Quantitative analysis of single muscle fibre action potentials recorded at known distances

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Summary   In vivo records of single fibre action potentials (SFAPs) have always been obtained at unknown distance from the active muscle fibre.

A new experimental method has been developed enabling the derivation of the recording distance in animal experiments. A single fibre is stimulated with an intracellular micropipette electrode. The same electrode is used thereafter for labelling with an auto-fluorescent dye, Lucifer Yellow. In this method there is no use of chemical fixation. The tissue structure is kept as well as possible. In cross-sections the fluorescent fibre is seen and its position is quantitized with respect to the tip of one or more recording wire electrodes.

Morphometric data, such as the recording distance and the fibre cross-sectional area, are used for the interpretation of parameters of the SFAPs (peak-peak amplitude, time between the first positive and negative peaks). The present results show that within 300 μm recording distance is not as dominant for the SFAP shape as expected.

The method offers also a direct check of the relation between the muscle fibre; diameter and the conduction velocity of the action potential. In the present small set of data there is no simple linear relationship.

Key words: Action potential; Fibre labelling; Fibre cross-section; Conduction velocity; Recording distance; Muscle fibre; SFEMG

In the description of single fibre action potentials (SFAPs) within 300 μm of an excited muscle fibre the tissue structure plays an important role (Albers et al. 1986, 1988a, b, c). Therefore, information concerning the undisturbed tissue structure is essential to the experimental study of SFAPs at this small scale.

This paper describes the techniques of labelling a single stimulated muscle fibre with an auto-fluo-rescent dye, the detection of this fibre and the derivation of the distance between the excited fibre and the extracellular recording electrode in unfixed and unstained cryosections of the muscle. The morphometric data (fibre dimensions, recording distance, tissue structure), obtained with the new labelling procedures, can be used for SFAP analysis. The restricted set of data obtained with the presented techniques offers another view of SFAPs than generally assumed.

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Materials and methods

Muscle preparation

Experiments were carried out in vivo on the m. extensor digitorum longus (EDL) of the rat (Wistar, male, 3–5 months old, 0.3–0.4 kg), using Nembutal anaesthesia. A detailed description of

the preparation of the EDL and anaesthesia during the experiment was given by Wallinga-De Jonge et al. (1980). Experiments were carried out at optimal twitch length and at a muscle temperature of 36–38°C.

Electrodes

Micropipette electrodes were pulled from glass capillaries with an internal filament. The outer diameter of the capillaries was 1.5 mm (Clark Electromedical Instruments, Type GC 150F-15). The tip diameter of the micropipettes was 2.0 ± 0.5 μm. The pipettes were filled with the autofluorescent dye Lucifer Yellow CH (the di-lithium salt, Sigma) (Stewart 1978), dissolved in a 0.5 M LiCl solution. The dye concentration was 50 mg/ml. Filling of the micropipettes was achieved using a fine injection needle. The resistance of these electrodes ranged from 4 to 15 MΩ.

SFAPs were recorded with a 14-channel wire electrode arrangement. The wire electrodes had a stainless steel core with a diameter of 25 μm, isolated with a 4 μm Karma coating. The wire tip was cut at an angle of about 45° and coated with a small silver layer.

Electrophysiological recording and labelling

The experimental procedure is illustrated in Fig. 1.

Ten to 14 wire electrodes were inserted into the muscle in linear arrays of 3 electrodes. The array orientation was normal to the fibre direction. The most cranial electrode group was about 5 mm away from the origin tendon and the total length over which the electrodes were placed was 4–5 mm. The wires were positioned at an angle of about 45° with respect to the surface of the muscle, with the tip pointing to the insertion.

The micropipette was tapped into the muscle with abrupt steps of 5 μm at a position at least 4 mm towards the insertion tendon from the most caudal wire electrode. An abrupt decrease of about 70 mV of the potential at the micropipette tip indicated penetration of a muscle fibre. The muscle fibre was stimulated by a hyperpolarizing current pulse with an amplitude of 250–350 nA and a duration of 30–40 msec. Successful stimulation of

the fibre resulted in 1–5 responses. If an SFAP with an amplitude larger than 1 mV was recorded at one or more extracellular wire electrodes, the stimulated muscle fibre was labelled by iontophoresis of Lucifer Yellow CH from the micropipette.

