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CHOLINERGIC NEUROMUSCULAR TRANSMISSION IN THE LONGITUDINAL MUSCLE OF THE GUINEA-PIG ILEUM

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SUMMARY

1. Brief transmural stimuli, 0·5–1 ms, initiated contractions of the longitudinal muscle taken from the guinea-pig ileum that were recorded isometrically. In separate preparations similar stimuli were found to initiate excitatory junction potentials which were recorded using intracellular recording electrodes. All of these responses were abolished by either tetrodotoxin, ω-conotoxin or hyoscine.

2. The contractions produced by increasing $[K^+]_o$ were blocked by nifedipine, 1×10^{-7} M; nicardipine, 1×10^{-7} M; verapamil, 1×10^{-5} M or diltiazem, 1×10^{-5} M. In these solutions brief stimuli continued to initiate contractions: this indicates that neuronally released acetylcholine continues to trigger a contraction when muscle voltage-dependent calcium channels appear to have been blocked.

3. When membrane potential recordings were made from the smooth muscle layer, brief transmural stimuli initiated excitatory junction potentials that triggered muscle action potentials. Although muscle action potentials were abolished by low concentrations of a range of organic calcium antagonists, excitatory junction potentials persisted and continued to initiate contractions of reduced amplitude.

4. When the internal concentration of calcium ions, $[Ca^{2+}]_i$, was measured using fura-2, brief transmural stimuli caused an increase in $[Ca^{2+}]_i$. Part of this response, which occurred at a time corresponding to the unblocked excitatory junction potential, persisted in the presence of the organic calcium antagonist nifedipine.

5. Two explanations appear possible. Neuronally released acetylcholine may simultaneously activate non-selective cation channels and cause the release of Ca^{2+} from an internal store. Alternatively, neuronally released acetylcholine may cause an increase in $[Ca^{2+}]_i$ which is separate from that which accompanies the activation of voltage-dependent calcium channels. At this stage there is little other anatomical or electrophysiological evidence to support this view.

INTRODUCTION

The longitudinal smooth muscle layer of the mammalian intestine is innervated by projections from the enteric nervous system. In most species stimulating the projections elicits an excitatory response, which is abolished by muscarinic receptor

antagonists (Zar & Goopta, 1983; Ito, Kuriyama & Parker, 1988; Bauer, Holzer & Ito, 1991). Most of the excitatory neurones innervating the longitudinal muscle layer are thought to release acetylcholine, ACh, which interacts with a muscarinic receptor to trigger contraction (Kaplita & Triggle, 1983). In many intestinal preparations cholinergic nerve stimulation initiates an excitatory junction potential (EJP) (Bennett, 1972; Bauer & Kuriyama, 1982; Bauer *et al.* 1991). Many of these responses are mimicked by applied ACh. In the longitudinal muscle layer of guinea-pig ileum, applied ACh causes a membrane depolarization and an increase in the rate of discharge of muscle action potentials, so triggering contraction (Bolton, 1979). This depolarization was shown to result from an increase in cation permeability. As with the responses to nerve stimulation, there is an appreciable delay before the onset of membrane depolarization following the ionophoretic application of ACh (Bolton, 1979). Therefore it is unlikely that the channels activated are directly gated by ACh.

More recently the effects of ACh on single smooth muscle cells have been examined (Benham, Bolton & Lang, 1985; Inoue, Kitamura & Kuriyama, 1987; Inoue & Isenberg, 1990*a*, *b*; Vogalis & Sanders, 1990). These reports have confirmed that applied ACh causes the opening of a set of non-selective cation channels which allow sodium ions, Na^+, calcium ions, Ca^{2+} and potassium ions, K^+, to diffuse down their electrochemical gradients (Inoue & Isenberg, 1990*a*). The gating of these channels is influenced by both the membrane potential and the internal concentration of Ca^{2+} (Benham *et al.* 1985; Lim & Bolton, 1988; Inoue & Isenberg, 1990*b*). In addition these studies on isolated single cells support the view that ACh activates a complex pathway which leads to channel opening (Inoue & Isenberg, 1990*a*, *b*; Komori & Bolton, 1990; Pacaud & Bolton, 1991). In addition, high concentrations of ACh cause the release of Ca^{2+} from internal stores (Himpens & Somlyo, 1988). Muscarinic receptor activation causes the liberation from the plasma membrane of the second messenger D-*myo*-inositol 1,4,5-trisphosphate, IP_3, which in turn leads to the release of stored Ca^{2+} (Bolton & Lim, 1989; Komori & Bolton, 1990, 1991).

Intestinal smooth muscle cells generate action potentials when depolarized. These action potentials result from the influx of Ca^{2+} (Bülbring & Kuriyama, 1963). Recordings from single isolated cells have described the properties of the voltage-dependent Ca^{2+} channels which are present in these cells (Lang & Paul, 1991). The channels are opened by membrane depolarization (Benham *et al.* 1985; Lang & Paul, 1991; Inoue & Isenberg, 1990*a*), are selective for Ca^{2+}, and are blocked by organic Ca^{2+} antagonists (Lang & Paul, 1991).

Taken together the observations suggest that ACh causes intestinal muscle to contract by first opening a set of non-selective cation channels. The resulting membrane depolarization activates voltage-dependent Ca^{2+} channels. The internal concentration of Ca^{2+}, $[Ca^{2+}]_i$, rises and after a delay a contraction is triggered (Himpens & Somlyo, 1988; Ito *et al.* 1988). Further, the rise in $[Ca^{2+}]_i$ might be augmented by ACh-induced release of Ca^{2+} from an internal store (Himpens & Somlyo, 1988).

The experiments reported here were undertaken to determine how neuronally released ACh causes contractions of the longitudinal muscle layer of the guinea-pig ileum. This preparation was chosen because in most regions of the small intestine of

this species, the responses to transmural stimulation are entirely abolished by atropine, unless trains of repetitive stimuli are applied (Niel, Bywater & Taylor, 1983). This indicates that a simple cholinergic projection can be studied. A preliminary account of this work has appeared (Cousins, Edwards, Hirst & Wendt, 1992).

