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NICOTINE INDUCES CALCIUM SPIKES IN SINGLE NERVE TERMINAL VARICOSITIES: A ROLE FOR INTRACELLULAR CALCIUM STORES

K. L. BRAIN, S. J. TROUT, V. M. JACKSON, N. DASS and T. C. CUNNANE*

Department of Pharmacology, Mansfield Road, University of Oxford, Oxford OX1 3QT, UK

Abstract—While nicotine is known to act at neuronal nicotinic acetylcholine receptors (nAChRs) to facilitate neurotransmitter release, the mechanisms underlying this action are poorly understood. Some of its effects are known to be mediated by presynaptic receptors. In the mouse vas deferens nicotine (10–30 μM) transiently increased the force of neurogenic contraction by 135 ± 25%, increased the amplitude of excitatory junction potentials by 74 ± 6% and increased the frequency of spontaneous excitatory junction potentials in four out of six preparations. Confocal microscopy and the calcium indicator Oregon Green 488 BAPTA-1 dextran were used to measure calcium concentration changes in the nerve terminals. Nicotine did not affect the action potential-evoked calcium transient but instead triggered small, random fluctuations ('calcium spikes') in intra-varicosity calcium concentrations at an average frequency of 0.09 ± 0.02 Hz. These were insensitive to tetrodotoxin at a concentration that blocked action-potential evoked calcium transients (300 nM). They were abolished by the nAChR blocker hexamethonium (100 μM) and by both ryanodine (100 μM) and caffeine (3 mM), agents that modify calcium release from intracellular stores.

We propose a novel mechanism whereby nicotine's action at nAChRs triggers calcium-induced calcium release from a ryanodine-sensitive calcium store in nerve terminals. This primes neurotransmitter release mechanisms and enhances both spontaneous and action potential-evoked neurotransmitter release.

Key words: vas deferens, synaptic transmission, calcium-induced calcium release (CICR), mouse, nicotinic acetylcholine receptor, excitatory junction potentials.

The principal psychoactive chemical in tobacco is nicotine and one of its actions is to potently enhance neurotransmitter release in the periphery by mechanisms that remain poorly understood. It is likely that presynaptic facilitatory nicotinic acetylcholine receptors (nAChRs) located on both central (McGehee et al., 1995) and peripheral (Rose et al., 1999) neurones underlie many of the drug's perceived effects and contribute to its liability for dependence. Presynaptic nAChRs are increasingly being exploited for the treatment of dementias (Nishizaki et al., 2000) and in nicotine replacement therapies to help stop smoking. Thus, there is great interest in nAChRs but their effects remain difficult to study, particularly those located on nerve terminals in intact preparations.

Neuronal nAChRs are known to be located on peripheral ganglia, the adrenal medulla and both peripheral and central nerve terminals. The actions of nicotine on sympathetic nerve terminals mediate, at least in part, its acute cardiovascular actions (Nedergaard and Schrold, 1977; Richardt et al., 1988; Haass and Kubler, 1997). Nicotine is known to increase the release of neurotransmitter following nerve stimulation in the rodent vas deferens (von Kügelgen and Starke, 1991; Todorov et al., 1991). The mouse vas deferens has a rich sympathetic innervation and has been successfully used in the past to measure changes in the intra-terminal calcium concentration (Brain and Bennett, 1997). For these reasons we chose to investigate the effects of nicotine on neurogenic contractions, intra-varicosity calcium dynamics and neurotransmitter release from postganglionic sympathetic nerve terminals in the mouse vas deferens.

*Corresponding author. Tel.: +44-1865-271612; fax: +44-1865-271853.

E-mail address: tom.cunnane@pharm.ox.ac.uk (T. C. Cunnane).

Abbreviations: BAPTA, 1,2-bis(2-aminophenoxylethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; $[Ca^{2+}]_v$, intra-varicosity calcium concentration; $\Delta[Ca^{2+}]_v$, change in intra-varicosity calcium concentration; CICR, calcium-induced calcium release; DMSO, dimethyl sulphoxide; EJPs, excitatory junction potentials; nAChRs, nicotinic acetylcholine receptors; n_p, number of experimental preparations; n_s, number of strings of varicosities; n_v, number of varicosities; SEJPs, spontaneous excitatory junction potentials; TTX, tetrodotoxin; VGCCs, voltage-gated calcium channels.

EXPERIMENTAL PROCEDURES

Vasa deferentia were removed from 8–12-week-old Balb/c mice (Harlan, UK), which had been humanly killed by cervical fracture. All efforts were made to minimise the number of animals used and their suffering; all experiments were in accordance with the European Communities Council Directives (86/609/EEC of 24 November 1986) and approved by the local ethics committee (Department of Pharmacology, University of Oxford, Oxford, UK). A midline incision was made to expose the abdominal viscera and the vasa deferentia were carefully dissected out and cleared of loosely adhering connective tissue. The prostatic third of each vas deferens was removed to ensure that no ganglia were present in the preparation. The tissue was

bathed in Krebs solution which contained (mM): NaCl 118.4, $NaHCO_3$ 25.0, NaH_2PO_4 1.13, $CaCl_2$ 1.8, KCl 4.7, $MgCl_2$ 1.3 and glucose 11.1. The pH and [O_2] were regulated by continuously bubbling the solution with 95% O_2/5% CO_2. All electrophysiological and confocal experiments were carried out in the presence of 1 μM nifedipine (an L-type calcium channel blocker) and 100 nM prazosin (a competitive α_1-adrenoceptor antagonist) to reduce movement artefacts induced by contraction. No attempt was made to block the purinergic component of contraction.

