects from one moment to the next, when we gave a steady dose which on the average kept, say, 10000 or 20000 ion channels open. If we have a steady average number of 10000 channels through which sodium ions flow at any given time, then on Poisson's simple statistical rule, we would expect the measured current to fluctuate with a standard deviation which is equal to the square root of 10 000, that is 1% of the mean value, and this would be within our grasp and within the resolving power of our recording method. This prediction proved to be correct (Fig. 4). We found that the action of acetylcholine in depolarizing the end plate is invariably associated with a characteristic voltage noise which we were able to measure and analyse.

This work was taken up successfully in several laboratories, and the method was considerably improved by the application of the voltage-clamp technique to measure the fluctuations in membrane current [10]. This work produced a large number of new and interesting clues, concerning the time course of the molecular action of a variety of acetylcholine-like agents and of its various antagonists like curare and other neuromuscular blocking agents. We concluded that acetylcholine molecules open ion channels which close after a life span of about 1 ms at 20 °C. This is quite characteristic of the transmitter acetylcholine itself; other related agonists, carbachol or acetylthiocholine, produce channels of shorter lifetime, maybe because these agents form a less stable compound with the receptor and dissociate after a shorter interval. Again, one substance, suberyldicholine reacts longer, and the ion channel remains open for a longer period.

The method by which these conclusions were reached, depended on a spectral Fourier analysis of the current fluctuations. The size of the channel and its electric conductance could be determined simply by finding the ratio between the variance and mean amplitude of the membrane current. The amplitude of the single channel current is of the order of 2 to 3 pA, and the conductance about $2 \times 10^{-11} \Omega^{-1}$, or 20 pS (that is, the electric resistance of a single channel is about 50000 MΩ). It produces a depolarization of about 0.25 μV. Thus, the molecular action is a few thousand times smaller than the quantal effect of a packet, it is a few hundred thousand times less than the effect of a nerve impulse at a single junction, and several hundred million times smaller than the discharge of the electric organ in elasmobranch fish. Nevertheless each channel, opened by the action of acetylcholine on a single membrane molecule, allows some ten thousand sodium ions to cross the cell membrane within 1 ms. The noise analysis enabled us to get new insight into the kintics of different agonists and blocking agents, and naturally it soon developed into a research field of its own which was taken up and intensively pursued in many laboratories.

But we had one reservation: our conclusions were reached on the basis of an indirect method. We had not been able to record the discrete molecular action by itself, but derived it from a statistical analysis of a massive multi-molecular fluctuation phenomenon, even though the underlying assumptions were of a very simple and plausible nature. It looked as though our aim was still some two orders of magnitude beyond the resolving power of the method which we employed. About two years ago, two research workers in Göttingen, E. Neher and B. Sakmann, at the Max-Planck-Institute for Biophysical Chemistry, succeeded brilliantly in doing just this. Their findings present a very important advance, and I will conclude by describing their technique and the results which they managed to obtain (Fig. 5).

Neher and Sakmann made up a special micropipette which was filled with a solution of acetylcholine or

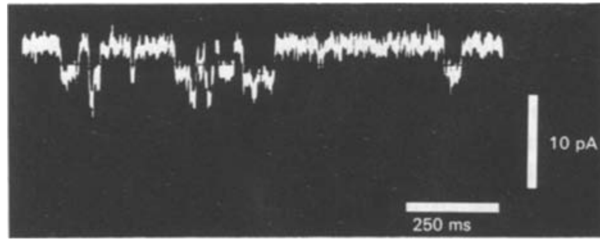

Fig. 5. 'Single-channel' current pulses, evoked by suberyldicholine in a small patch of frog muscle membrane [11]

similarly acting drugs and pressed gently over the sensitive area of the muscle membrane. The same pipette was also utilized to record the current pulses produced by the acetylcholine molecules acting on the underlying piece of membrane. I do not want to go into the electronic details of the technique. Sufficient to say that it involved an ingenious application of the voltage-clamp method, whereby the membrane potential is stabilized by negative feedback of a current; it is a null-point method in which the feedback arrangement automatically compensates and so provides a direct measurement of the membrane current. The whole experiment depended principally upon establishing a fairly tight seal between the glass wall of the pipette and the cell membrane. To achieve this, the authors had to prepare the cell surface by a special chemical cleansing procedure. They used a combination of proteolytic enzymes to remove traces of collagen from the outside. Furthermore, the noise fluctuations which resided in the recording system had to be reduced far beyond what had previously been achieved. The authors did this by choosing recording conditions which gave channels of the longest attainable life span. They made ingenious use of information which earlier experiments had provided. It was known that long-life channels can be obtained by certain experimental manipulations; for example, prior surgical denervation of muscle fibres causes them to develop new acetylcholine receptors with 'long-channel' properties. In addition, working at low temperature, using suberyldicholine instead of acetylcholine, hyperpolarizing the muscle membrane, all this helps to change the kinetics of the channel and to lengthen the duration of its open state. By combining these methods, one can produce channels which stay open for 30 to 40 ms instead of 1 ms and this, in turn, enabled the experimenters to cut down the frequency bandwidth of the recording system by a factor of 30, which reduced the undesirable background fluctuations to less than one fifth. In this way, Neher and Sakmann were able to observe the molecular effects themselves. The little square pulses in Fig. 5, about 3 pA in amplitude, were evoked by a low concentration of suberyldicholine in the sealed-on pipette. The pulses are of the rectangular type, and their individual durations vary at random around a mean value. All this had been predicted from the noise measurements, but could not be proved until the single 'shot effects' could be visualized directly. In many important respects, as regards the amplitude of the pulses, their duration, time course, the characteristic differences between several different agonists, these results have confirmed earlier conclusions, but it is clear that this new method opens up a new approach to a study of the kinetics as well as of the molecular mechanism of drug actions.

It has already thrown some interesting light on the mechanism of certain acetylcholine antagonists. These have been divided into so-called 'competitive' inhibitors, that is, drugs which one presumes combine with the acetylcholine receptor at the same attachment sites as the agonist molecule, and so block the access to the latter. There are other 'non-competitive' substances which apparently interfere with the kinetics of the ion channel after it has been opened and can even cause it to oscillate rapidly between open and blocked states [12]. It is possible now to ask a whole new range of questions concerning the molecular basis of synaptic transmission to which a few years ago no experimental approach seemed feasible. The new techniques will be not only of great theoretical interest to those who wish to study the properties of biological membranes, but of equal importance ultimately to those who are concerned with the practical, clinical application of membrane-active drugs.

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