METHODS

Guinea-pigs, of either sex, weight 180–500 g, were killed by stunning and exsanguination. A length of ileum was taken, unless stated otherwise, at a distance greater than 20 cm from the ileocaecal junction and transferred to physiological salt solution of composition (mM): NaCl, 120; KCl, 5·0; $NaHCO_3$, 25; NaH_2PO_4, 0·1; $CaCl_2$, 2·5; $MgCl_2$, 2·0; glucose, 11·0) bubbled with 95% O_2–5% CO_2. To isolate the longitudinal muscle layer, a 3 cm length of intestine was slit along the mesenteric border and pinned out mucosal surface downwards in a dissecting chamber containing physiological saline. Two fine strands of longitudinal muscle were teased away at the mesenteric and anti-mesenteric borders. One end of the flat sheet of longitudinal muscle was dissected free and gripped with a pair of wide-jawed forceps. The entire flat sheet was pulled gently away from the underlying layer of circular muscle. This dissection gave flat sheets of longitudinal muscle, complete with adhering myenteric plexus, measuring about 2 cm in length and about 0·8 cm in width. In the electrophysiological and tension studies the preparations were trimmed down to have lengths of 1·5 cm and widths of 0·6 cm; in the experiments involving a determination of $[Ca^{2+}]_i$, the preparations used were 1·0 cm long and 0·6 cm wide.

In electrophysiological experiments, preparations were placed in a shallow recording chamber, the base of which consisted of a microscope coverslip coated with Sylgard (Dow Corning Corporation, Midland, MI, USA) silicone resin. The preparations were pinned out by placing fine pins, cut from 50 μm tungsten wire, around the border of the isolated sheet of muscle. Transmural stimuli were delivered using two platinum electrodes, one positioned in a well in the silicone resin and the second positioned above the muscle layer. Intracellular recordings were made using conventional techniques with fine glass microelectrodes (resistance 150–240 MΩ) filled with 0·5 M KCl. All membrane potential records were low-pass filtered, cut-off frequency 1 kHz, digitized and stored on disk for later analysis. The preparations were continuously perfused with oxygenated physiological saline, at a rate of 6 ml min^{-1} (bath volume 1·5 ml) and maintained at 25 °C. In many experiments drugs were added to the preparation by changing the inflow line from the control solution to one containing the appropriate concentration of drug. Where the cable properties of the longitudinal muscle layer were determined, the muscle sheet was impaled with two independent electrodes, one to pass current and one to record membrane potential changes. Cable properties were determined using the method described by Bywater, Campbell, Edwards & Hirst (1990).

In experiments where muscle contractions were recorded, preparations of longitudinal muscle and adhering myenteric plexus were placed in a tissue bath with one end tied to a rigid tissue holder and the other attached to an isometric force transducer. Preparations were set up with a resting tension of 100 mN and allowed to equilibrate for 30 min before the start of each experiment. Enteric nerves were stimulated by drawing the preparations through a pair of platinum ring electrodes, positioned at the centre of the preparation. Brief transmural stimuli, pulse width 0·5–1 ms, applied voltages 20–90 V, initiated contractions. The strength and time course of contractions produced either by transmural stimulation or by the addition of drugs were measured. The responses were digitized and stored on disk for analysis.

In a third series of experiments, contractile responses and the changes in $[Ca^{2+}]_i$ were simultaneously measured. In these experiments preparations of longitudinal muscle, along with the attached myenteric plexus, were mounted by tying one end to a rigid holder and the other to a force transducer (Harvard). The preparations were positioned in an organ bath on the stage of an inverted fluorescence microscope. The muscle strips were loaded with the fluorescent Ca^{2+} indicator fura-2 by incubation in a solution containing the acetoxymethylester of fura-2 (fura-2 AM; 5 μM) and 0·01% Pluronic F-127 to aid dispersal of the fura 2 AM. The muscle was incubated in the fura-2 AM solution for 3 h at room temperature following which it was washed thoroughly in fresh Ca^{2+}-free physiological saline solution to remove extracellular fura-2 AM. Tissue fluorescence, at 510 nm, was continuously monitored with a photomultiplier tube during alternate (50 Hz) excitation at 340

and 380 nm, achieved by passing the excitation light through a rotating filter wheel. Following subtraction of background fluorescence (determined for each preparation prior to loading) the ratio of the fluorescence at 340 nm excitation to that at 380 nm ($R_{340/380}$) was taken as an indicator of $[Ca^{2+}]_i$. An internal calibration was performed on each muscle at the completion of the experiment to allow for estimation of $[Ca^{2+}]_i$ using the formula described by Grynkiewicz, Poenie & Tsien (1985). The maximum fluorescence ratio (R_{max}) was obtained by exposing the muscle to a 10 μM concentration of the Ca^{2+} ionophore, ionomycin, in physiological saline solution containing 1·5 mM Ca^{2+}. Immediately after determining R_{max} the solution was replaced with Ca^{2+}-free solution containing 2 mM EGTA and the minimum fluorescence ratio (R_{min}) was determined. The dissociation constant for Ca^{2+}-fura-2 was taken to be 224 nM (Grynkiewicz *et al.* 1985). Brief transmural stimuli were applied by a pair of platinum electrodes placed at the centre of the preparation. Force transients, emission during excitation at 340 and 380 nm and the 340/380 nm ratio were measured and stored on disk.

Drugs used in this study were, tetrodotoxin, atropine, hyoscine, nicardipine, verapamil, diltiazem and nifedipine (all obtained from Sigma Chemical Co., USA), ω-conotoxin (obtained from Peninsula Chemicals, Belmont, CA, USA) and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP), pirenzipine dihydrochloride, methoctramine hydrochloride (Research Biochemicals, Natick, MA, USA). Fura-2 AM, ionomycin, ethylene glycol-*bis* (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid and Pluronic F-127 (obtained from Molecular Probes, Eugene, OR, USA). All drugs were dissolved in distilled water except nifedipine and nicardipine, which were dissolved in absolute alcohol, and fura-2 AM and ionomycin, which were dissolved in dimethyl sulphoxide. In all experiments involving nifedipine, solutions were freshly made and protected from the light using aluminium foil wrapping. Applied drugs were allowed to equilibrate with the tissue for at least 10 min.

