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THE NEUROMUSCULAR JUNCTION REVISITED: Ca^{2+} CHANNELS AND TRANSMITTER RELEASE IN CHOLINERGIC NEURONES IN *XENOPUS* NERVE AND MUSCLE CELL CULTURE

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Summary

Although the entry of calcium ions into the presynaptic nerve terminals through voltage-gated Ca^{2+} channels is now universally recognized as playing an essential role in evoked transmitter release at the neuromuscular junction (NMJ), and indeed in chemical synapses generally, we have as yet very little direct knowledge of the Ca^{2+} channels of the presynaptic terminals. In this work, making use of co-cultured nerve and muscle cells from *Xenopus* embryos, we studied the NMJ formed between the soma of identified cholinergic neurones and myoball, which allowed the use of patch-clamps on both the pre- and postsynaptic components. Both whole-cell and single-channel recordings of Ca^{2+} channels in the presynaptic cell were made. We found only one type of voltage-gated Ca^{2+} channel with high-voltage activation and slow inactivation characteristics, allowing its classification either as the L or the N type. The channels were susceptible to block by met-enkephalin but not to block by nifedipine or to enhancement by Bay K 8644. This combination of pharmacological properties favours their classification as the N type. Preliminary observations on the correlation between calcium currents and transmitter release disclosed a strikingly rapid run-down of the evoked release with unchanged calcium currents and spontaneous release during whole-cell recording, indicating a specific wash-out effect on some link between calcium entry and evoked transmitter release.

Introduction

The senior author (TPF) regrets that owing to unforeseen visa problems he was not able to take part, as he had intended, in the Batam Discussion Meeting on Synapse Formation that, being initially conceived by John Treherne shortly after he attended a similar meeting in Beijing University in 1988, has now sadly become a memorial to him. We are glad still to be able to contribute a paper to this volume which is, of course, also in honour of his memory. To its senior author this paper marks the beginning of a revisit to the NMJ, once his major research field (Feng,

Key words: neuromuscular junction, calcium channels, transmitter release, *Xenopus* nerve–muscle culture.

1988). There are threads of ideas which in his mind quite naturally connect the past with the present. One thread originates from his early studies on various effects of calcium on neuromuscular transmission that pointed, for the first time, to a specific relationship between calcium and transmitter release by the motor nerve endings (Feng, 1937), and led to the present work. As a result of the basic research of Bernard Katz, Ricardo Miledi, Rodolfo Llinas and others during the last several decades, it is now universally recognized that the entry of calcium ions through voltage-gated Ca^{2+} channels in the plasma membrane of the presynaptic nerve endings when they are depolarized by action potentials is an essential step in evoked transmitter release at the NMJ and, indeed, in chemical synapses generally. However, although we now know a great deal about voltage-gated Ca^{2+} channels in many cells, including nerve cells (see Tsien *et al.* 1988), we have practically no direct knowledge of the Ca^{2+} channels of motor nerve endings or other presynaptic terminals (Smith and Augustine, 1988). Their small size and the inaccessibility of their synaptic surface are severe obstacles to any attempt at a direct study of their ion channels. Even so, with the various new and powerful techniques, such as the patch-clamp, that have recently become available, it now seems worthwhile to attempt to gain more direct knowledge of the Ca^{2+} channels of the motor nerve endings and to understand further the links between calcium entry and transmitter release. We report here some initial results of our efforts in this direction.

The *Xenopus* neuronal soma–myoball system in culture

As yet there is no known NMJ that allows direct study of the Ca^{2+} channels of their presynaptic terminals with the patch-clamp technique, and we have to resort to more or less artificial preparations. In 1987 when one of us (TPF) was visiting the Department of Anatomy and Neurobiology in Washington University, St Louis, and the Jerry Lewis Neuromuscular Research Center in UCLA, he had, with the help of friends in these places, looked in a preliminary way into the possibility of using motor nerve endings, freed by enzymatic treatment, from snake and frog nerve–muscle preparations. Motor nerve endings freed or exposed in this way are useful for a variety of investigations, and a good example of their use has been given by Grinnell *et al.* (1989), but the possibility of making a direct study of the ion channels of the freed nerve endings using the patch-clamp technique needs further exploration. For the present work we turn first to *Xenopus* embryonic nerve and muscle cell culture and make use of the soma of an identified cholinergic neurone and a myoball manipulated into contact with it, the system used by Mu-ming Poo and his collaborators (Chow and Poo, 1985; Xie and Poo, 1986). Such a cell pair provides a model of the neuromuscular junction that allows the application of a patch-clamp on both the pre- and postsynaptic components and so enables the Ca^{2+} channels of the presynaptic neurone to be studied and the acetylcholine (ACh) release to be monitored simultaneously.