Contraction studies

Isometric recordings were made from vasa deferentia suspended in 5 ml organ baths using Letica transducers coupled to a MacLab A/D converter and recording system (AD Instruments, Hastings, UK) to allow recording of the data for subsequent off-line analysis. Platinum ring electrodes (positioned around the proximal end) were used to elicit contractions with trains of five stimuli at 10 Hz (pulse width 0.3 ms, supramaximal voltage of 40 V) applied every 30 s. Preparations were allowed to equilibrate for 1 h under a resting tension of 9.8 mN before beginning the experiment. Drugs were added directly to the organ baths and were removed by flushing with multiple bath changes of Krebs solution over 10 s.

Electrophysiology experiments

Conventional intracellular recording techniques were used to record excitatory junction potentials (EJPs) in smooth muscle cells as a measure of ATP release from postganglionic nerves (see Brock and Cunnane, 1992). Preparations were pinned to the Sylgard (Dow-Corning, Reading, UK) covered base of a 3 ml Perspex organ chamber and gently stretched to their approximate *in situ* length. Tissues were superfused with Krebs solution at a rate of 2 ml min^{-1} and drugs were applied by changing the perfusion solution to one containing the drug at the required final bath concentration. Trains of stimuli (rectangular pulses, 0.01–0.1 ms, supramaximal voltage) were delivered through Ag/AgCl electrodes positioned around the proximal end of the vas deferens. The stimulus pattern was controlled by a digital stimulator (Applegarth Instruments, Oxford, UK) coupled to an optically isolated stimulus unit.

Changes in membrane potential were recorded using microelectrodes connected to the input headstage of an Axoclamp 2B with a Ag/AgCl wire. Signals were digitised and recorded using the same MacLab system used in the contraction experiments. Microelectrodes were fabricated from capillary glass tubing containing an inner glass microfilament (outer diameter 1.5 mm; Clark Electromedical, Reading, UK) using a Flaming-Brown P87 electrode puller (Sutter Instruments, Novato, CA, USA) and filled with 5 M potassium acetate (tip resistances were 60 to 120 MΩ).

Calcium imaging experiments

Postganglionic sympathetic nerve terminals were loaded with a calcium indicator dye as previously described (Brain and Bennett, 1997). The cut prostatic end of the vas deferens was placed in a saturated solution of Oregon Green™ 488 1,2-bis(2-aminophenoxylethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA)-1, dextran linked with a relative molecular weight of 10 kDa (Molecular Probes Inc., OR, USA), in 2.5% Triton X-100 phosphate-free Krebs solution. This indicator was chosen for its high affinity and strong fluorescent signal using the 488 nm wavelength line of an argon laser. The vasa deferentia were left in the dark for 5 h in the presence of the indicator, followed by a 3–6-h wash, to allow time for the indicator to load.

The prostatic end of the vas deferens was secured between a pair of Ag/AgCl electrodes and stimulated with an isolated stimulator (Digitimer DS2) using a pulse width of 0.06–0.2 ms. The applied voltage was adjusted to give a reliable response following a single stimulus, as determined by a change in fluorescent signal in a string of varicosities. The stimuli were electronically synchronised with confocal microscope scanning. Fluorescent images were collected with an inverted Leica TCS-NT laser scanning confocal microscope with an argon laser (excitation wavelength 488 nm) and a 515 nm long pass emission filter. All sampled fields of view included at least two varicosities from the same string, sampled with a temporal resolution of 0.2 s. Drugs were introduced into the bath by changing the reservoir from the one in which the perfusing solution was drawn to one containing the required final concentration of the drug, yielding a solution change time of 1 min.

Data analysis

Images were analysed with Scion Image (available from http://www.scioncorp.com/), using custom-written macros. Background signal was corrected for by subtraction. In some experiments, spikes in the intra-varicosity calcium concentration ($[Ca^{2+}]_v$) were observed. These were counted with an automated algorithm, which defined a spike as the following:

- the fluorescent signal from a varicosity increased by three standard deviations (measured during a control period) above the average of that from the three previous frames (i.e. the previous 0.6 s), and,
- was not immediately preceded by a spike (i.e. within 0.2 s), and,
- the fluorescent signal was greater than that on the immediately preceding frame.