RESULTS

Contractile responses produced by brief transmural stimuli

Isolated preparations of longitudinal muscle were set up with a resting force of 100 mN. Most preparations, sixty-one from seventy-nine preparations ($n = 67$, where on this and on every other occasion the 'n value' refers to the number of animals from which the preparations were taken), contracted spontaneously; the remaining preparations were quiescent. We have no explanation why some preparations generated spontaneous activity and others did not. When a number of preparations were made from a given animal each showed a similar behaviour pattern, i.e. all preparations from one animal were either spontaneously active or quiescent. In all preparations single, brief supramaximal transmural stimuli, pulse width 0·5–1 ms of applied voltage 60–90 V, initiated contractions. In spontaneously active preparations, the force increased to a peak some 2–3 s after the stimulus and returned to baseline over 30 s with a number of small force oscillations (Fig. 2*Ba*). In spontaneously active preparations (23 preparations, $n = 19$) contractions initiated by a single stimulus had mean peak amplitudes of $6{\cdot}05 \pm 0{\cdot}64$ mN. The responses began after a delay, measured as the separation between the stimulus pulse and the time to 10% of peak amplitude, of $0{\cdot}94 \pm 0{\cdot}05$ s with the peak force increases occurring some 2–3 s after the initiation of the response (rise time, measured as the time from 10 to 90% of peak amplitude: $2{\cdot}43 \pm 0{\cdot}46$ s): contractions had a half-width, measured as the time between 50% peak amplitude on rising and falling phases, of $8{\cdot}29 \pm 1{\cdot}09$ s. In quiescent preparations (10 preparations, $n = 8$), the responses had smaller peak tensions ($1{\cdot}37 \pm 0{\cdot}22$ mN). Although they occurred after a similar delay ($0{\cdot}88 \pm 0{\cdot}06$ s), the responses had faster rise times ($1{\cdot}18 \pm 0{\cdot}07$ s) and decayed monotonically to baseline within 10–15 s (Fig. 1*Aa*, *Ba* and *Ca*). These responses were abolished by tetrodotoxin, 1×10^{-6} M ($n = 2$), by ω-conotoxin,

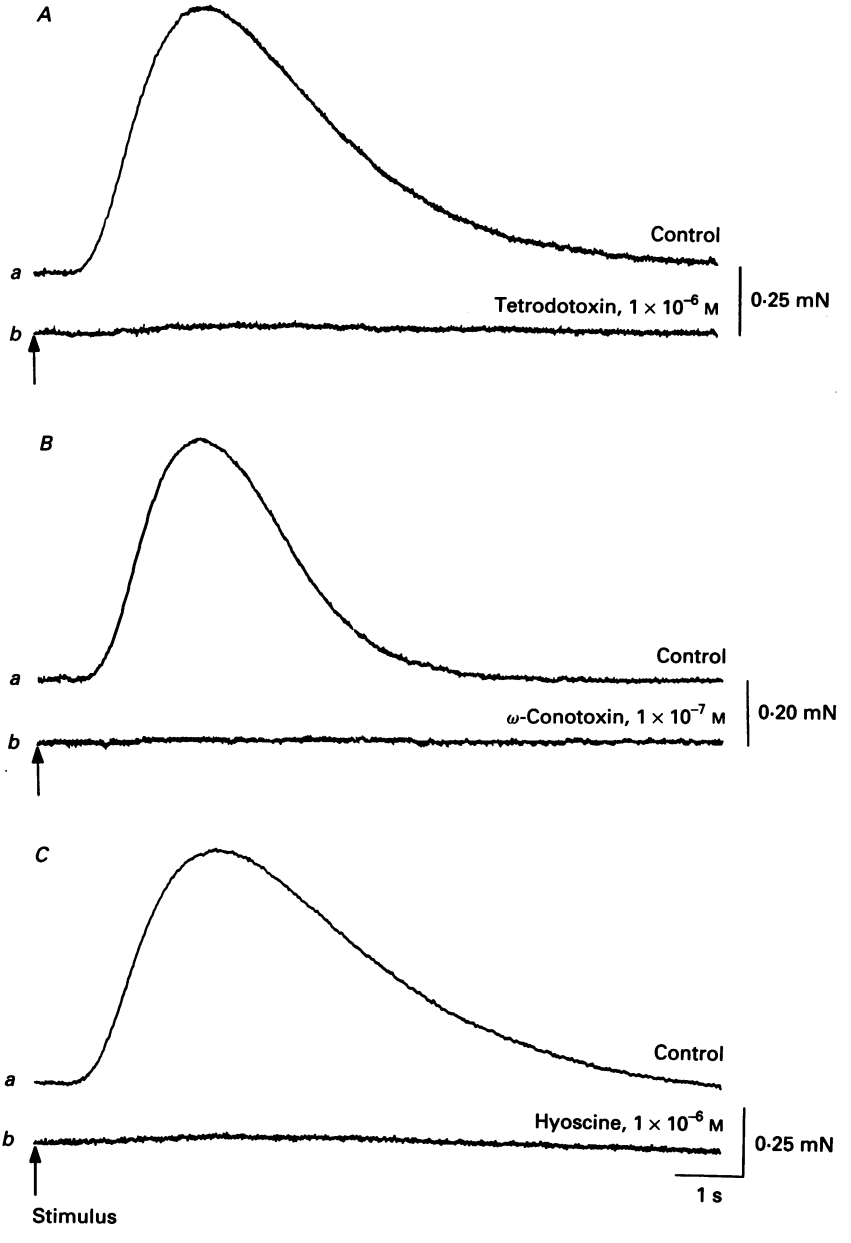

Fig. 1. Effect of tetrodotoxin, ω-conotoxin and hyoscine on contractile responses of the longitudinal muscle of guinea-pig ileum. Each trace shows the response to a single supramaximal brief, 0·5 ms, stimulus. *A* shows the responses recorded from tissue before (*Aa*) and after (*Ab*) the addition of tetrodotoxin, 1×10^{-6} M. *B* shows the responses recorded from tissue before (*Ba*) and after (*Bb*) the addition of ω-conotoxin, 1×10^{-7} M. *C* shows the responses recorded from tissue before (*Ca*) and after (*Cb*) the addition of hyoscine, 1×10^{-6} M. It can be seen that each of these three agents completely abolished the responses produced by single brief transmural stimuli, suggesting that the stimuli selectively excited enteric cholinergic neurones. These responses have been recorded from quiescent preparations.