Through the whole-cell recording patch-clamp micropipette it is also possible to manipulate the intracellular chemical composition of the presynaptic cell. The potential usefulness of this experimental design is quite obvious. In addition, this culture system seems to provide attractive opportunities for studying possible developmental changes of Ca^{2+} channels and transmitter release accompanying the formation and maturation of the NMJ.

Cultures of *Xenopus* nerve and muscle cells were prepared essentially as described by Spitzer and Lamborghini (1976), Young and Poo (1983) and Xie and Poo (1986). The neural tube and the associated myotomal tissue of 1-day-old *Xenopus* embryos (stage 19–22, Nieuwkoop and Faber, 1967) were dissociated in Ca^{2+}- and Mg^{2+}-free saline supplemented with EGTA. The cells were plated on clean glass coverslips. The culture medium contained 10% (vol/vol) Leibovitz medium L15, 2% (vol/vol) foetal bovine serum and 200 i.u. ml^{-1} gentamycin sulphate. A whole-cell patch-clamp electrode carrying a myoball was used first as a probe to identify cholinergic neurones and then to monitor transmitter release when a neurone soma and a myoball were brought together and formed a NMJ. For the study of the Ca^{2+} channels of identified cholinergic neurones, both whole-cell recording and single-channel recording from cell-attached patches were carried out. Two patch-clamp amplifiers (V. Pantani, Yale University, USA) were used. An Atari computer served both to control the execution of the experiments and to handle the data. A tape recorder was also available for storing some of the data for playing back onto a storage oscilloscope.

Calcium channels in the soma of identified cholinergic neurones: electrophysiological and pharmacological properties

We began by studying the Ca^{2+} channels of the soma of identified cholinergic neurones by using whole-cell patch-clamp recording to investigate whether there was more than one type of voltage-gated Ca^{2+} channel and, if so, what types they were. Fig. 1A shows a series of Ca^{2+} current records in response to 200 ms depolarizing voltage pulses from a holding potential of −100 mV, increasing the depolarization in steps of 10 mV, with the first pulse from −100 mV to −80 mV and the last from −100 mV to +70 mV. From Fig. 1A two points are clear. (1) With depolarizations up to −40 mV, there is practically no activation of the Ca^{2+} channels; opening of the channels only begins to be noticeable when depolarization reaches −30 mV, maximum current occurring at 0 to +10 mV, after which the currents become smaller with further depolarization, undergoing a reversal in sign at about +50 mV. The corresponding I–V curve is shown in Fig. 1B. (2) Once activated, the currents are well maintained for the duration of the 200 ms long pulses, i.e. the inactivation is uniformly slow. Comparing the above characteristics of calcium currents in the cholinergic neuronal soma we are studying with the calcium currents of the dorsal root ganglion (DRG) neurones described by Fox *et al.* (1987*a*,*b*), the differences are obvious: the kinetic characteristics of the