Iontophoresis was carried out using hyperpolarizing current pulses with an amplitude of 40 nA. The pulses had a duration of 2 sec and a duty cycle of 50%. The minimum time these current pulses had to be applied in order to achieve reliable fibre labelling appeared to be 20 min. During iontophoresis the resting membrane potential and the resistance of the pipette tip were monitored. In most experiments the membrane potential slowly increased and the pipette tip resistance remained constant. An abrupt increase in membrane potential indicated that the pipette tip no longer remained in the stimulated fibre. A sudden increase in resistance indicated electrode blocking, which nearly always appeared to be irreversible.

After labelling the micropipette was retracted. The positions of the wire electrodes at which SFAPs were recorded were marked by electrophoretic deposition of silver from the wire tip by application of a current of 1 μA during 15 sec. These wires were cut carefully just above the surface of the muscle. The intramuscular part of the wire served as an additional indication of the recording position. The other electrodes were removed from the muscle.

The muscle was quickly frozen avoiding change in muscle length and stored at a temperature of at most $-90°$C. The time passing between termination of iontophoresis and freezing of the muscle was about 30 min.

Morphological procedure

Serial frozen cross-sections of 10 μm thick were cut with a Walter Dittes cryostat at a temperature of $-25°$C. The cryostat was extended with a micrometer device with an accuracy of 5 μm. Sectioning of the muscle was done from insertion towards origin. In this way the silver from the electrode tip was sectioned first, before the electrode itself was touched. Sectioning the wires often caused burrs in the knife. Damage to the section could be reduced by frequent repositioning of the knife.

The stimulated muscle fibre was detected by auto-fluorescence of Lucifer Yellow CH in the original cryosections (stimulating wave length: 430 nm, emission wave length: 540 nm). The fluorescent fibre was followed easily up to the non-fluorescent part of the stimulated fibre. There the fibre was traced from section to section up to and including the sections with silver deposits. The radial distance between the centres of the labelled fibre and a silver deposit was measured, as well as the longitudinal distance between the site of stimulation and the recording sites.

For morphometric determinations photographs of unfixed, unstained sections were used (to exclude distortion induced by histological staining procedures). The diameters of a number of fibres around the labelled fibre were estimated, using the method of the best fitting circle (Zuurveld et al. 1985) with a resolution of 4 μm. The cross-sectional area of the labelled fibre was determined using a grid with a resolution of 10 μm^2.

After morphometric evaluation the sections were stained with a Sirius red staining (modified after Sweat et al. 1964) for further analysis of the tissue structures, especially around the stimulated fibre (such as connective tissue strands, blood vessels and nerves).

Serial cryosections were used to determine the fibre type histochemically with: succinate dehydrogenase (SDH: EC 1.3.99.1, Nachlas et al. 1957), α-glycerol-phosphate-dehydrogenase (GPOX: EC 1.1.99.5, Wattenberg and Leong 1960, modified for GPOX, Jobsis 1971) and myofibrillar ATPase (ATPase: EC 3.6.1.3, Dubowitz and Brooke 1973), pre-incubation pH 4.35. The classification of fibres was according to the scheme of Wirtz et al. (1983).

Action potential analysis

The SFAPs were low pass filtered (f_c = 40 kHz, 12 dB/octave) and amplified. Records were stored digitally after sampling at a rate of 100 kHz and A-D conversion (10 bits resolution, 8192 samples/channel). Because of the position of the recording electrodes (at least 4 mm from the stimulation micropipette and some millimetres from the tendon) nearly all SFAPs were triphasic. Only these triphasic SFAPs were analysed. Characteristics derived were: the peak-peak amplitude (the
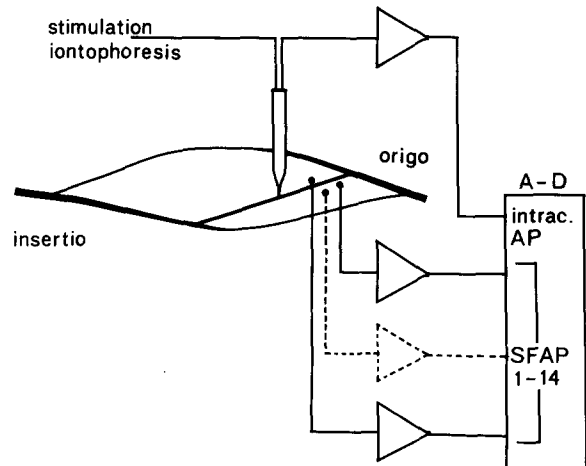