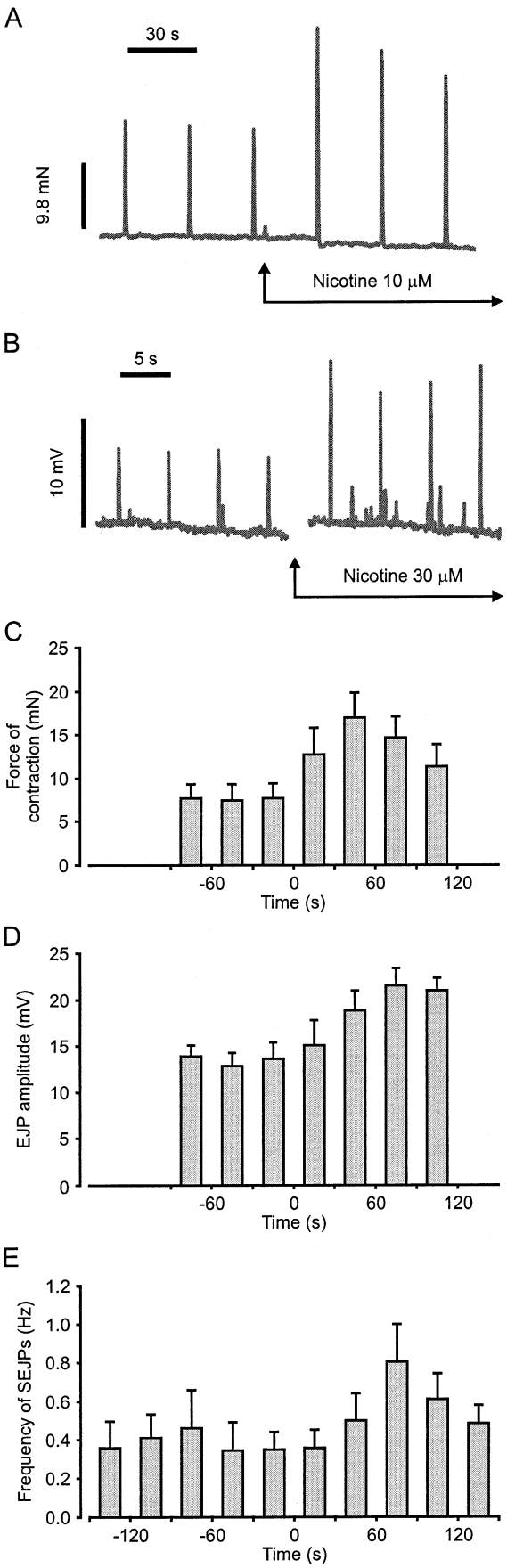
This definition of a 'spike' prevents double counting of large amplitude events, but limits the spike frequency to a maximum of 2.5 Hz (which is well above the maximum frequencies recorded in the present work). Using this definition, even Gaussian noise will have a non-zero spike frequency. Hence, the frequencies reported are net spike frequencies (unless otherwise noted), obtained by subtracting the spike frequency under control conditions from the spike frequency under a given experimental condition. When detecting calcium spikes, images were captured for 26 s every 2 min. This protocol prevents excessive photobleaching and phototoxicity.

In some experiments, values of calcium concentration were estimated using a calibration previously described for this preparation (Brain and Bennett, 1997). Given the approximations that must be made in calibrating the response with a non-ratioable indicator, these calcium concentration values are only indicative.

In the relevant experiments, a stock solution of ryanodine dissolved in dimethyl sulphoxide (DMSO) was used such that the final DMSO concentration was 0.2%. A stock 10 μM tetrodotoxin (TTX) solution was prepared and aliquoted before storing at −20°C; this ensured that the drug only passed through one freeze–thaw cycle. Stock nifedipine (10 mM in ethanol) and prazosin (1 mM in Krebs) solutions were diluted on the day of the experiment. Caffeine solutions were prepared on the day of each experiment. Ryanodine and caffeine were obtained from Research Biochemicals International (Natick, USA); nifedipine and prazosin were obtained from Sigma-Aldrich (Poole, Dorset, UK). TTX was obtained from Alomone Labs (Jerusalem, Israel). Data are expressed as the mean ± standard error. Statistical significance was evaluated using two-tailed, paired Student's *t*-tests; significant results were those with $P < 0.05$.

RESULTS

The effect of nicotine on neurogenic contractions evoked by a train of 10 stimuli at 10 Hz was investigated in mouse vas deferens from which the sympathetic ganglia had been removed. Nicotine (10 μM) increased the force of the neurogenic contraction by 135 ± 25% ($n = 6$; $P < 0.01$; Fig. 1A,C). The potentiating effect peaked about 30 s after the application of nicotine, after which



the force of contraction returned towards its resting level. The time taken to return to control levels was not measured as nicotine was removed from the bathing fluid after about 2 min contact to minimise desensitisation; consistent repeat responses to nicotine were obtained when successive nicotine applications were given at 1 h apart.

In order to confirm that the actions of nicotine were prejunctional, the effects of nicotine on EJPs were investigated. In the presence of nicotine (30 μM) the amplitude of evoked EJPs increased by $74 \pm 6\%$ ($n = 6$; $P < 0.05$; Fig. 1B,D). The amplitude of the EJP reached a peak about 1 min after exposure to nicotine and then declined towards resting levels. In four out of six experiments an increase in the frequency of spontaneous EJPs (SEJPs; for example, see Fig. 1B) was observed, while in the remaining two preparations there was no significant change. In the subset of preparations which did show an increase in spontaneous activity, the average frequency of SEJPs increased from 0.38 ± 0.03 Hz (in the 150 s before adding nicotine) to 0.60 ± 0.07 Hz (in the 150 s immediately following addition of nicotine; $P < 0.05$). The frequency of SEJPs then declined towards control levels (Fig. 1E).

To investigate the actions of nicotine on calcium dynamics, postganglionic sympathetic nerve terminals were loaded by orthograde transport with the calcium indicator Oregon Green™ 488 BAPTA-1 dextran. In the present experiments, changes in $[Ca^{2+}]_v$ ($\Delta[Ca^{2+}]_v$) from the resting level were measured. A single action potential evoked an increase in the fluorescent intensity of the indicator in sympathetic varicosities which formed part of the complex anatomy of a single, multiply branching, sympathetic neurone (Fig. 2A). The action potential-evoked increase in fluorescent intensity occurred almost simultaneously in all varicosities studied and reflects an increase in $[Ca^{2+}]_v$. The increase in $[Ca^{2+}]_v$ evoked by field stimulation was abolished by TTX (300 nM; $0.4 \pm 0.3\%$ of the control amplitude; $P = 0.21$), from 32 varicosities ($n_v = 32$), on 10 strings ($n_s = 10$) from 5 preparations ($n_p = 5$). Hence, these evoked changes in neuronal calcium concentration were triggered by a sodium-dependent nerve action potential.