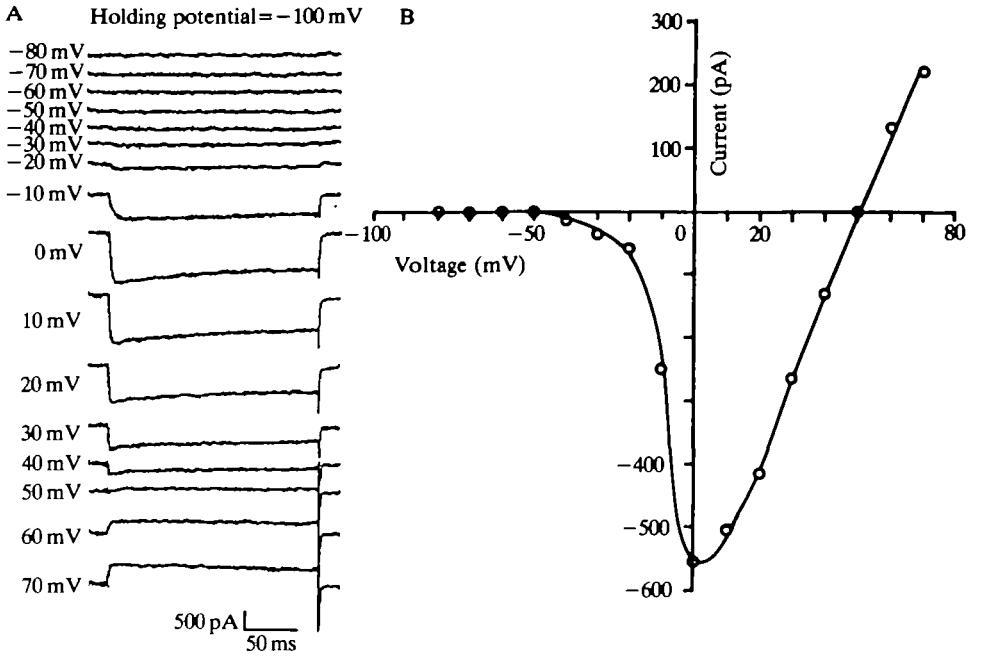

Fig. 1. (A) Whole-cell Ca^{2+} currents of the soma of an identified cholinergic neurone elicited by 200 ms depolarizing voltage pulses all from a holding potential of −100 mV, increasing in steps of 10 mV. All current signals shown in this and other figures are after subtraction of linear leak and capacity current. External solution (in mmol l^{-1}): TEA-Cl, 115; $CaCl_2$, 5; TTX, 0.001; Hepes, 10; pH 7.6 adjusted with TEAOH. Pipette solution (in mmol l^{-1}): CsCl, 90; Cs_4–EGTA, 10; $MgCl_2$, 5; ATP, 2; Hepes, 20; pH 7.4 adjusted with CsOH. Test pulses given every 5 s. (B) Corresponding I–V curve.

calcium currents of the DRG neurones, as given by Fox *et al.*, clearly show the presence of more than one type of calcium channel, while the current characteristics of our neurone give no evidence of multiple channel types. In particular there is no sign of the presence of calcium channels in our neurone with properties resembling the T-type channels of the DRG neurones. Fig. 2 shows a series of Ca^{2+} current records produced by longer depolarizing voltage pulses. The holding potential is −80 mV. The successive pulses are from −10 mV to +50 mV, spaced at 10 mV intervals as indicated. The purpose of this figure is to show that, although the time course of inactivation varies with the depolarizing voltage, in each record the decay time course can be fitted by an exponential with a single time constant. This provides additional evidence that the Ca^{2+} currents of the neurone under study are given by a single type of Ca^{2+} channel.

Fig. 3 shows the effects of replacing Ca^{2+} with Ba^{2+} and also compares the effects of two different concentrations of the two cations. The larger current with Ba^{2+} instead of Ca^{2+} and the relative shifts in the positions of the peak current are all to be expected for Ca^{2+} channels activated by high voltage, which the Ca^{2+}

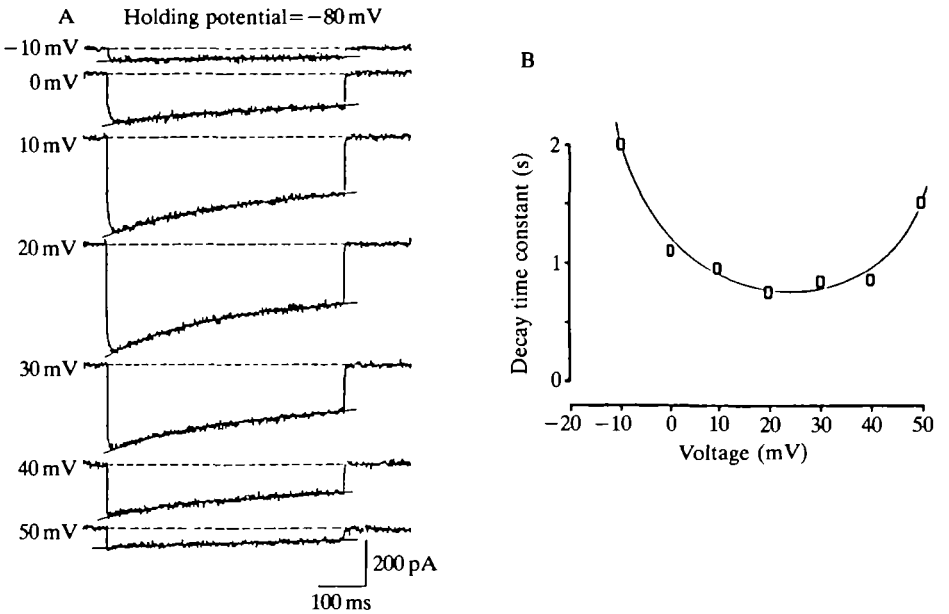

Fig. 2. (A) Ca^{2+} currents elicited by 500 ms depolarizing pulses all from a holding potential of −80 mV. Test pulses given every 10 s. The inactivation time course varies with the activation voltage, but in each case can be fitted by a single exponential. (B) Plots of the time constants of the exponentials against the activation voltages.

channels under study evidently are. We also examined the I–V curve obtained with 90 mmol l^{-1} Ba^{2+}, instead of 5 mmol l^{-1} Ba^{2+}, in the bath. With the much larger Ba^{2+} concentration the peak current became much larger and its position shifted further in the direction of more positive voltage. We mention this observation here as in our single-channel recordings the higher Ba^{2+} concentration was used. An example of our single-channel recordings is shown in Fig. 4. Here the same characteristics of the Ca^{2+} channels, i.e. activation by high voltage and slow inactivation, are equally as evident as in whole-cell recordings.