Fig. 1. A scheme of the experimental procedure. SFAPs were recorded with a 14-channel wire electrode arrangement. They were low pass filtered (f_c = 40 kHz, 12 dB/oct) and amplified by DC-coupled amplifiers. Records were stored digitally after sampling at a rate of 100 kHz and A-D conversion (10 bits resolution, 8192 samples/channel). Only 3 electrodes have been drawn; each one represents a group of 3 electrodes in a linear array, normal to the fibre direction.

potential difference between the first positive maximum and the minimum), V_{tt}; and the time between the first positive maximum and the minimum, Δt.

Conduction velocity derivation

During cryosectioning the longitudinal positions of the sections were measured with a micrometer device built into the microtome. The cross-sections with the silver deposits were noted. The conduction velocity was derived from the latency between two simultaneously recorded SFAPs and the longitudinal distance between their recording positions. The zero crossing between the first positive and the negative phase of the SFAP was used to determine the latency between 2 action potentials (Stålberg 1966).

Results

It is generally assumed that V_{tt} decreases and Δt increases with recording distance. The values of V_{tt} versus Δt of all recorded triphasic SFAPs in 17
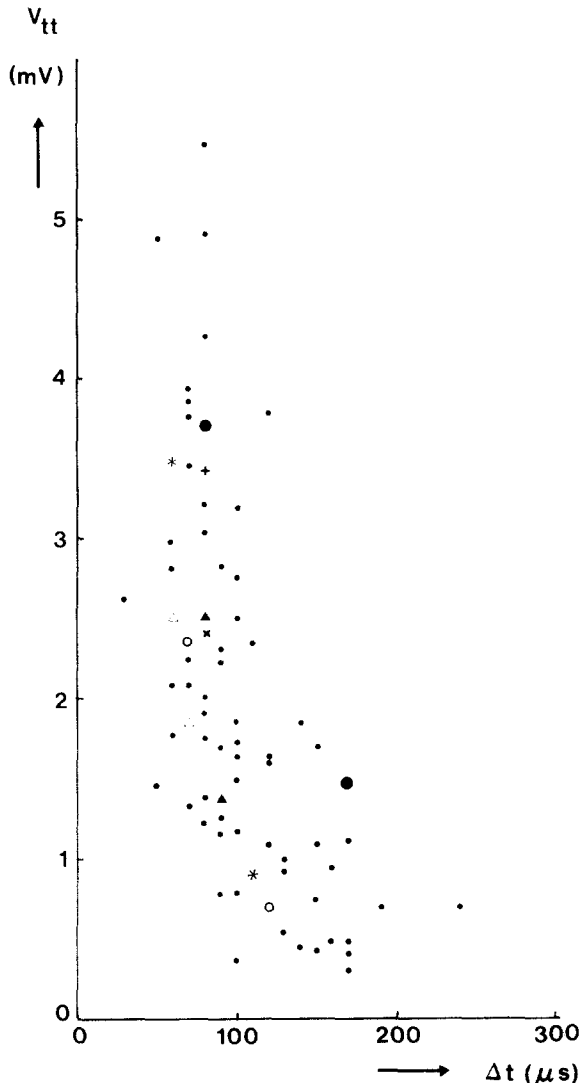
Fig. 2. The amplitude, V_{tt}, of 80 triphasic SFAPs plotted against their Δt (time between the first positive maximum and the negative maximum). The results belonging to the experimental data given in Table I have been marked.

experiments are plotted in Fig. 2. The plot covers a large area. The data measured at known distances are highlighted in the figure. After description of the morphometrical results we will give the V_{tt} as function of the recording distance and the fibre cross-sectional area.

In the labelled muscle fibres fluorescence was observed over a length of 2–4 mm. During storage

at low temperature the dye remained within the labelled muscle fibre, despite the fact that no chemical fixation was applied. At room temperature Lucifer Yellow slightly diffused due to water condensation on the surface of the section. Stain diffusion was prevented by freeze drying of the section and avoiding condensation on the slides. Since exposure to daylight would cause fading of the fluorescence, the sections were kept in the dark as much as possible.