In the absence of stimulation, the basal calcium concentration in the terminals has been estimated (Brain and Bennett, 1997) to be about 70 nM. Spontaneous calcium

Fig. 1. The effect of nicotine on transmitter release in mouse vas deferens. (A) An example trace illustrating the effect of nicotine (10 μM) on electrically evoked contractions (trains of five stimuli, pulse width 0.3 ms, 15 V, at 10 Hz repeated every 30 s) recorded isometrically under an initial tension of 9.8 mN. (B) An example trace of electrically evoked (1 stimulus, width 0.1 ms, 15 V, every 5 s) EJPs in the absence and presence of nicotine (30 μM). The signals in the presence of nicotine were recorded about 60 s after adding nicotine to the bath. (C) A histogram showing the average effect of nicotine (10 μM) on electrically evoked contractions in six tissues. Nicotine was added at 0 s. (D) Average amplitude of EJPs evoked by electrical stimulation, as in (B), in six tissues. EJPs were evoked once every 5 s and the signals were accumulated in 30-s periods for the histogram. Nicotine was added at 0 s. (E) A histogram of the frequency of occurrence of SEJPs observed over 30-s periods in six tissues.

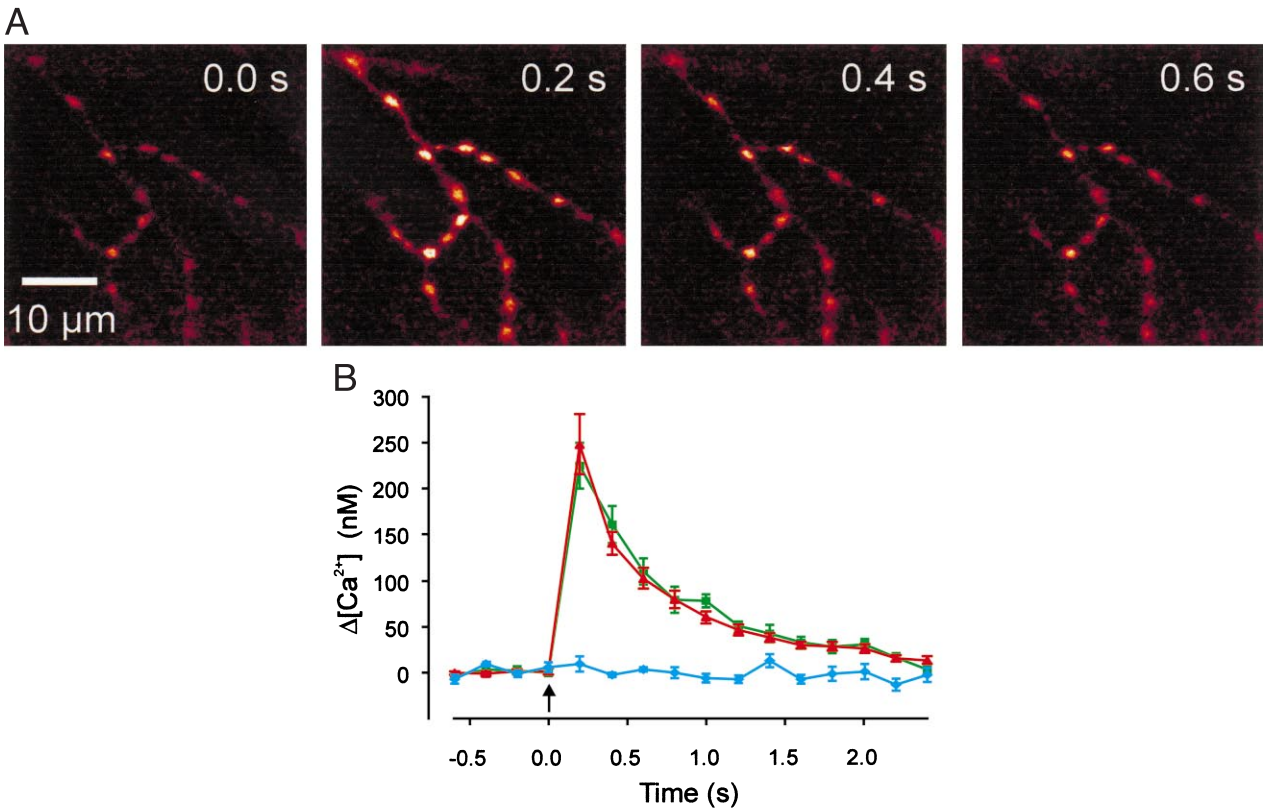


Fig. 2. The change in intracellular calcium concentration to a single action potential within a sympathetic nerve terminal. (A) Four consecutively recorded images of the same terminal before and after an action potential. This terminal shows a complex branching pattern. The swellings along the terminal are sympathetic varicosities. The times marked are the time after the stimulus. Within the terminal, the fluorescent signal increases as the calcium concentration increases. The width of each frame is 40 μm. (B) Changes in the $[Ca^{2+}]_v$ in a single varicosity in the absence of electrical stimulation (blue diamonds), in response to a single action potential (green squares) and in response to single action potential in the presence of nicotine (30 μM; red triangles). Each trace is the average of four recordings taken 1 min apart. The stimulus occurred just before $t = 0.2$ s (marked with an arrow). Nicotine does not significantly modify the $\Delta[Ca^{2+}]_v$ in response to a single action potential.

spikes, similar to those previously reported (Brain and Bennett, 1997), were observed only rarely under control conditions. Generally, strings of varicosities displayed a stable basal $[Ca^{2+}]_v$ within the noise level of the recording system. When nicotine (30 μM) was applied to aganglionic preparations, there was a small increase in the resting calcium concentration of 17 ± 3 nM ($n_v = 48$, $n_s = 15$, $n_p = 8$; $P < 0.01$). However, we could detect no effect of nicotine on the amplitude of the calcium transient evoked following single action potentials ($-3 \pm 3\%$; $n_v = 13$, $n_s = 5$, $n_p = 3$; Fig. 2B; $P = 0.36$).