1×10^{-7} M (3 preparations, $n = 2$), or by the muscarinic antagonists hyoscine, 1×10^{-6} M ($n = 2$), pirenzipine, 1×10^{-6} M ($n = 5$), methoctramine, 1×10^{-6} M ($n = 5$) and 4-DAMP, 1×10^{-6} M ($n = 5$). Figure 1 illustrates responses from quiescent preparations. These observations indicate that the responses resulted from the

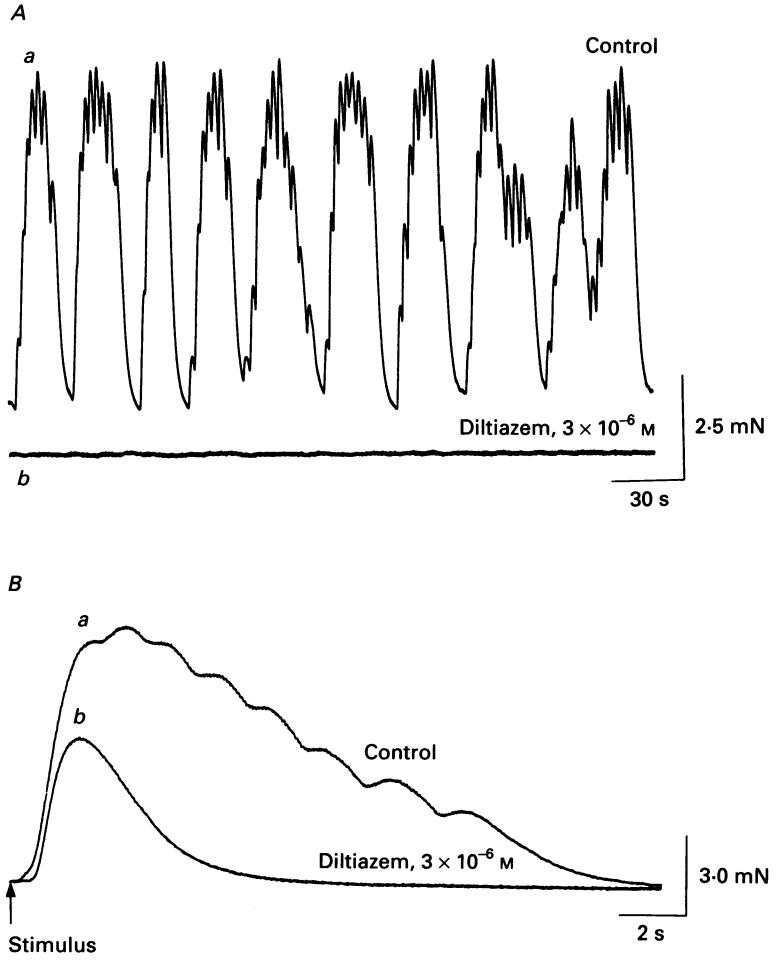

Fig. 2. Effect of diltiazem on myogenic (*A*) and evoked (*B*) responses of longitudinal smooth muscle. The trace *Aa* shows a series of spontaneous myogenic contractions recorded in control solution; these were rapidly abolished, *Ab*, by adding diltiazem, 3×10^{-6} M to the physiological saline. *B* shows contractile responses produced by a single brief transmural stimulus recorded from the same preparation in control solution during a pause in the on-going myogenic activity (*Ba*) and after adding diltiazem, 3×10^{-6} M to the physiological saline (*Bb*).

activation of the cholinergic nerves that innervate this muscle layer. Neither tetrodotoxin, ω-conotoxin, nor the muscarinic antagonists affected spontaneous myogenic activity in the spontaneously active preparations. These agents did not change the resting force in quiescent preparations.

Effect of organic calcium antagonists on spontaneous activity and responses to cholinergic nerve stimulation

Spontaneous myogenic activity was rapidly abolished by adding either nifedipine, 1×10^{-8} M ($n = 4$), 3×10^{-8} M ($n = 1$); nicardipine, 1×10^{-7} M ($n = 2$); diltiazem,