In Fig. 3a an example of a fluorescent fibre is given. Fig. 3b shows a neighbouring cryosection in bright field.

In 12 out of 14 'fibre typed' experiments the stimulated fibres were FG (according to the classification of Peter et al. 1972), IIB (according to Brook and Kaiser 1970) or type Y (according to the scheme of Wirtz et al. 1983). In 2 cases the labelled fibre appeared to be of a slightly more oxidative type (type J) in the Wirtz scheme. Here it is not possible to indicate the type in the other classifications, because the type is intermediate between IIB and IIA and FG and FOG. The clear preference of the method for type Y fibres can be caused by the Y fibre prevalence in the peripheral layer of the muscle and by their relatively large dimensions (Zuurveld et al. 1985).

A considerable variation in cross-sectional fibre dimensions was found in the EDL (see Figs. 3a, b and 4a). The fibre diameters of the whole fibre population ranged from 21 to 75 μm in the dorsal/medial part of the muscle. In unstained sections the mean fibre diameter of 100 fibres around the labelled fibre varied in 8 experiments from 41 to 51 μm.

Fig. 4a shows a silver deposit indicating an extracellular recording position. Silver deposits were observed in 1 or in 2–3 adjacent sections and the transverse dimensions varied according to this (from 10 to 30 μm). The possible error in the recording distance increases with the size of the silver deposit. The reliability of the recording distance is estimated to vary between 10 and 30 μm. The extracellular action potential recorded at the position indicated in Fig. 4a is shown in Fig. 4b. The recording distance was 80 $\pm$ 10 μm.

The morphological, histochemical and electrophysiological data of SFAP experiments at known
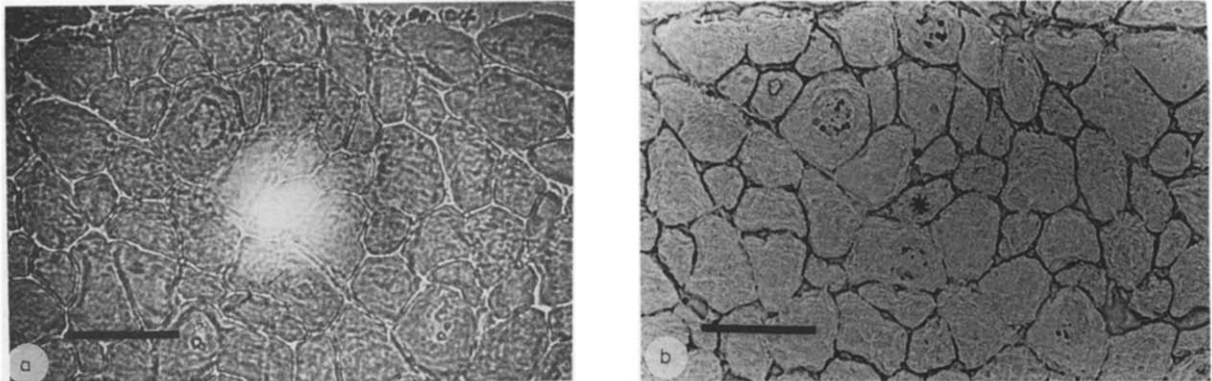
Fig. 3. a: fluorescence microphotograph of a part of a cryosection. The lightly coloured fibre is the one labelled with Lucifer Yellow CH. b: light microphotograph of a cryosection of the same muscle, taken at about the same position as in a. The labelled fibre, shown in a, is indicated with an asterisk. The bar represents 100 μm.

distances are summarized in Table I. Note that all records represented were made from muscle fibres which had rested quite a long time before the stimulation. The amplitude of the SFAP decreased, with one exception (exp. 85), with distance to the fibre. The data showed that the amplitude of an SFAP was not set dominantly by the recording distance (Fig 5a). A normalization of the amplitude with the fibre area did not eliminate the differences between the experiments (Fig. 5b).

The Δt (time between the first positive and the negative maximum) increased with the recording distance, again with the results of exp. 85 as exception. The range of values of Δt for the records at about the same distance (3 values for 190 and 200 μm) was large (60–170 μsec).