Exposure to nicotine (30 μM) also induced discrete, randomly distributed (both spatially and temporally) fluctuations in $[Ca^{2+}]_v$, as shown in Fig. 3A–C. These shall be referred to as 'calcium spikes'. Characteristically, calcium spikes had a fast rising phase, with a falling phase similar to that observed following action potential-evoked $\Delta[Ca^{2+}]_v$ transients (Fig. 3D). In most cases even adjacent varicosities responded independently (but see Fig. 4C). Using the spike counting algorithm described in the Experimental procedures, the net spike frequency per varicosity was 0.09 ± 0.02 Hz ($n_v = 27$, $n_s = 8$, $n_p = 5$; $P < 0.01$) averaged over 2–8 min of nicotine exposure. After approximately 8 min, the net spike frequency declined towards zero (for example, see Fig. 3E). The non-zero spike count in the control period is attributed entirely to noise, as a stochastic model in which the standard deviation of the signal was identical with that observed experimentally produced a similar frequency and shape of 'control' spikes (data not shown). The calcium spikes could also be elicited by 2 μM nicotine, although at a reduced net spike frequency of 0.020 ± 0.004 Hz ($n_v = 24$, $n_s = 7$, $n_p = 3$; $P < 0.01$) averaged between 2 and 16 min of nicotine exposure. Clearly, it is necessary to establish whether calcium spikes resulted directly from the activation of nAChRs, or whether they were due to non-specific actions of nicotine on nerve terminals. These questions were addressed in two ways. First, we demonstrated that, like nicotine, the specific nAChR agonist epibatidine (100 nM) could also potently elicit calcium spikes. These were similar to calcium spikes elicited by nicotine and had a net rate of occurrence of 0.10 ± 0.014 Hz ($n_v = 19$, $n_s = 6$, $n_p = 3$; $P < 0.01$) when averaged during the period from 2 to 8 min of exposure to epibatidine. Second, we demonstrated that the effects of nicotine were abolished by the nAChR channel blocker hexamethonium. Pretreatment with hexamethonium (100 μM) for 20 min prevented nicotine (30 μM) from inducing calcium spikes (-0.003 ± 0.005 Hz net frequency compared to control,