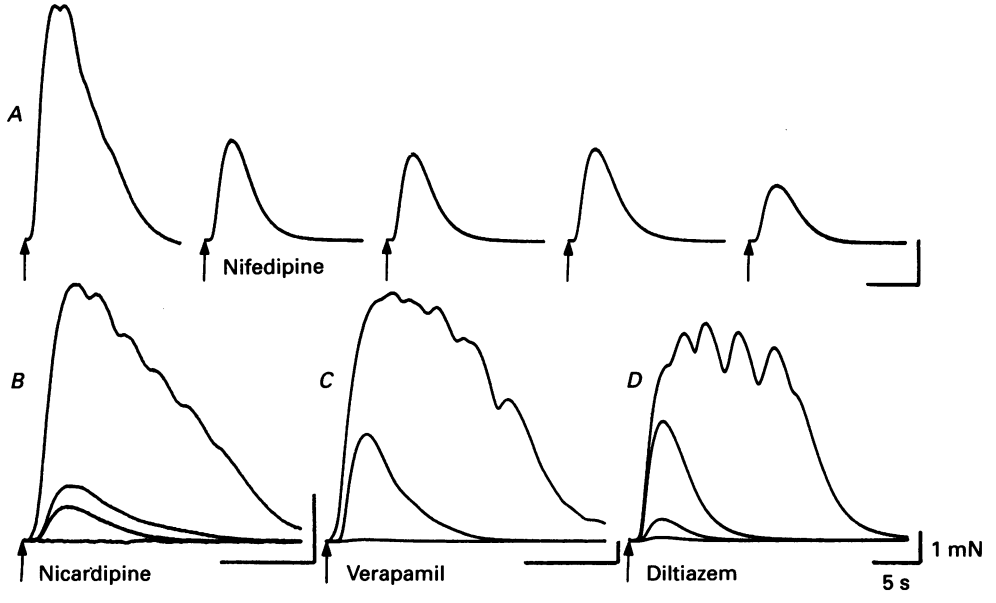

Fig. 3. Effect of the calcium antagonists, nifedipine (*A*), nicardipine (*B*), verapamil (*C*) and diltiazem (*D*) on the contractile responses of longitudinal smooth muscle produced by single brief transmural stimuli. The upper panel, *A*, shows the responses of a tissue in control solution and in progressively increasing concentrations of nifedipine-containing solutions, 1×10^{-7} M, 1×10^{-6} M, 1×10^{-5} M and 1×10^{-4} M; *B* shows the effects of increasing concentrations of nicardipine-containing solution, 5×10^{-6} M, 1×10^{-5} M, 5×10^{-5} M; *C* shows the effects of increasing concentrations of verapamil-containing solution, 3×10^{-6} M, 1×10^{-5} M and *D*, diltiazem, 1×10^{-6} M, 1×10^{-5} M, 1×10^{-4} M on the contractile response produced by nerve stimulation. For each antagonist, concentrations which blocked voltage-dependent Ca^{2+} channels, reduced but did not abolish the contractile responses. However, whereas responses persisted in very high concentrations of nifedipine they were abolished in high concentrations of either nicardipine, verapamil or diltiazem. In each trace, the time calibration bar represents 5 s and the force calibration represents 1 mN.

1×10^{-6} M ($n = 5$), 3×10^{-6} M ($n = 2$); or verapamil, 3×10^{-6} M (6 preparations, $n = 3$), 1×10^{-5} M ($n = 1$) to the physiological saline. In quiescent preparations, the resting force was unchanged by each of these Ca^{2+} antagonists. In the presence of any of these four antagonists at the above concentrations, single brief supramaximal stimuli continued to initiate contractions of the longitudinal muscle. In spontaneously active preparations, the oscillations in force on the falling phases of the responses were abolished and the peak increase in force reduced. The amplitudes of responses were reduced to about 50% of their control values: nifedipine, 3×10^{-8} M,

$36 \pm 6\%$ ($n = 5$); nicardipine, 1×10^{-7} M, $71 \pm 7\%$ (6 preparations, $n = 5$); verapamil, 3×10^{-6} M, $47 \pm 7\%$ (7 preparations, $n = 4$); diltiazem, 3×10^{-6} M, $46 \pm 7\%$ (9 preparations, $n = 7$). Again the responses that persisted were abolished by either tetrodotoxin, 1×10^{-6} M ($n = 2$); ω-conotoxin, 1×10^{-7} M (3 preparations, $n = 2$) or hyoscine, 1×10^{-6} M ($n = 2$). Figure 2 shows the effect of diltiazem, 3×10^{-6} M, on spontaneous activity (Fig. 2*Aa* and *Ab*) and evoked responses (Fig. 2*Ba* and *Bb*).

When the concentration of nifedipine was increased, the amplitudes of the contractions produced by brief transmural stimuli were little changed until concentrations of 3 to 10×10^{-5} M were applied (Fig. 3*A*). At these concentrations, contractions were reduced in amplitude but never abolished. Thus a contraction could always be recorded even in the highest concentration tested, that is nifedipine, 1×10^{-4} M. A similar observation has been made on the contractile responses of the longitudinal and circular muscle layers of the human colon produced by brief transmural stimuli (Zar & Goopta, 1983). In contrast the other three antagonists, nicardipine, verapamil and diltiazem all abolished the contractile responses produced by brief transmural stimulation. To do this, very high concentrations of each antagonist were required (Fig. 3*B*, *C* and *D*). The concentrations required to abolish contractile responses to brief transmural stimuli were: nicardipine, 5×10^{-5} M (7 preparations, $n = 6$); verapamil, 3×10^{-5} M (7 preparations, $n = 4$) and diltiazem, 3×10^{-4} M (9 preparations, $n = 7$).

Effect of organic calcium antagonists on responses produced by increasing the external concentration of potassium ions

The possibility that the organic Ca^{2+} antagonists were ineffective at blocking ileal smooth muscle voltage-dependent Ca^{2+} channels was tested. The external concentration of potassium ions, $[K^+]_o$, was rapidly increased from control (5 mM) to 20, 40 and 80 mM. The contractions were recorded in control solutions and then in solutions containing gradually increasing concentrations of the organic Ca^{2+} antagonists. The response to each increase in $[K^+]_o$ was abolished by nifedipine at a concentration of 1×10^{-7} M (12 preparations, $n = 9$). The results from these experiments are summarized in Fig. 4, which also shows the relationship between membrane potential and $[K^+]_o$ determined in five preparations in the presence of nifedipine, 1×10^{-7} M. An increase in $[K^+]_o$ to 20, 40 and 80 mM depolarized the muscle cells by $11{\cdot}7 \pm 1{\cdot}2$ mV, $22{\cdot}3 \pm 0{\cdot}9$ mV and $36{\cdot}5 \pm 1{\cdot}3$ mV respectively.

In parallel experiments the effects of nicardipine, verapamil or diltiazem on the contractile responses produced by increases in $[K^+]_o$ were determined. Nicardipine at a concentration of 1×10^{-7} M blocked the responses to each increase in $[K^+]_o$ in six from seven preparations ($n = 7$); in one preparation a concentration of 3×10^{-7} M was required. Verapamil at a concentration of 3×10^{-6} M (2 preparations from 5 animals), abolished the responses to each increase in $[K^+]_o$; in the remaining three preparations a concentration of 1×10^{-5} M was required. Diltiazem at a concentration of 1×10^{-5} M ($n = 5$) abolished the responses to each increase in $[K^+]_o$.

Each of these four organic Ca^{2+} antagonists completely abolishes the contractions produced by increasing $[K^+]_o$ in the range 20–80 mM at a much lower concentration than that required to abolish the contractions evoked by cholinergic nerve stimulation.