Often a positive correlation has been suggested between the conduction velocity of the AP along the fibre and the fibre diameter. For 5 experiments this relation could be derived directly. This
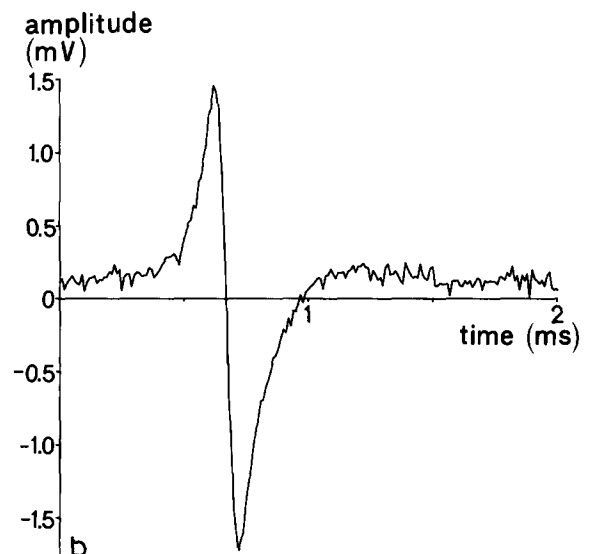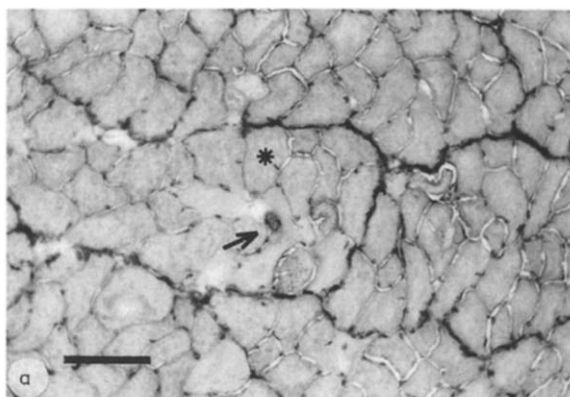

Fig. 4. a: cryosection stained with Sirius Red. The black dot (indicated with an arrow) is the silver deposit of the wire electrode used to record an SFAP. The stimulated and labelled fibre is indicated with an asterisk. The bar represents 100 μm. b: single fibre action potential recorded at the position indicated in a.

small set of observations did not enable a clear conclusion about the relation between conduction velocity and fibre size.

Preceding activity can influence the action potential shape. When a series of responses of a fibre occurred after the injection of current the Δt

values were derived at all relevant electrodes. Slight changes either with positive or with negative sign during the train of action potentials were found. The directions of change in Δt in the records at different electrodes were similar.

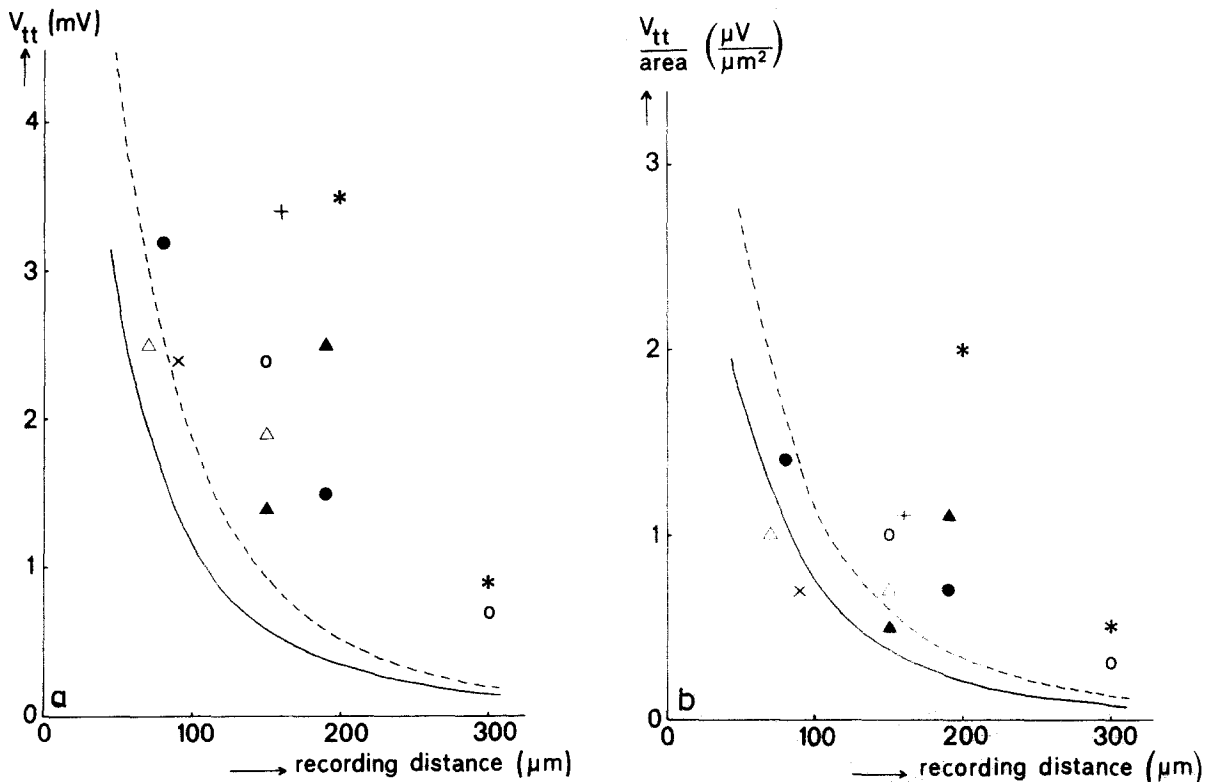The conduction velocity of the action potential

