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THE ORIGIN OF THE SKEWED AMPLITUDE DISTRIBUTION OF SPONTANEOUS EXCITATORY JUNCTION POTENTIALS IN POORLY COUPLED SMOOTH MUSCLE CELLS

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Abstract—The skewed amplitude distribution of spontaneous excitatory junction potentials (sEJPs) in the mouse vas deferens and other electrically-coupled smooth muscle syncytia has been attributed to electrically-attenuated depolarizations resulting from the spontaneous release of quantized packets of ATP acting on remote smooth muscle cells (SMCs). However, in the present investigation surface SMCs of the mouse isolated vas deferens were poorly electrically coupled, with input resistances (176±18 MΩ, range: 141–221 MΩ, n=4) similar to those of dissociated cells. Furthermore, the amplitude of evoked EJPs was more variable in surface compared with deeper SMCs (F test, F=17.4, P<0.0001). Using simultaneous electrophysiology and confocal microscopy to investigate these poorly-coupled cells, it is shown that α-latrotoxin-stimulated sEJPs correlate, in timing (median delay ranged from −30 to −57 ms, P<0.05 in all experiments, n=5) and amplitude (Pearson product moment correlation, ρ>0.55 and P<0.001), with purinergic neuroeffector Ca^{2+} transients (NCTs) in SMCs. The temporal correlation between sEJPs of widely ranging amplitude with NCTs in the impaled SMC demonstrates that all sEJPs could arise from neurotransmitter action on the impaled cell and that the skewed distribution of sEJPs can be explained by the variable effect of packets of ATP on a single SMC. The amplitude correlation of sEJPs and NCTs argues against the attenuation of electrical signal amplitude along the length of a single SMC. The skewed sEJP amplitude distribution arising from neurotransmitter release on single SMCs is consistent with a broad neurotransmitter packet size distribution at sympathetic neuroeffector junctions.

Key words: calcium imaging, confocal microscopy, neurotransmission, smooth muscle, ATP, sEJP.

The quantal basis of neurotransmission at the skeletal neuromuscular junction was identified by del Castillo and Katz (1954), in their reanalysis of experiments by Fatt and Katz (1952). They concluded that "transmission at a nerve-muscle junction takes place in all-or-none 'quanta' whose sizes are indicated by the spontaneously occurring miniature (end-plate potential) discharges." The narrow, Gaussian amplitude distribution of the miniature end-plate potentials (their Fig. 7), the invariance of this distribution as the Ca^{2+} concentration changed and the existence of a multimodal histogram of excitatory postjunctional potentials imply that evoked neurotransmitter release causes integer multiples of a consistent unitary electrical response. The distribution of packet sizes released from sympathetic postganglionic terminals has been much harder to study because the electrical syncytium that arises through the coupling of smooth muscle cells (SMCs) makes it difficult to distinguish local from distant neurotransmitter release sites during intracellular recording. To tackle this problem, several different approaches have been tried. By differentiating the excitatory junction potential (EJP) it is possible to identify intermittent 'discrete events' buried within the depolarization phase (Blakeley and Cunnane, 1979), which indicate the intermittent release of neurotransmitter packets. Some of these discrete events had sizes comparable to the amplitude of the derivative of spontaneous excitatory junction potentials (sEJPs), which argues that the same types of packets contribute to both events, but does not indicate that evoked release is an integral multiple of unitary packets. A further approach is to measure local extracellular potential changes arising following both spontaneous and evoked neurotransmitter release: the excitatory junction currents (sEJCs and EJCs; Brock and Cunnane, 1987). This approach demonstrates intermittent neurotransmitter release with a broad packet size. A development of this approach to study visualized varicosities under conditions of low release probability with small electrode tips (Macleod et al., 1994) demonstrated that, at 1 mM Ca^{2+}, the truncated amplitude distribution of EJPs was broad (their Fig. 3B), arguing against packets of uniform size; that packet size was not uniform was implicit in the model they used to fit the results at higher external Ca^{2+} concentrations, where the basic packet size varied as a gamma variate (Robinson, 1976). Further evidence for a broad amplitude distribution of the fundamental packet size at the autonomic neuroeffector junction has come from the use of laser-scanning confocal microscopy to image purinergic neuroeffector Ca^{2+} transients (NCTs) on an impulse-to-impulse basis at individual neuroeffector junctions (Brain et al., 2002, 2003). NCTs arise when intermittently-released packets of ATP activate local $P2X_1$ receptors, causing a local increase in smooth muscle Ca^{2+} concentration. The finding that these local Ca^{2+} transients are amplified by Ca^{2+}-induced Ca^{2+} release in the SMC,