Excitatory junction potentials recorded from the longitudinal muscle layer in response to brief transmural stimuli

Intracellular recordings were made from isolated preparations of the longitudinal muscle layer: care was taken to avoid making recordings from the fine strands of

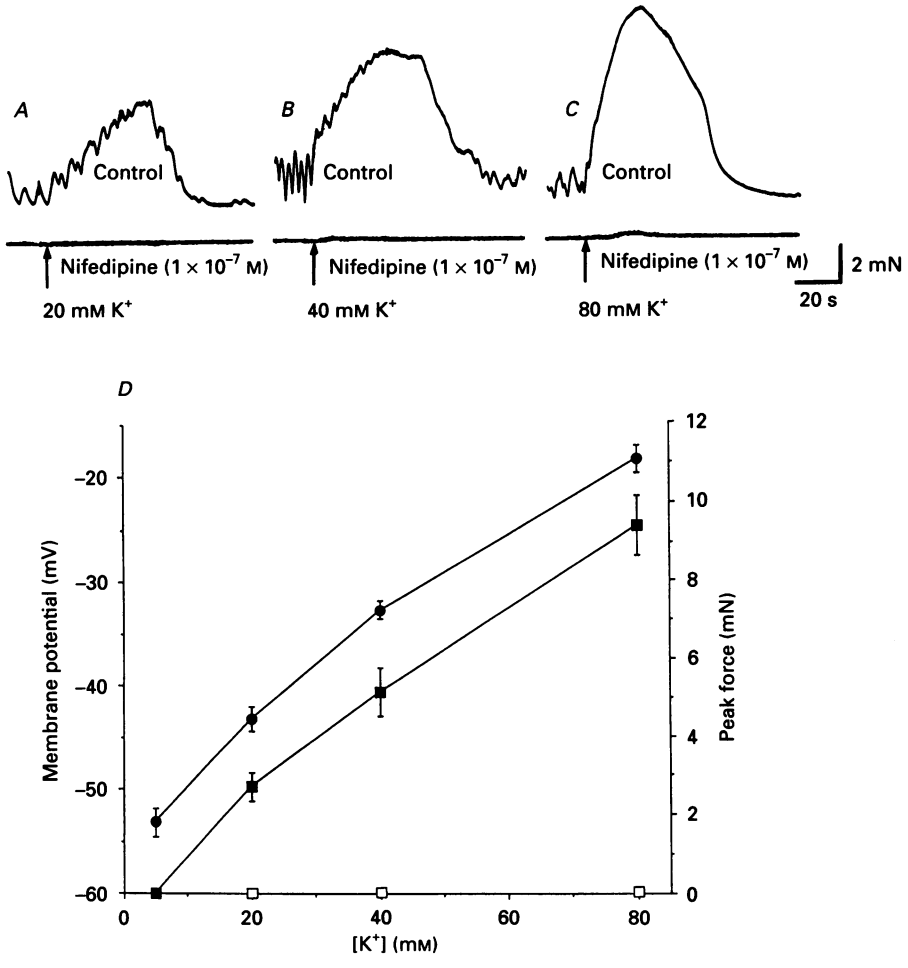

Fig. 4. Effect of nifedipine on contractions of ileal longitudinal muscle produced by increasing $[K^+]_o$. The responses, recorded before and after adding nifedipine, 1×10^{-7} M, which were produced by increasing $[K^+]_o$ to 20, 40 and 80 mM are shown in *A*, *B* and *C* respectively. *D* shows the relationship between peak force and $[K^+]_o$ determined from 12 preparations from 9 animals. After the addition of nifedipine, 1×10^{-7} M, these contractile responses were abolished. The graph also shows the relationship between $[K^+]_o$ and membrane potential determined from a separate group of 5 preparations. ●, membrane potential (mV); ■, peak force (control) (mN); □, peak force (nifedipine) (mN). Vertical lines represent ± 1 S.E.M.

circular muscle which occasionally remained attached. In some preparations an on-going discharge of action potentials was recorded from the longitudinal layer (see Bolton, 1979). In these preparations, a brief transmural stimulus evoked a membrane

depolarization or EJP which triggered one or a burst of muscle action potentials (see Bauer & Kuriyama, 1982). Such responses were associated with a brisk contraction which usually dislodged the recording electrode. In other preparations, the membrane potential was stable, with values of about −50 mV; a brief transmural stimulus initiated an EJP that also triggered an action potential and a brisk contraction.

In preparations that showed myogenic activity, both the spontaneous discharge of action potentials and bursts of action potentials superimposed on evoked EJPs were abolished by adding nifedipine, 1×10^{-8} M, to the physiological saline. In quiescent preparations, nifedipine, 3×10^{-8} M, also abolished action potentials superimposed on evoked EJPs. In either type of preparation, after the muscle action potentials had been abolished, a brief stimulus evoked an EJP. Visual inspection of the muscle sheet indicated that each EJP was followed by a contraction. The contractions were often sufficiently brisk to dislodge the recording electrode.

EJPs and associated contractions persisted when the concentration of nifedipine was further increased in the range 1×10^{-7} M to 1×10^{-4} M. To characterize the time courses and properties of EJPs, nifedipine, 1×10^{-5} M, was routinely added to the physiological saline to reduce muscle contraction and allow stable electrode penetrations. In this solution the membrane potential was invariably stable and lay in the range −34 to −60 mV (−50·3 ± 1·0 mV; $n = 38$). In these preparations brief supramaximal transmural stimuli, pulse widths 0·5–1·0 ms, applied voltages 15–65 V, initiated EJPs (see Fig. 5) which had maximum amplitudes in the range 1·3–17·6 mV (6·9 ± 0·7 mV; $n = 44$). The membrane depolarization started after a delay of about 500 ms. When measured as the separation between the stimulus pulse and the time to 10% of peak amplitude the delay lay in the range 430–730 ms, (560 ± 10 ms; $n = 44$). EJPs had rise times, when measured as the time from 10–90% of peak amplitude, in the range 200–530 ms (370 ± 10 ms; $n = 44$) and half-widths, the time between 50% peak amplitude on rising and falling phase, of 410–1016 ms (720 ± 20 ms; $n = 44$). Excitatory and inhibitory junction potentials recorded from other smooth muscle preparations have a more rapid onset than offset, with the offset being readily described by single exponentials (Bennett, 1972; Hirst & Edwards, 1989). However, we were unable to describe adequately the decay of EJPs recorded from this muscle layer with single exponentials. Unlike EJPs recorded from other tissues the rates of rise and decay of EJPs, recorded from ileal cells, were similar (see Fig. 5*Aa*, *Ba* and *Ca*; and Bauer *et al.* 1991).

