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Transmitter release induced by injection of calcium ions into nerve terminals

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Calcium ions injected into the presynaptic nerve terminal in the giant synapse of the squid, evoked transmitter release while similar doses of Mg and Mn were ineffective. The transmitter release induced by intracellular application was still observed when Ca was replaced in the external fluid by Mn, in spite of the fact that this abolished transmitter release in response to presynaptic depolarization.

As far as is known, all 'chemical' synapses cease to transmit impulses if calcium ions are absent from the external medium. It has been shown that in these conditions, nerve impulses continue to invade the nerve terminals but fail to release transmitter; furthermore, the synapse may be reactivated locally by applying calcium ions from a micropipette. All this indicates that calcium ions are directly involved in the process whereby depolarization of the presynaptic nerve membrane leads to transmitter release (Katz & Miledi 1965, 1969*a*; Miledi & Slater 1966).

In a previous study on the giant synapse of the squid deprived of calcium, it was observed that intracellular injection of calcium did not restore the nerve impulse's ability to release transmitter, even though a similar focal application of calcium outside the presynaptic membrane had the expected effect of restoring transmitter release in that part of the synapse (Miledi & Slater 1966). However, it was still possible that intracellular injection of Ca^{2+} caused an asynchronous release of transmitter quanta, which may have remained undetected because, in the giant synapse, single transmitter packages evoke miniature synaptic potentials (m.s.ps) which are generally too small to be detected (Miledi 1967). Further experiments on this question are reported in this note.

The mantle–ganglion preparation of *Loligo vulgaris* was used as described previously (Miledi 1967; Katz & Miledi 1967). The preparation was perfused with oxygenated natural, or artificial, sea water with tetrodotoxin (2×10^{-7} g/ml) added to abolish nerve impulses. Three intracellular microelectrodes were generally used: one in the postaxon for monitoring synaptic potentials and thus transmitter release, and two in the presynaptic axon. Of the latter, one electrode was filled with $CaCl_2$ (0.1 to 0.5 M) and placed within the synaptic region.

When a Ca pipette was inserted into the presynaptic terminal there was usually an increase in the baseline noise, and a small steady depolarization, recorded by the postsynaptic electrode. In suitably small squid, in which discrete m.s.ps could

be resolved (cf. Miledi 1967), the postsynaptic depolarization and associated voltage fluctuations were clearly seen to be caused by an increase in the frequency of these potentials. This effect could be greatly reduced by applying a small negative bias to the Ca^{2+} pipette, thus preventing the efflux of calcium ions from it. The bias hyperpolarized the terminal by a few millivolts; but the reduction of transmitter release did not depend on this potential change for no such effect was seen when a similar hyperpolarization was imposed from a K-filled microelectrode inserted upstream. If the bias was subsequently removed, the postsynaptic axon became again slightly depolarized (figure 1). Experiments of this type indicate that intracellular application of calcium ions can indeed cause asynchronous release of transmitter.

FIGURE 1. Postsynaptic depolarization caused by leakage of Ca^{2+} into the presynaptic terminal of a squid giant synapse. The retention bias on the Ca pipette was switched off at the first arrow and back on at the second. All experiments in the presence of tetrodotoxin.

Further information was obtained by injecting pulses of calcium into nerve terminals, as in the experiment illustrated in figure 2. A postsynaptic depolarization was detectable with Ca pulses which caused small depolarizations of the presynaptic axon, but these depolarizations of the terminal were well below the 'threshold' level for phasic transmitter release (cf. Katz & Miledi 1967; Kusano 1970; Miledi 1967). If the intensity of the Ca^{2+} pulse was increased the postsynaptic potential occurred earlier, and its rate of rise and amplitude increased, until something resembling a plateau was reached beyond which the postsynaptic potential did not grow further but was merely prolonged. In these cases, the synaptic potential declined slowly so that nearly a minute had to elapse, after a 1 s pulse, before the depolarization returned to control levels.

The slow postsynaptic potential evoked by the injection of calcium into the terminal usually had some faster components superimposed. These peaks came on in a regular pattern as the intensity of the Ca^{2+} pulse was increased. The most likely explanation of all this is that as the pulse of Ca^{2+} is increased, the rate of transmitter release in the vicinity of the Ca pipette reaches a maximum, while more distant areas – with different capabilities for transmitter release – are progressively brought into action as calcium ions diffuse away from the site of an injection.