Judging only by the above electrophysiological observations, it is possible to classify the Ca^{2+} channels of the neuronal soma we are studying as either the L or the N type. For a definite identification, it is also necessary to take into consideration their pharmacological properties.

So far we have studied the effects of two cations, Cd^{2+} and Ni^{2+}, two dihydropyridines, nifedipine and Bay K 8644, ω-conotoxin (ω-CgTX) and met-enkephalin. The results are collected in Table 1. The previously known actions of these agents on the neuronal N- and L-type Ca^{2+} channels are also quoted in Table 1 for comparison. Special attention is called to the lack of effect of nifedipine and Bay K 8644 and the striking blocking effect of met-enkephalin on the Ca^{2+} channels of the *Xenopus* neurone soma. This combination of pharmaco-

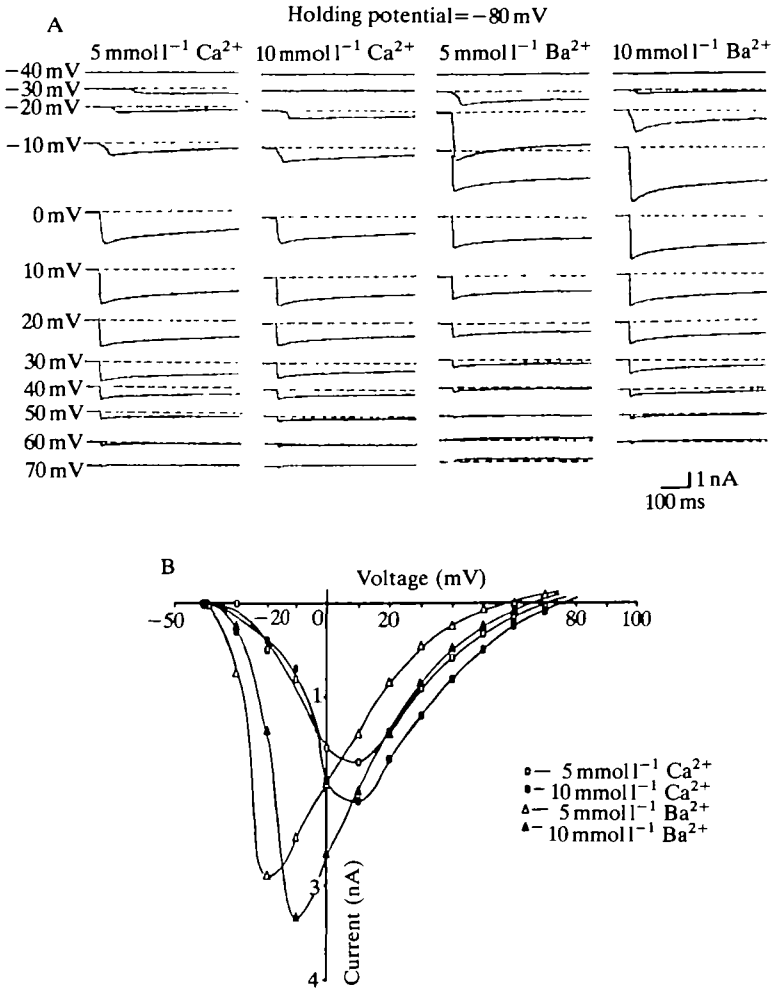

Fig. 3. (A) Comparison of Ca^{2+} currents elicited by the same series of depolarizing pulses when the external solution (otherwise the same as given in the legend to Fig. 1) contained 5 mmol l^{-1} Ca^{2+}, 10 mmol l^{-1} Ca^{2+}, 5 mmol l^{-1} Ba^{2+} instead of Ca^{2+} or 10 mmol l^{-1} Ba^{2+} instead of Ca^{2+}. (B) Corresponding I–V curves.

logical properties seems to be a clear indication that the Ca^{2+} channels under study can be classified as the N type.