The delay measured in these studies is somewhat longer than that reported by Bauer & Kuriyama (1982). The difference probably reflects the difference between the temperatures at which the two sets of observations were made. When the bath temperature was increased, the delay was shortened and the time course of the EJPs was more rapid. In five preparations, EJPs recorded at 25 °C had a delay of 510 ± 30 ms ($n = 5$). In the same preparations, the delay determined at 35 °C was 290 ± 30 ms ($n = 5$). At 25 °C, EJPs had a mean rise time of 340 ± 30 ms and half-width of 740 ± 110 ms ($n = 5$). At 35 °C the rise time and half-width were 180 ± 10 ms and 390 ± 30 ms respectively ($n = 5$).

When recordings were made from different positions either along or across the axes of the muscle fibre bundles, as seen under the microscope, EJPs of similar shape and

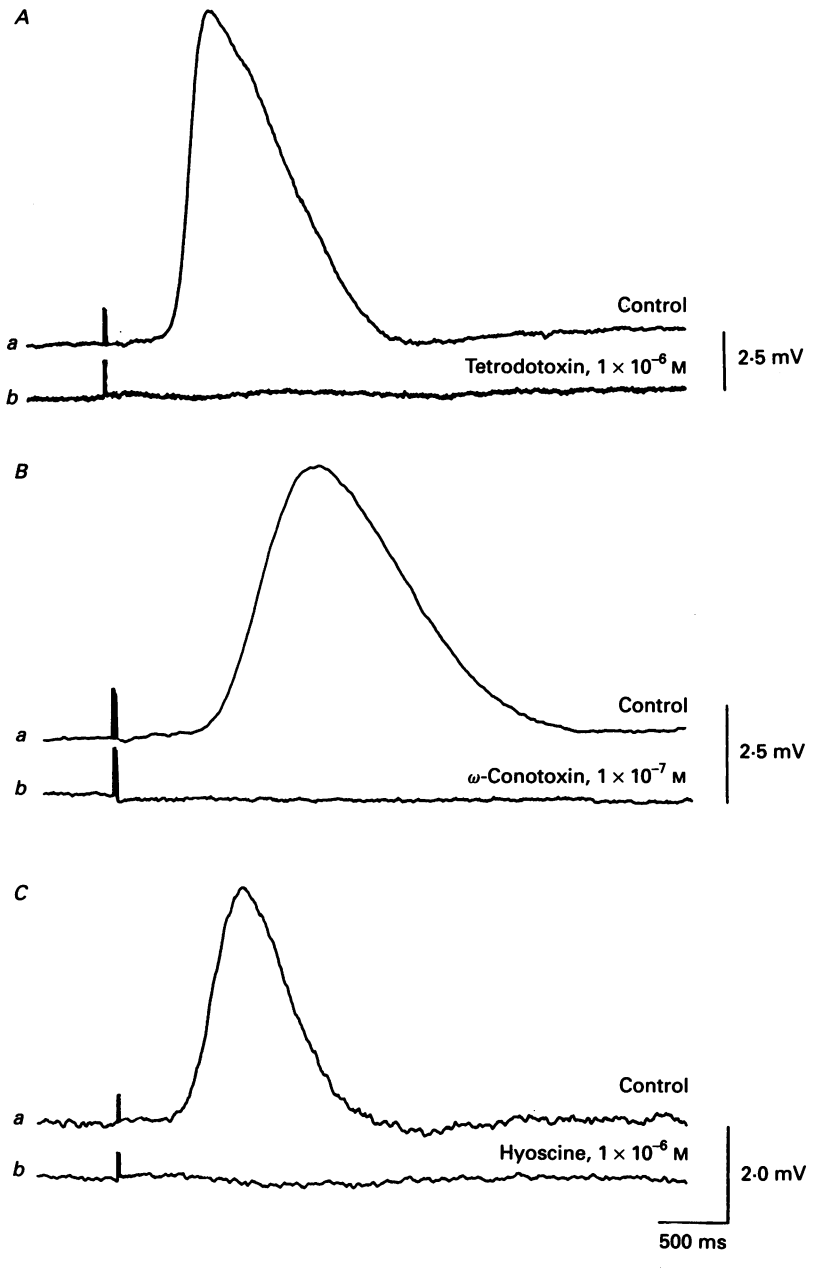

Fig. 5. Effect of tetrodotoxin, ω-conotoxin and hyoscine on EJPs recorded from longitudinal muscle of guinea-pig ileum. Each trace shows the responses produced by single supramaximal brief, 0·5–1·0 ms, stimuli. *A* shows an EJP abolished by tetrodotoxin, 1×10^{-6} M. *B* shows an EJP abolished by ω-conotoxin, 1×10^{-7} M. *C* shows an EJP abolished by hyoscine, 1×10^{-6} M. In each preparation nifedipine, 1×10^{-5} M, was present throughout. The time calibration refers to all traces.

time course were recorded. This suggests that the cholinergic innervation is widely distributed and that during an EJP the preparation is isopotential. Membrane potential changes that might have corresponded to the spontaneous release of packets of transmitter, were not detected.