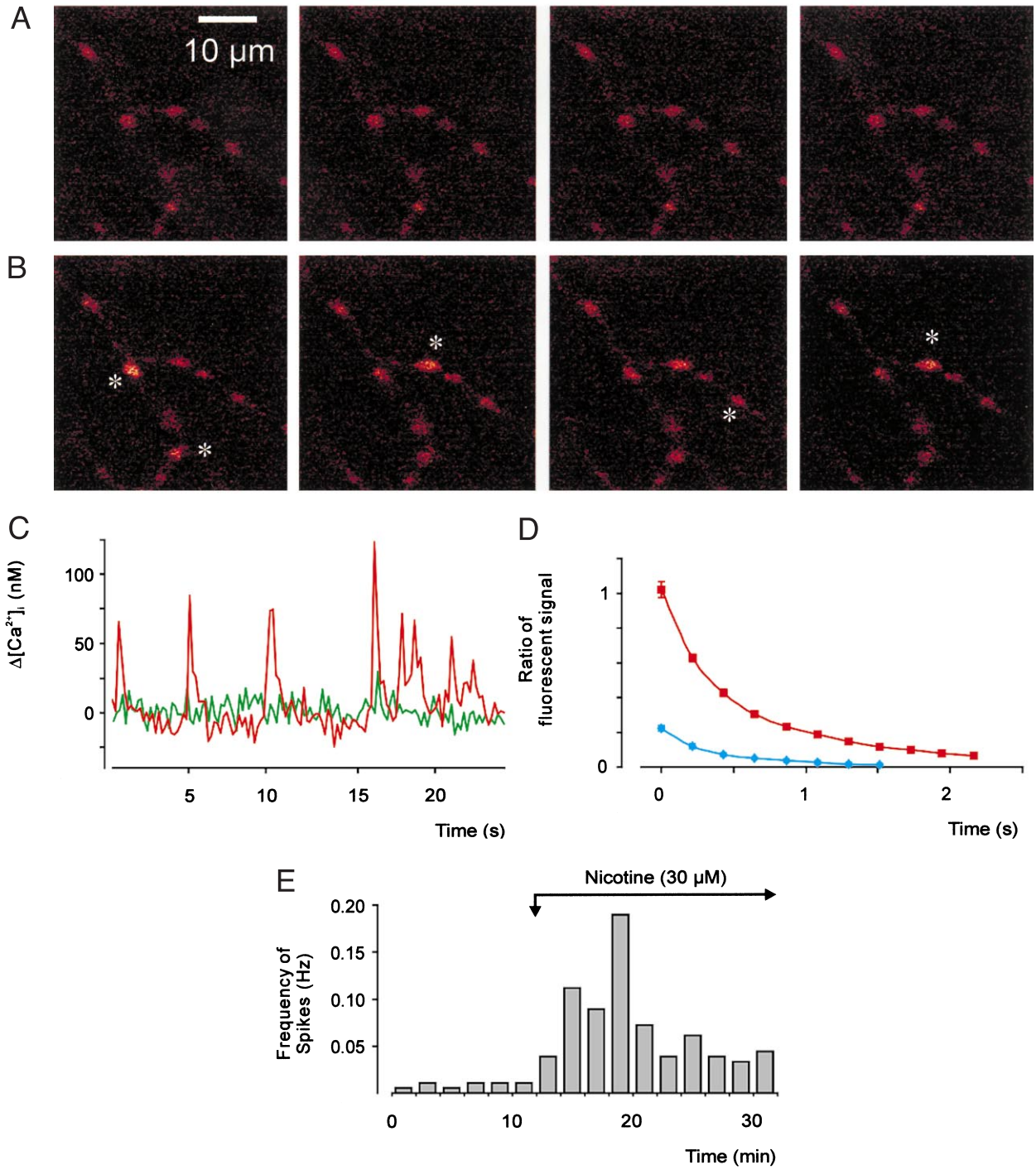


Fig. 3. Effects of nicotine on basal calcium. (A) The top panel shows four successive scans, each recorded 200 ms apart, in the absence of any stimulus. The $\Delta[Ca^{2+}]_v$ within the varicosities is relatively constant. The width of each frame is 40 μm. (B) The lower panel shows four successive scans, each recorded 200 ms apart, in the same terminal, but now in the presence of nicotine (30 μM). Calcium spikes can be observed (marked with an asterisk). The width of each frame is 40 μm. (C) Traces of 25 s of continual sampling of resting $\Delta[Ca^{2+}]_v$ in the absence (green) and presence of nicotine (30 μM; red). (D) A comparison of the kinetics of nicotine-induced calcium transients (blue diamonds) with that of the action potential-evoked calcium transient (red squares). The relative change in the fluorescent signal during the transient, compared with that over the preceding second, is shown. All calcium spikes which were not followed by a second calcium spike within 2 s were averaged (average of 105 spikes from $n_v = 20$, $n_p = 4$). The action potential-evoked transient is an average over the subset of these varicosities in which the response to single action potentials was recorded (from $n_v = 17$, $n_p = 3$). (E) A histogram showing the calcium spike frequency over the course of an experiment in seven varicosities from one terminal. The arrow indicates application of nicotine and the bar represents the duration of applied nicotine.