In summary, we conclude that the Ca^{2+} channels of the soma of the *Xenopus* cholinergic neurones we are studying are all of the same type, the N type. Barish (1989) recently reported that 'Three components of Ca current corresponding to T-, N- and L-types are found in neurons that differentiate in cultures of dissociated *Xenopus* neural plate cells.' The reason for this apparent discrepancy between Barish's observations and ours remains to be explained. We plan to go into this matter later when taking up developmental studies of Ca^{2+} channels in cultured *Xenopus* neurones.

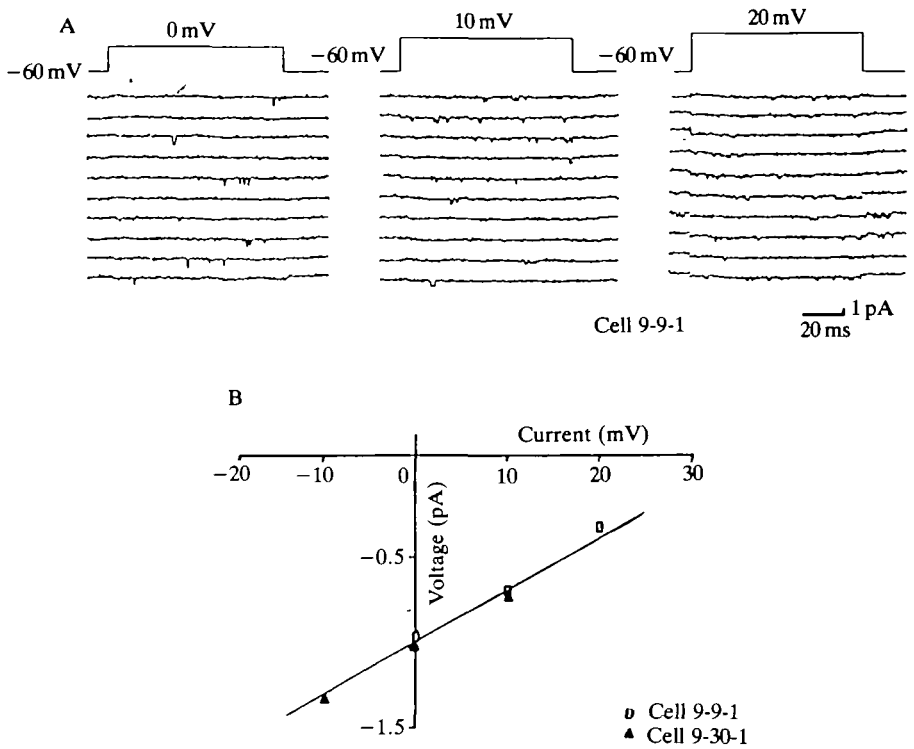

Fig. 4. (A) Single-channel Ca^{2+} currents recorded from a cell-attached patch. Three groups of consecutive current records taken at 5 s intervals are shown below their respective associated voltage protocols. Head-stage amplifier feedback resistance 10 GΩ; current signals filtered at 1 kHz. External solution (in mmol l^{-1}): CsCl, 90; Cs_4–EGTA, 10; $MgCl_2$, 5; Hepes, 20; pH 7.4 adjusted with CsOH; pipette solution (in mmol l^{-1}): $BaCl_2$, 90; TEACl, 10; Hepes, 10; pH 7.6 adjusted with $Ba(OH)_2$. (B) Single-channel slope conductance (25 pS) derived from current records including the one shown in A.

Transmitter release at the soma–myoball junction

Evoked release at the soma–myoball junction

It has been amply shown by Poo and his collaborators (Chow and Poo, 1985; Xie and Poo, 1986; Evers *et al.* 1989) that the different regions of a cultured *Xenopus* spinal neurone – the growth cone, the neurite and the soma – are all capable of spontaneous transmitter release, giving rise to what they call spontaneous synaptic current (SSC) in the myoball contacting any of the three regions. Usually the frequency and the mean amplitude of the SSCs found at the soma are significantly lower than at the neurite or growth cone (Evers *et al.* 1989). The evoked release giving rise to evoked synaptic current (ESC) at the soma–myoball junction, however, has not previously been studied. As we are interested in correlating Ca^{2+} current recorded in the soma with its transmitter release, we have made a special study of the ESC at the soma–myoball junction. We found that, to get

Table 1. *Pharmacological properties of the Ca^{2+} channels of the* Xenopus *embryonic cholinergic neurones compared with those of the N- or L-type Ca^{2+} channels in other nerve cells*

Agent	Concentration (μmol l^{-1})	*Xenopus*	N	L
Cd^{2+}	30	Partial block	Block	Block
	200	Block		
Ni^{2+}	100	Little effect	Little effect	Little effect
Nifedipine	10	No block	No block	Block
	100	No block	No block	Block
Bay K 8644	5	No effect	No effect	Enhancement
ω-CgTX	10	Block, irreversible	Block, irreversible	Block, irreversible
Met-enkaphalin	40	Block, reversible	Block	

The data cited for the N- or L-type Ca^{2+} channels are all from Fox *et al.* (1987*a*,*b*), except that for met-enkephalin (Barish, 1989).
ω-CgTX, ω-conotoxin.