The question arises whether the response to injected calcium is a direct effect on transmitter release. It could be that an increase in intracellular Ca^{2+} leads to an increased Ca permeability and an influx of Ca from the external medium might be responsible for transmitter release. Another possibility is that the injected Ca^{2+}

crosses the membrane and acts on its outside surface. Some clues are obtained by repeating the internal injection of Ca^{2+} after replacing the outside calcium by manganese, since it is known that this prevents the release of transmitter normally induced by depolarization of the presynaptic membrane (Katz & Miledi 1969*b*). An example is shown in figure 3: the injection of Ca^{2+} still evoked transmitter

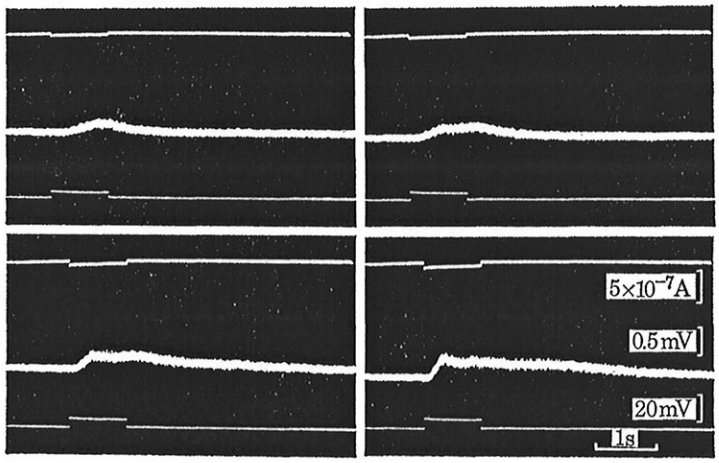

FIGURE 2. Effect of intracellular Ca pulses of increasing intensity on the giant synapse. Top traces monitor current flowing through Ca pipette inside nerve terminal. Middle traces monitor postsynaptic potential. Lower traces record presynaptic axon membrane potential.

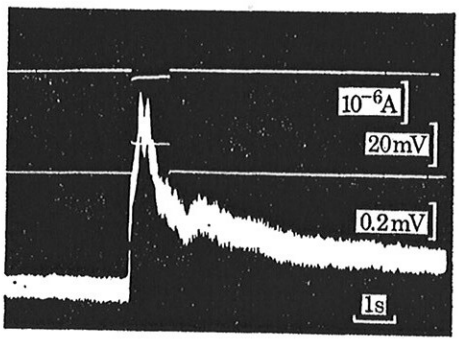

FIGURE 3. Effect of injecting a pulse of Ca into a presynaptic nerve terminal after replacing Ca by Mn (11 mM). Same preparation as for figure 2.

release even though the release in response to presynaptic depolarization had been suppressed. Lanthanum ions in the external fluid, which also abolish transmitter release evoked by presynaptic membrane depolarization (Miledi 1971), again did not prevent the transmitter release induced by the intracellular injection of Ca. All these experiments, therefore, suggest that the injected calcium is acting intracellularly, perhaps on the inner side of the presynaptic membrane.

Another interesting question concerns the specificity of calcium ions. At frog neuromuscular junctions and squid synapses, strontium can replace external Ca^{2+} in the process of transmitter release (Miledi 1966; Katz & Miledi 1969*b*). It has now been found that injection of Sr^{2+} into the presynaptic axon had effects similar to those of Ca^{2+}. Intracellular pulses of magnesium or manganese were practically ineffective in causing transmitter release. On the contrary, when a Ca pipette and a Mn pipette were both inserted in the synaptic terminal, pulses of Mn led to a slight reduction in the amount of transmitter released by a pulse of Ca. A similar antagonism was observed in one experiment where Mg and Ca were injected from a multi-barrel pipette.

In summary, these experiments showed that intracellular application of Ca^{2+} causes release of transmitter, and that this occurs in quantal form. The synaptic potentials evoked by intracellular pulses of Ca are small when compared with those evoked by a nerve impulse. However, account should be taken of the fact that a nerve impulse releases thousands of quanta, over the whole synapse, within a few milliseconds (Miledi 1967), while the response to injected Ca has a much longer duration and represents the release from only 0.1 to 0.2 of the synapse.

The size and time characteristics of the components in the responses to injection of Ca, were analysed with the statistical treatment used to study the molecular components of acetylcholine action at neuromuscular junctions (see Katz & Miledi 1972). After analysis of both the variance and the power spectral distribution, it was found that the Ca responses consisted of a summation of high-frequency m.s.ps of about 10 to 25 μV amplitude and 2 ms decay time constant, values which were also expected from previous work in which discrete m.s.ps were observed (cf. Miledi 1967). In the experiment illustrated in figure 1, the amplitude of the m.s.ps calculated from the mean and variance of the depolarization was 12.5 μV and the time constant of decay derived from Fourier analysis of the 'noise' was 1.8 ms. In the same experiment the maximum rate of release, evoked by a 1 s pulse of Ca, was 6.7×10^4 quanta/s. A rough estimate of the efficacy of injected Ca was that about 10^5 to 10^6 Ca ions were needed per transmitter quantum released. This is a large value, but not very surprising since most of the Ca injected is probably taken up by mitochondria (cf. Baker, Hodgkin & Ridgway 1971; Krnjević & Lisiewicz 1972). In fact the amplitude and time course of transmitter release by intracellular injections of Ca reflect factors such as diffusion of Ca, its uptake by mitochondria, binding to release sites and extrusion.

Experiments of the type described, lend strong support to the hypothesis that Ca ions act as a transmitter releasing factor on the inside of the terminal (Katz & Miledi 1967) and will help to study the various stages of the release process which follow entry of Ca into the terminal.

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