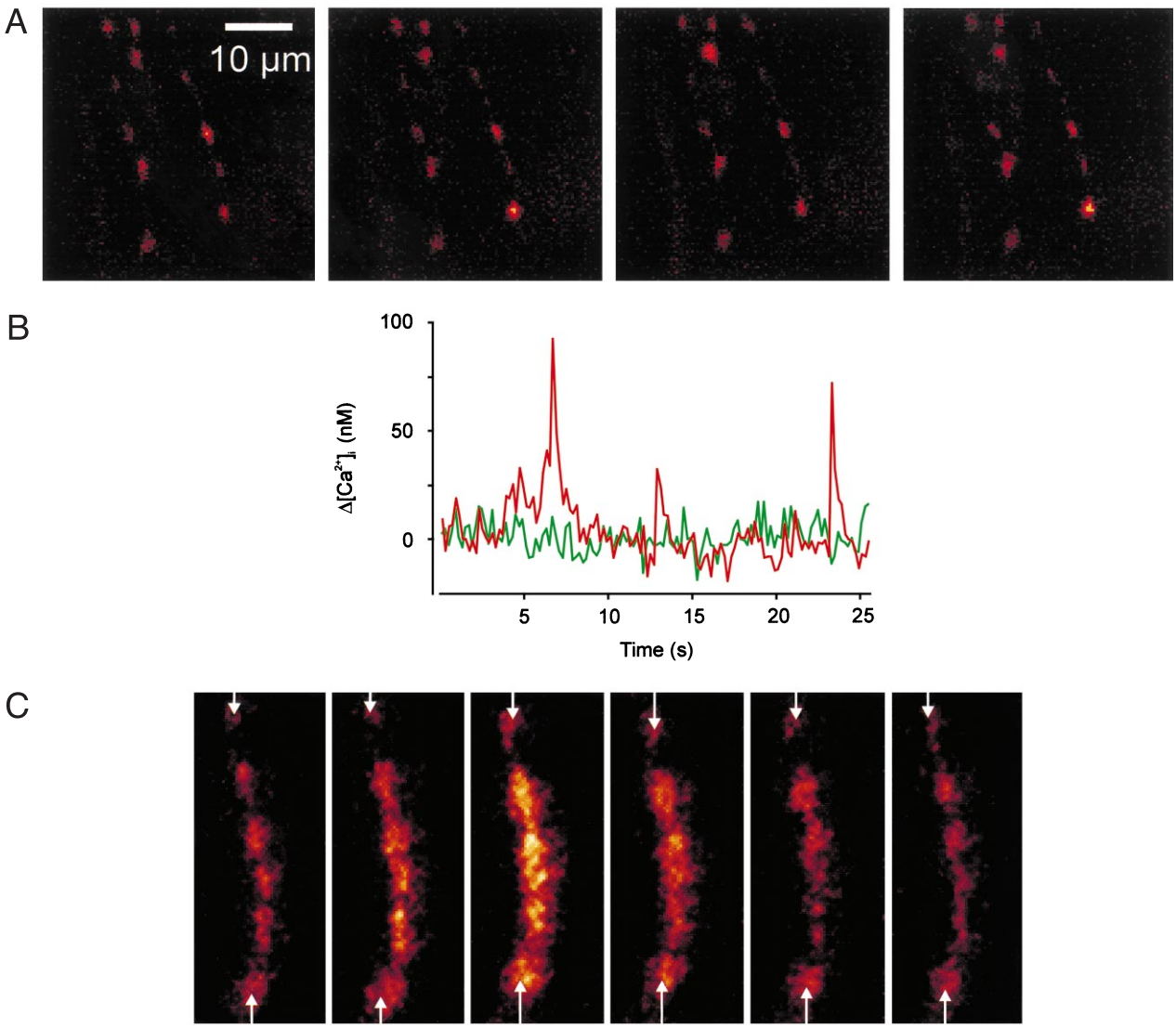


Fig. 4. Nicotine induced calcium transients are not blocked by TTX. (A) Sets of consecutive scans in the presence of TTX after the application of nicotine (30 µM). The width of each frame is 35 µm. (B) 25-s traces of resting $\Delta[Ca^{2+}]_v$ sampled continuously in the absence (green) and presence (red) of nicotine, both in the presence of TTX (300 nM). Under these experimental conditions the calcium transients evoked by the nerve action potential were abolished. (C) Sequential nicotine-induced (2 µM) rise in calcium in several adjacent varicosities. A local rise in $[Ca^{2+}]_v$ is followed by local contraction, suggesting that the elevation in calcium concentration is associated with local transmitter release. The arrows mark the centre of two of the varicosities. The height of each image is 8 µm.

$P = 0.6$; -0.004 ± 0.005 Hz compared to hexamethonium alone; $n_v = 15$, $n_s = 6$, $n_p = 3$; $P = 0.43$). In two experiments, hexamethonium and nicotine were washed out of the bath and the preparation was left to recover for 1 h. Re-exposure to nicotine, now in the absence of hexamethonium, led to a significant increase in the net spike count with a frequency of 0.03 ± 0.01 Hz ($n_v = 10$, $n_s = 4$; $P < 0.05$).

In the presence of the voltage-gated sodium channel blocker TTX (300 nM), nicotine (30 µM) induced a net spike frequency of 0.17 ± 0.03 Hz ($n_v = 15$, $n_s = 5$, $n_p = 2$; $P < 0.01$; Fig. 4A,B). The effectiveness of TTX blockade was established prior to the application of nicotine by confirming that action potential-evoked $\Delta[Ca^{2+}]_v$ transients were abolished, even when the stimulus intensity was increased over threshold by more than three-fold.

An important question addressed was whether the nicotine-induced calcium transients are associated with neurotransmitter release. As shown in Fig. 4C, the presence of nicotine caused an increase in fluorescence of five neighbouring varicosities and a simultaneous contraction in the presence of the α_1-adrenoceptor antagonist prazosin (0.1 µM). This contraction is the result of neurotransmitter release, that in turn elicits a local contraction of the smooth muscle cells innervated by these varicosities.

By increasing the sensitivity of the calcium-induced calcium release (CICR) mechanism to cytoplasmic calcium, caffeine can deplete the CICR stores of calcium (Sitsapesan and Williams, 1990). There was no significant increase in the net spike count after varicosities were exposed to caffeine (3 mM) for 20 min (-0.01 ± 0.01 Hz; $n_v = 16$, $n_s = 6$, $n_p = 3$; $P = 0.17$) compared to control

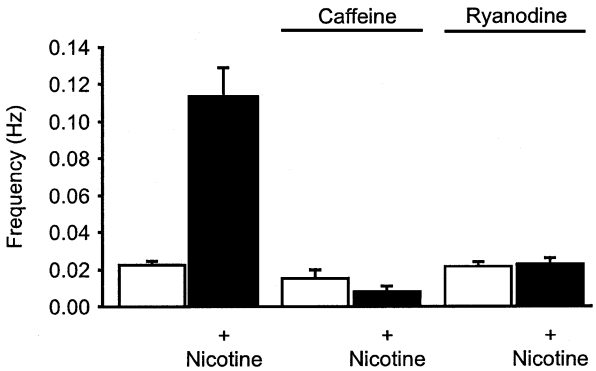


Fig. 5. A histogram showing the effects of pretreatment with caffeine or ryanodine on nicotine induced increases in calcium spike frequency. The first two columns show a histogram of the average spike count, in all such experiments, before and after exposure to 30 μM nicotine. The third and fourth columns show the effects on a different set of terminals of the application of caffeine (3 mM) alone, or caffeine and nicotine (30 μM). The last two columns show the effects of prolonged application of ryanodine (100 μM) before and after the application of nicotine (30 μM). Clearly, both caffeine and ryanodine abolished the ability of nicotine to increase the frequency of calcium spikes.

conditions (Fig. 5). It is noteworthy that no transient increase in the spike frequency was detected when caffeine was first applied. When nicotine was added after varicosities had been exposed to caffeine for 20 min, no calcium spikes were elicited (-0.01 ± 0.01 Hz; $n_v = 16$, $n_s = 6$, $n_p = 3$; $P = 0.15$; Fig. 5). In one experiment caffeine and nicotine were washed out of the bath, and the preparation was left to recover for 1 h. Re-exposure to nicotine, now in the absence of caffeine, led to a trend towards a significant net spike count with a frequency of 0.08 ± 0.04 Hz ($n_v = 5$, $n_s = 2$; $P = 0.08$). The role of intracellular calcium stores was further explored using the CICR blocker ryanodine, which either locks the CICR channel in a sub-conductance state leading to depletion of stores (Rousseau et al., 1987) or direct block of the channel (McPherson et al., 1991). After a control period of recording, each preparation was exposed to ryanodine for 90 min, during which time trains of 10 stimuli at 10 Hz were given every 30 s. This stimulus protocol was deemed necessary because the action of ryanodine is use-dependent. Importantly, we also established that ryanodine had no effect on action potential propagation in the secretory terminals at the highest concentration employed (100 μM). After this prolonged exposure to ryanodine, nicotine (30 μM) failed to induce calcium spikes (Fig. 5; net frequency 0.002 ± 0.003 Hz; $n_v = 18$, $n_s = 6$, $n_p = 3$; $P = 0.63$). In a separate control experiment, where the vehicle alone (DMSO) was added instead of the ryanodine solution, nicotine was still able to induce calcium spikes.