ESCs reliably from the soma–myoball junction, the choice of neurone was important. Our experience was that the somas of neurones that had already grown long neurites were generally less capable of responding to stimuli, i.e. of giving ESCs. For the study of evoked release from the soma we therefore chose neurones that had only just begun to grow processes.

An example of ESCs elicited by stimuli (1 V and 0.5 ms square pulses delivered through a polished micropipette with a tip diameter of 2–4 μm in direct contact with the soma) given once every 5 s for several minutes after establishing the soma–myoball contact is given in Fig. 5. The ESCs are irregular in size, very similar to those given by the growth cone–myoball or neurite–myoball junction described by previous workers (Xie and Poo, 1986; Sun and Poo, 1987).

Ca^{2+} currents and transmitter release by the presynaptic cell in the soma–myoball system

To conduct correlated studies on calcium currents and transmitter release requires simultaneous whole-cell patch-clamp recordings from the pre- and postsynaptic cells. This demands very careful experimentation, involving first forming a whole-cell recording patch-clamp microelectrode on a myoball, then manipulating the myoball into contact with the soma of a neurone, and finally, after verifying that the neurone is cholinergic from its ability to give rise to SSCs, forming another whole-cell patch-clamp microelectrode on the soma. The completion of this whole chain of delicate manoeuvres remained, even after considerable practice, quite a strenuous effort with frequent failures. From the successful experiments we have made, a special note may be made of the following two points.

(i) Within the first 1–2 min after starting the usual whole-cell recording, there was, with a series of depolarizing pulses of different amplitudes, a general

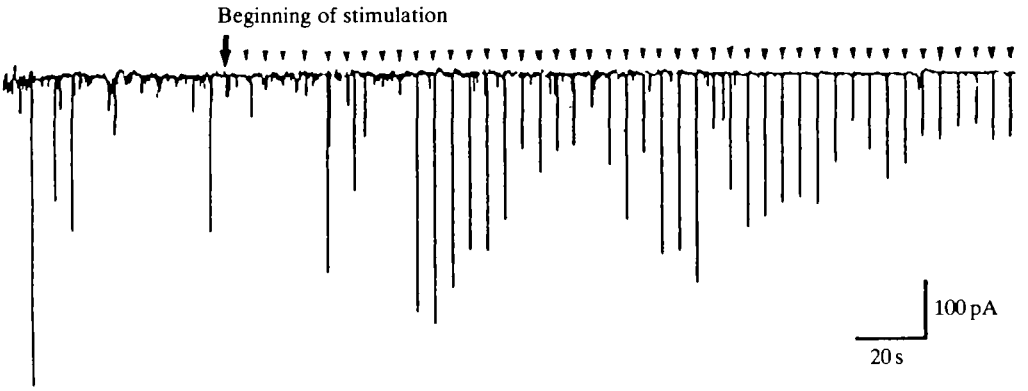

Fig. 5. An example of evoked synaptic currents (ESCs, peak amplitudes) at a soma–myoball junction elicited by stimuli (1 V and 0.5 ms square pulses) once every 5 s delivered through a polished micropipette with a tip diameter of 2–4 μm applied to the neuronal soma. Every stimulus is marked. There are some spontaneous synaptic currents before and in between the stimuli. The myoball was clamped at −62 mV through the whole-cell recording patch-clamp pipette. Pipette solution (in mmol l^{-1}): KCl, 106.5; $MgSO_4$, 1.3; $CaCl_2$, 1; K_4–EGTA, 11; NaCl, 0.67; Hepes, 5; pH 7.4 adjusted with KOH.

correlation between the size of the Ca^{2+} current and the size of the ESC elicited each time, but there were many irregularities. This is to be expected, as the ESCs elicited by constant stimuli are irregular in size, as already shown in Fig. 5. An example of correlated observation on Ca^{2+} currents and transmitter release evoked by 20 ms depolarizing pulses is given in Fig. 6, which shows similar irregularities. The basis of such irregularities is still a puzzle. The optimal experimental conditions for conducting correlated studies on Ca^{2+} currents and evoked transmitter release remain to be worked out. Attention may first be called to an unexpected phenomenon interfering with such studies.