Fig. 5. a: the SFAP amplitude versus recording distance with respect to the center of the active fibre. b: the SFAP amplitude/cross-section area versus recording distance. The experimental observations are indicated by the symbols given in Table I. Examples of simulations are represented by the curves (———, homogeneous model; - - - - -, inhomogeneous model). The parameter values (for background see Albers et al. 1988c) are:

intracellular conductivity	$0.750\ (\Omega m)^{-1}$
extracellular conductivity	$1.800\ (\Omega m)^{-1}$
parallel homogeneous conductivity	$0.855\ (\Omega m)^{-1}$
transverse homogeneous conductivity	$0.095\ (\Omega m)^{-1}$
fibre radius	$25\ \mu m$
intracellular volume fraction	0.9
membrane capacitance/surface area	$0.01\ Fm^{-2}$
membrane conductance/surface area	$1\ \Omega^{-1}\ m^{-2}$
conduction velocity	$3\ m/sec$

TABLE I

Summary of histochemical/morphometrical data of stimulated fibres and their SFAPs at known recording distances.

Exp.	Symbol	Fibre area (μm^2)	Fibre type (Wirtz et al. 1983)	El. code	Recording distance (μm)	V_{tt} (mV)	V_{tt}/ area (μV/ μm^2)	Δt (μsec)	Cond. vel. (m/sec)	Error cond. vel. (m/sec)
77	+	3100	Y	7	160	3.4	1.1	80	–	–
79	●	2300	Y	12	80	3.2	1.4	100	8.5	1.6
				1	190	1.5	0.7	170		
81	×	3300	Y	10	90	2.4	0.7	80	–	–
82	○	2400	J	13	150	2.4	1.0	70	6.1	0.5
				9	300	0.7	0.3	120		
85	▲	2200	Y	4	150	1.4	0.6	90	4.0	0.3
				13	190	2.5	1.1	80		
88	△	2600	Y	7	70	2.5	1.0	60	3.4	0.7
				6	150	1.9	0.7	70		
89	★	1700	J	8	200	3.5	2.0	60	6.9	1.2
				12	300	0.9	0.5	110		

along the muscle fibre was determined as well. If there was any variation of the conduction velocity during a train of responses, it remained very small.

Discussion

The decrease of the amplitude of single fibre action potentials recorded at increasing distances from an active isolated muscle fibre in saline is well known (Håkansson 1957). When an active fibre is surrounded by other fibres and tissue components with complicated electrical conductivity properties the action potential cannot be measured directly at known distance. Modelling of this situation with muscle fibres but without other structures is already very complicated (Albers et al. 1986, 1988a, b, c). A relation between action potential amplitude and estimated recording distance is given by Ekstedt (1964) and Gath and Stålberg (1978). The measurements were made with a multielectrode array in a needle. The largest amplitude was assumed to occur at the electrode closest to the active fibre. A very reasonable assumption, because all results showed a smooth decline of the amplitude to both sides along the electrode array. The distances between the various recording positions in the multielectrode were known, but the absolute distances between the

electrodes and the fibre could only be estimated. The size of the electrode shaft (diameter about 0.5 mm) was large with respect to the fibre dimensions (about 70 μm) and the SFAP recording distances, smaller than 300 μm. This might have introduced a relative uniform condition masking the conductivity properties of the tissue.