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Abbreviations: α-SNAP, α–soluble *N*-ethylmaleimide-sensitive factor attachment protein; BAPTA-1 AM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid–1 acetoxymethyl ester; EJP, excitatory junction potential; LTX, α-latrotoxin; NCT, neuroeffector calcium transient; NF449, 4,4′,4″,4‴-[carbonylbis[imino-5,1,3-benzenetriyl bis (carbonyl-imino)]]tetrakis(benzene-1,3-disulfonic acid); PSS, physiological salt solution; sEJP, spontaneous excitatory junction potential; SMC, smooth muscle cell.

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however, means that the broad amplitude distribution of NCTs at a single junction cannot be used to imply a broad distribution of basic packets size (Brain et al., 2003).

The mouse vas deferens, unlike that of the guinea pig, provides a good model to study quantal neurotransmission because there is already good evidence that the SMCs are poorly electrically coupled; in particular, it has not been possible to detect electronic potentials spreading from cell-to-cell or across the mouse vas deferens (Holman et al., 1977).

By combining confocal imaging with simultaneous intracellular electrophysiological recording in the mouse isolated vas deferens, it is possible to establish whether a correlation occurs between a traditional electrophysiological approach to monitoring neurotransmitter release, the EJP, and recently-developed optical approaches. Moreover, the combined techniques permit a study of the effective neurotransmitter packet size at the autonomic neuroeffector junction.

EXPERIMENTAL PROCEDURES

Ca^{2+} indicator loading

Eight- to 12-week-old Balb/c mice (Harlan, Bicester, Oxfordshire, UK) were killed by cervical fracture and both vasa deferentia removed. Efforts were made to minimize the number of animals used and their suffering; all experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and European Communities Council Directive 86/09/EEC. The connective tissue around each vas deferens was carefully dissected in order to obtain clear images of SMCs and to remove any ganglia located close to the prostatic end.

Each vas deferens was then exposed to 10 μM Oregon Green 488 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid–1 acetoxymethyl ester (BAPTA-1 AM) (Invitrogen, Paisley, Renfrewshire, UK) in 1% dimethyl sulfoxide/0.2% pluronic F-127 (Sigma-Aldrich, St. Louis, MO, USA) in physiological salt solution (PSS) for 2 h at 36 °C. Each tissue was then cut longitudinally to create a flat sheet and rinsed in PSS, bubbled with 95% O_2/5% CO_2, for at least 10 min. Tissues were pinned flat, serosal side up in a Sylgard-lined organ bath, and mounted on the stage of an upright confocal microscope. The PSS contained (mM): NaCl 118.4, $NaHCO_3$ 25.0, NaH_2PO_4 1.13, KCl 4.7, $CaCl_2$ 1.8, $MgCl_2$ 1.3 and glucose 11.1. The pH was maintained at 7.4 and the solution oxygenated by continuous bubbling with 95% O_2/5% CO_2.

Confocal microscopy

The vas deferens was placed in a chamber that was continuously superfused with standard PSS (bath temperature 33–34 °C). Images were acquired with a Leica SP2 upright confocal microscope (Leica Microsystems, Milton Keynes, UK). A series of 100 or 200 frames was captured at approximately 5 or 13.5 Hz to generate one image set. Such sets were acquired once every minute. Between 8 and 12 such sets were acquired for each SMC.