(ii) With the usual whole-cell recording method, within 2–3 min after rupturing the cell-attached patch during the formation of the whole-cell recording mode, while the depolarizing voltage pulses still produced the same Ca^{2+} currents, the correlated evoked transmitter release began to decline. It disappeared altogether within 4–5 min. When this happened, the spontaneous transmitter release giving rise to SSCs continued without significant change. There is thus a rapid differential run-down of the evoked release, with unchanged calcium current and spontaneous release. Recording the Ca^{2+} current required the elimination of the potassium current, so the micropipette was filled with a solution containing caesium instead of potassium. We wondered whether the caesium entering the cell might be responsible for the run-down of the evoked release and so, in a number of experiments, we forsook the recording of Ca^{2+} currents. We filled the whole-cell recording micropipette with a solution containing KCl to replace CsCl as the main constituent, and followed the change of the evoked release with time. It was found

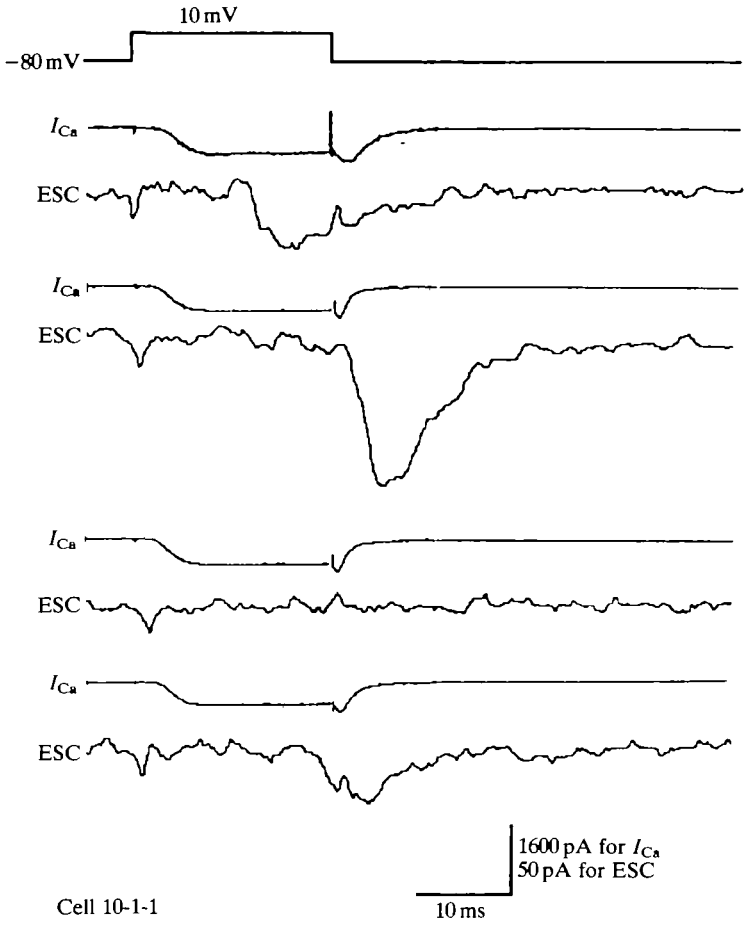

Fig. 6. Simultaneous records of the presynaptic Ca^{2+} current (I_{Ca}) and the postsynaptic evoked synaptic current (ESC) at a soma–myoball junction produced by 20 ms voltage pulses from −80 to +10 mV applied to the soma through the whole-cell recording patch-clamp pipette, showing the irregularity of the ESCs with practically the same I_{Ca}. External solution (in mmol l^{-1}): NaCl, 111; KCl, 3; $CaCl_2$, 5; tetrodotoxin, 0.02; tetraethylammonium, 5; Hepes, 10; pH 7.6 adjusted with NaOH; pipette solution (in mmol l^{-1}): CsCl, 90; Cs_4–EGTA, 10; $MgCl_2$, 5; ATP, 2; pH 7.4 adjusted with CsOH.

that there was the same rapid run-down of the evoked release as when caesium was in the micropipette. Thus, it appeared that the run-down was probably due to the loss of some cytoplasmic compounds from the cell rather than to the introduction of some harmful substance into the cell. This idea was further tested by using a modified whole-cell patch-clamp recording method introduced by Horn and Marty (1988). In this method, nystatin, a pore-forming antibiotic, is added to the micropipette solution. After the micropipette has established a gigaohm seal on the cell, the patch of membrane separating the micropipette from the cell interior