DISCUSSION

The contraction studies demonstrate that nicotine acts within this tissue to increase the force of neurogenic contraction, an action previously attributed mainly to increased noradrenaline release (McGrath, 1978). The increase in the amplitude of EJPs and the increase in the frequency of SEJPs suggest that nicotine also acts on prejunctional sympathetic terminals to increase the release of the co-transmitter ATP, which is the neurotransmitter generating EJPs in the mouse vas deferens (Stjärne and Åstrand, 1984). Similar conclusions were reached from transmitter release studies in guinea-pig vas deferens (von Kügelgen and Starke, 1991; Todorov et al., 1991).

An increase in resting calcium concentration in response to nicotine has been reported in the neuronal processes of cultured chick sympathetic neurones (Dolezal et al., 1995), cultured central neurones (McGehee et al., 1995), astrocytes (Sharma and Vijayaraghavan, 2001) and rat mossy fibre presynaptic terminals (Gray et al., 1996). The present work is the first report of the effect of nicotine on resting calcium concentrations within a mature peripheral nerve terminal. This increase alone may be sufficient to affect transmitter release according to contemporary versions of the residual calcium hypothesis (Zucker, 1989), and is consistent with the known paired-pulse facilitation of transmission that occurs in this tissue (Bennett and Florin, 1975).

The observation that hexamethonium blocks these nicotine-induced calcium spikes suggests that the calcium spikes require cation entry though the nAChR pore. The most likely candidates, based on their relatively high conductance through the channel (Rathouz et al., 1996; Burnashev, 1998), are sodium (which could cause a local depolarisation) and calcium. It was important to explore the possibility that local depolarisation and subsequent firing of action potentials could occur as it has been previously reported that nicotine can elicit antidromic nerve action potentials in cardiac sympathetic nerve terminals (Bevan and Haeusler, 1975). The inability of TTX to block the calcium spikes in these experiments suggests that local active depolarisation is not responsible in sympathetic nerve terminals. TTX-insensitive nicotine-induced transmitter release has been reported in several preparations (Lou et al., 1992). It is possible, but unlikely, that Na^+ influx through the nAChR pore could passively depolarise the varicosity and open voltage-gated calcium channels (VGCCs). This would imply that the membrane potential was significantly different among adjacent varicosities, as most calcium spikes were confined to one varicosity.

It is noteworthy that even low concentrations of nicotine (2 μM) could induce almost synchronous calcium spikes in varicosities on the same terminal branch, which sometimes produced a local contraction. This shows that the 'calcium spikes' sometimes precede neurotransmitter release (see Fig. 4C). This concentration of nicotine is close to that reported within the serum of smokers (< 0.5 μM; Armitage et al., 1975; Russell et al., 1980). It should also be noted that in the steady state, the concentrations of nicotine in the lungs, brain and heart are significantly higher than that in the serum, at least in the rabbit (Benowitz and Jacob, 1987). These findings suggest that calcium spikes may well play a role in mediating the acute effects of nicotine during smoking.

We have previously reported that CICR plays a role in

action potential-evoked neurotransmitter release in rodent vas deferens (Smith and Cunnane, 1996). The potential interaction of nicotine with intracellular calcium stores has been investigated in adrenal chromaffin cells (Foucart et al., 1995) and chick ciliary neurones (Rathouz et al., 1995), but no association has been found. It has been shown that cholinergic transmission in intact ciliary ganglia induces CICR in the spines of ciliary neurones (Shoop et al., 2001) and that nicotine induces CICR in astrocytes (Sharma and Vijayaraghavan, 2001). It has been suggested that calcium influx though postsynaptic nAChRs located on guinea-pig outer hair cells, implied from changes in the activity of compound action potentials, can trigger a ryanodine-sensitive calcium release from intracellular stores over tens of seconds (Sridhar et al., 1997). In the present study we observed that basal calcium levels rose by about 20% with a similar time course to that 'indirectly measured' in outer hair cells. However, our results with caffeine and ryanodine provide the first direct evidence of an interaction between CICR stores and nicotine (a) in the form of rapid 'calcium spikes' and (b) at the level of a single varicosity in secretory nerve terminals.

It is interesting to note that the calcium imaging technique used in these experiments allows one to indirectly follow the propagation of a single action potential through a branching nerve terminal, confirming that intermittence of neurotransmitter release, at the level of the single varicosity, is not due to failure of the action potential to invade the secretory terminals (Brock and Cunnane, 1988, 1992).

It will be of great interest to correlate measurements of SEJPs and calcium spikes by simultaneously confocal microscopy and intracellular recording. At present, this work is hampered by the technical difficulties of simultaneous high-resolution imaging and intracellular recording in a relatively thick tissue.

In summary, these results suggest that nicotine binds to the neuronal nAChRs on sympathetic nerve terminals and triggers the influx of calcium through the ligand-gated channel and/or VGCCs. The release of calcium from intra-varicosity calcium stores is then triggered, either directly or indirectly. Should these results hold true for other secretory terminals, it is probable that CICR from intra-terminal stores plays an important role in the actions of nicotine at presynaptic nerve terminals.

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