Surface SMCs do not lie perfectly orthogonal to the optical axis of the microscope, and therefore measurement of SMC lengths required this finding to be taken into consideration. When measuring cell lengths, a series of high-resolution (1024×1024 pixels) images was taken at intervals along the *z* axis. An average was then taken of the resultant series (‘*z*-stack’) of images, and the *x*–*y* projected length was calculated using a polygon tool function of Canvas (version 9, ACD Systems, Miami, FL, USA). The *z*-component was calculated by measuring the focal plane position at each end of the cell, so that a more precise length in three-dimensional space could be calculated using Pythagoras’ Theorem.

Image analysis

Image analysis was performed with the stack profile function of the Leica LCS software or using an Image J (http://rsb.info.nih.gov/ij/download.html) plug-in written by R. J. Amos. In the first frame of the image series a region of interest was established which encompassed the portion of a SMC visible within the confocal plane. The fluorescent signal in this region was measured over time throughout the image set. Data were exported to Excel (Microsoft, Redmond, WA, USA) for formatting and then to Spike 2 (Cambridge Electronic Design, Cambridge, UK) for analysis in conjunction with electrophysiological recordings.

Electrophysiology

Conventional intracellular recording techniques were used to record sEJPs in SMCs (see Brock and Cunnane, 1992). Each vas deferens was superfused with PSS and drugs were applied by swapping the perfusion solution to one containing the drug at the required final bath concentration. Preparations were perfused with prazosin (100 nM; Sigma-Aldrich) and α-latrotoxin (LTX) (25 pM; Sigma-Aldrich) for 30 min prior to recordings. The low concentration of latrotoxin was used to induce a mildly elevated rate of spontaneous neurotransmitter release so that a sufficient number of events could be detected within a fixed imaging period, but not so high that the majority of events could not be independently measured. Microelectrodes (tip resistances of 140–160 MΩ) were filled with Texas Red (2 mM filtered in 5 M potassium acetate; weight 625 Da; Invitrogen). The membrane potential was measured with an Axoclamp 2B (Axon Instruments, Sunnyvale, CA, USA) in bridge mode, in conjunction with a frame-coupled TTL output from the microscope to allow temporal correlation of electrophysiological and confocal recordings.

Recordings of electrically-evoked EJPs were achieved by applying rectangular pulses (0.6 ms duration; voltage amplitude at twice the threshold for eliciting EJPs, typically around 20 V) delivered through Ag/AgCl electrodes positioned around the prostatic end of the vas deferens. In some experiments, ‘non-surface’ SMCs were impaled using an almost vertical electrode approach (in the absence of confocal microscopy) with no attention paid to remaining close to the serosal surface.

In additional experiments, surface SMCs were voltage-clamped at various values of alternating depolarization and hyperpolarization with respect to their resting membrane potential (mean -65.1 ± 2 mV) for 200 ms, with approximately 10 s between steps. The input resistance was calculated for the linear part of the voltage–current relationship (around the resting membrane potential). Both inward and outward currents were identified during depolarizing steps (in two of four cells), and in one cell, an inward current was observed following a hyperpolarizing step (data not shown). These phenomena were not further investigated as part of the present study.

The voltage and current were digitized (at 5 kHz and 1 kHz, respectively) with a PowerLab system (ADInstruments, Chalgrove, UK). Recordings of sEJPs were exported from Chart 4.2 (ADInstruments) for analysis with Spike 2. The amplitudes of EJPs were calculated using Chart 4.2 (ADInstruments).

The microelectrode was always within the field of view of the confocal image. On completion of each experiment, the SMC recorded from was labeled by injecting Texas Red by iontophoresis (current injection, +2 nA, 15 s).

Analysis of correlations between NCTs and sEJPs

Analysis of the amplitudes of sEJPs and NCTs was performed on data where confocal images were acquired at 5 Hz. To obtain a