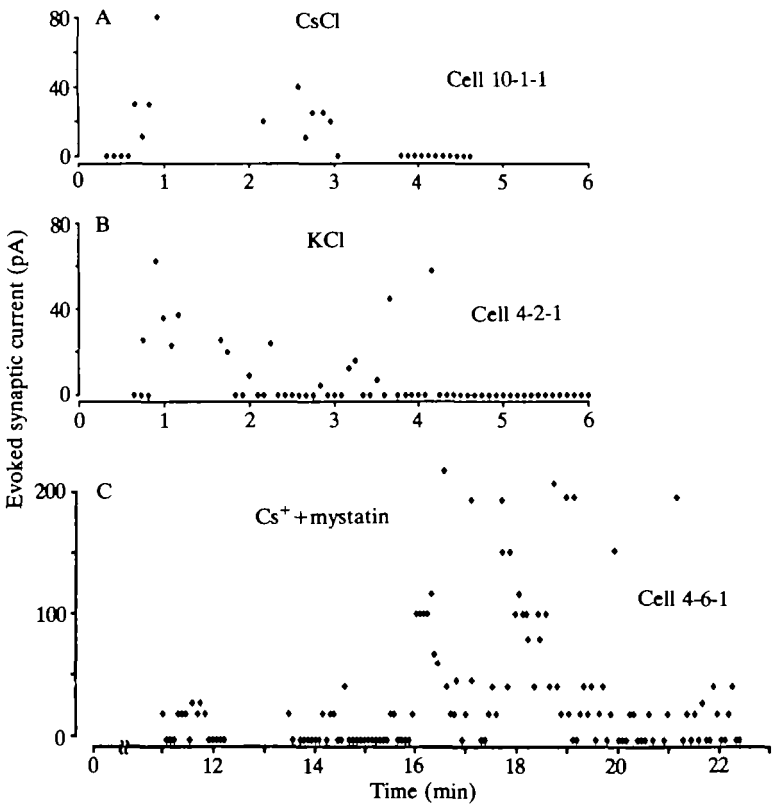

Fig. 7. The rapid decay of the evoked synaptic current (ESC) with the usual whole-cell recording method whether the micropipette solution contained CsCl (A) or KCl (B), and the prevention of the decay (C) with the nystatin whole-cell recording method (see text for explanation). Note the difference in scale for both the ordinate and abscissa between C and A and B: In C much larger ESCs appeared with longer observation times which, however, may not be typical. In C the zero of the abscissa roughly represented the time when the nystatin in the micropipette began to act on the patch of cell membrane sealing the micropipette tip; it usually took about 10 min for its pore-forming effect to develop sufficiently for observations to be started. Composition of solutions: external solution and pipette solution in A, same as in Fig. 6; external solution in B and C (in mmol l^{-1}): NaCl, 111; KCl, 3; $CaCl_2$, 5; tetrodotoxin, 0.01; pH 7.6 adjusted with NaOH; pipette solution in B (in mmol l^{-1}): KCl, 110; K_4–EGTA, 1; $MgCl_2$, 5; ATP, 2; Hepes, 20; pH 7.4 adjusted with KOH; pipette solution in C (in mmol l^{-1}): CsCl, 110; Cs_4–EGTA, 1; $MgCl_2$, 5; ATP, 2; Hepes, 20; 100 $\mu g\,ml^{-1}$ nystatin, pH 7.4 adjusted with CsOH.

is not ruptured by suction, as is the usual procedure, but is made permeable to cations by the antibiotic to provide the electrical continuity required for whole-cell recording. The permeabilized patch, while allowing the free flow of electric current, prevents the loss of cytoplasmic constituents. We have made preliminary observations with this method of whole-cell recording. Fig. 7 compares the change with time of the ESCs elicited by depolarizing pulses applied to the soma through

the whole-cell recording micropipette containing caesium, potassium or caesium plus nystatin. It shows that with the nystatin method of whole-cell recording the quick run-down of the evoked release could be prevented.

We hope that with the nystatin whole-cell recording method we shall be able to carry out more detailed studies on the relationship between Ca^{2+} currents and transmitter release. It should also be noted that the wash-out effect, apparently specifically affecting the evoked transmitter release, seems to be an important phenomenon in itself. Its analysis might help to disclose or define some essential link in the chain of molecular interactions leading from calcium entry to transmitter release.

We are grateful to Drs R. W. Tsien and F. J. Sigworth for valuable help in the course of equipping our patch-clamp laboratory, to Professor H. H. Chuang for a constant supply of *Xenopus* embryos from his aquarium and to Professor Kunitaro Takahashi for a gift of nifedipine and ω-conotoxin.

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