The advantage of the method presented here is that it enables the detection of a stimulated muscle fibre in original cryosections by means of a fluorescent dye, while in the experiments small wire electrodes are used. Tissue shrinkage is negligible and morphology is reasonably well preserved. Moreover, enzyme histochemistry can be performed allowing fibre typing.

Still, in the analysis of the results we have to consider several possible sources of error. Consecutively we shall discuss: direct tissue damage from the electrode(s); burrs on the microtome knives caused by sectioning of the wire electrodes; the size of the silver deposit; reliability of the shape and size of the active fibre; other structures in the tissue in the recording area (e.g., blood vessels and connective tissue layers).

Damage to tissue structure by the insertion into the muscle of the micropipette and wire electrodes is inevitable. Even when the stimulation micropipette and recording electrodes are positioned carefully following the longitudinal course of the

superficial fibres, a large number of electrodes are necessary to create a realistic opportunity to record SFAPs close to the stimulated fibre.

The cutting of the microtome knife through the silver deposit and the wire electrodes often causes burrs on the knife. We make the choice of cutting with the electrodes still present because the electrode cross-sections serve as markers for the silver deposit in the preceding cross-sections. Otherwise a silver deposit can be easily missed. It is an exception when the silver deposit or wire is dragged through the muscle cross-section by the sectioning procedure. The structures of 4–5 neighbouring sections are reasonably similar. So the morphological analysis is done on the cross-sections preceding the ones with the silver deposits.

The silver deposits are observed in 1–3 adjacent sections. The shape of the deposit is not spherical. The uncertainty about the centre of gravity is not constant in the various experiments. The reliability of the recording distance is estimated to be from 10 to 30 μm. Especially when the recording electrodes are close to each other the error can be quite apparent. The uncertainty in the position of the silver deposit determines the error in the conduction velocity (see Table I).

In the literature the diameter of the muscle fibre is often given; other data about the size and shape of the fibres are seldom mentioned. Values of the mean diameter of rat EDL fibres show considerable variation; differences in the histological procedures are at least partially responsible for this. Fibre type distribution patterns in the muscle cross-section (Armstrong and Phelps 1984; Zuurveld et al. 1985) will contribute as well. The results presented in Table I are all in the dorsal/medial part of the EDL; the values are within the range of literature values.

We expect that the spatial geometry of the active fibre with respect to the recording electrode has a large effect on the action potential. Attention to this point will be paid in future experimental results and in simulations.

The real distribution of current injected by the active fibre will be determined by the conductivity properties of the components, taken in consideration in the model (see parameters listed in Fig. 5) and by the presence of other structures between the rather regularly packed muscle fibres, e.g., connective tissue layers and blood vessels. An extended set of experimental data is necessary to derive conclusions about their effects.

In 4 out of 5 experiments (the ones with more than one simultaneous records of the same active fibre) the generally assumed decrease of the SFAP amplitude with recording distance was found, but the discrepancy in values between the experimental and simulated characteristics is intriguing (Fig. 5a). In the formula representing the SFAP amplitude, the cross-sectional area of the active fibre occurs (e.g., Plonsey 1974; Albers et al. 1988a, formula 9). Håkansson (1957) finds a linear relation experimentally between SFAP amplitude and the cross-sectional area in saline. The agreement between experiment and simulation in our data is enhanced if the amplitude/cross-sectional area is considered instead of the amplitude (Fig. 5b). These results can be further normalized by dividing through the square of the conduction velocity value (see Albers et al. 1988a, formula 9). A worsening of agreement between experiments and simulations and a further scattering in the experimental results is, however, the result.

In conclusion, the advantage of the method presented in this paper is that it provides direct information concerning the recording distance, together with information about the actual tissue structure. The electrophysiological data of a single muscle fibre, intracellularly (Wallinga-De Jonge et al. 1985) and extracellularly (as described in this paper), and the morphological data of that fibre and the surrounding tissue together could support the SFAP simulations. We learn from this study that the comparison between detailed experimental and simulated data demands further research. Thereafter, we can start discussion about the real effects of tissue structures, shape and size of the active fibre.

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