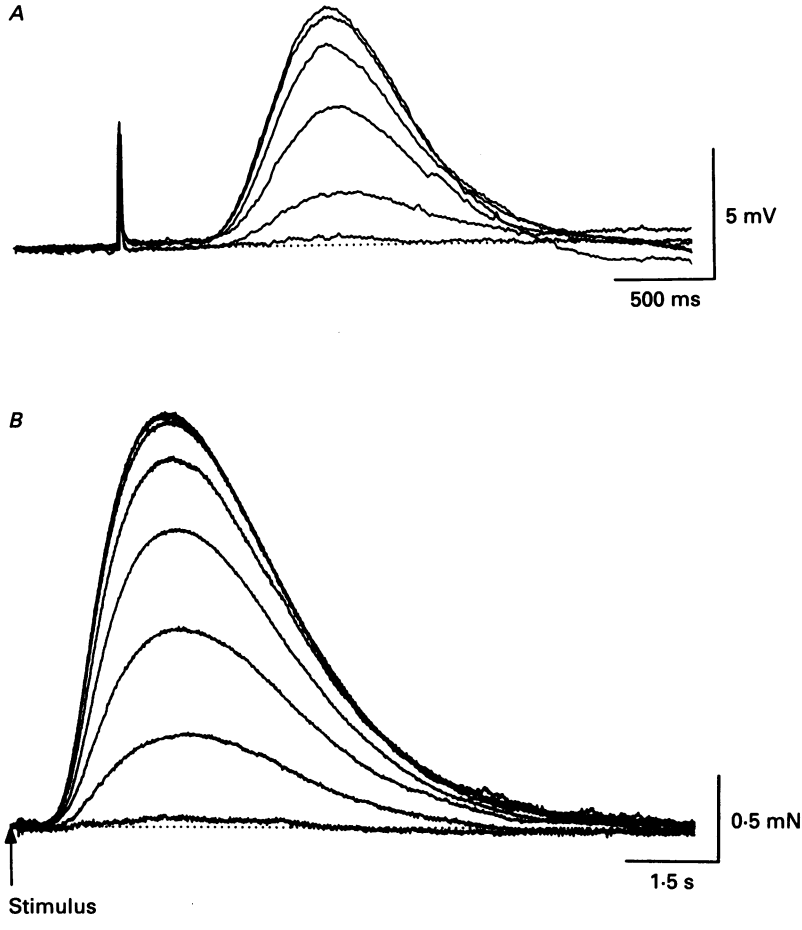

Fig. 6. Effect of varying the strength of stimulation on the amplitudes of EJPs and contractions recorded from longitudinal muscle. The upper series of traces (*A*) shows successive EJPs recorded as stimulus strength was increased in increments of 5 V from 15 to 40 V, pulse width 1 ms. The lower series of traces (*B*) shows successive contractile responses recorded from a separate preparation when the stimulus strength was increased from 15 to 50 V in increments of 5 V, pulse width 1 ms. In each preparation nifedipine, 1×10^{-5} M, was present throughout.

Like the contractions, EJPs were abolished by adding tetrodotoxin, 1×10^{-6} M, to the physiological saline (Fig. 5*Aa* and *b*), indicating that they resulted from the selective activation of nerve fibres. Similarly, they were abolished by the addition of ω-conotoxin, 1×10^{-7} M (Fig. 5*Ba* and *b*), suggesting that they resulted from the neuronal release of transmitter (Kerr & Yoshikami, 1984). Further, EJPs were

abolished by the addition of either hyoscine, 1×10^{-6} M, or atropine 1×10^{-6} M, indicating that they depended upon the activation of muscarinic receptors (Fig. 5*Ca* and *b*). Some EJPs were followed by a slow depolarization, others were followed by a slow hyperpolarization; both types were often apparent in the same preparation. We attributed these slow after-potentials to movement artifacts; they were abolished by any one of tetrodotoxin, ω-conotoxin or hyoscine which blocked both the EJPs and associated movements. In a few preparations taken from terminal ileum, that is within 10 cm of the ileo-caecal junction, the EJP was preceded by a small amplitude (1–2 mV) inhibitory junction potential (see also Bywater & Taylor, 1986). Such inhibitory junction potentials persisted in the presence of hyoscine.

The amplitudes of EJPs changed as the stimulus strength was varied. A threshold stimulus evoked an EJP with a small amplitude (Fig. 6*A*). As the stimulus strength was increased, the amplitude of the EJP increased until a maximal response was detected. We assume that as the stimulus strength is increased progressively greater proportions of cholinergic fibres are stimulated until all fibres are excited. When the preparation was viewed, a threshold stimulus, which evoked an EJP of small amplitude, was seen to evoke a small contraction. As the stimulus strength was increased, the size of the contraction increased. Thus we could not find a stimulus strength where we could detect either a movement without an associated depolarization or conversely a depolarization without a movement. In corresponding experiments where force development was measured, the size of contractions, also recorded in nifedipine, 1×10^{-5} M, increased as the stimulus strength was increased until a maximum response was evoked (Fig. 6*B*).

Passive electrical properties of longitudinal muscle layer and time course of transmitter action during an EJP

Individual smooth muscle cells of the intestine are coupled together to form an electrical syncytium (Abe & Tomita, 1968). The syncytial nature of the longitudinal muscle layer was reconfirmed by the use of two independent microelectrodes, one to pass current and one to record potential (cf. Abe & Tomita, 1968). Whatever the orientation of the electrodes, provided that their separation was less than 100 μm, a current pulse passed through one electrode caused an electrotonic potential to be recorded at the second electrode. However, the electrotonic potentials recorded with electrodes placed at right angles to the orientation of the smooth muscle bundles were consistently smaller in amplitude than those detected with the electrodes orientated parallel to the muscle bundles for the same electrode separation (Fig. 7).

To determine the passive electrical properties of the longitudinal muscle layer the two electrodes were first placed, at a known separation, parallel to the long axis of the bundles of smooth muscle fibres; the electrotonic potential produced by a current pulse will be termed axial. Subsequently the recording electrode was withdrawn from the muscle layer and reinserted, with a known separation, at right angles to the long axis of the smooth muscle bundles; the electrotonic potential produced by the same current pulse is termed transverse. Even with quite small electrode separations, transverse electrotonic potentials were preceded by appreciable electrotonic delays (Fig. 7*B*). These observations indicate that the electrical length constants in the axial and transverse direction differ markedly. The differences between axial and

transverse current flow were extreme with the decrement of electrotonic potentials being so marked that with transverse separations of some 200–300 μm, an electrotonic potential was often not detected. In contrast axial electrotonic potentials could be detected at some 800 μm.

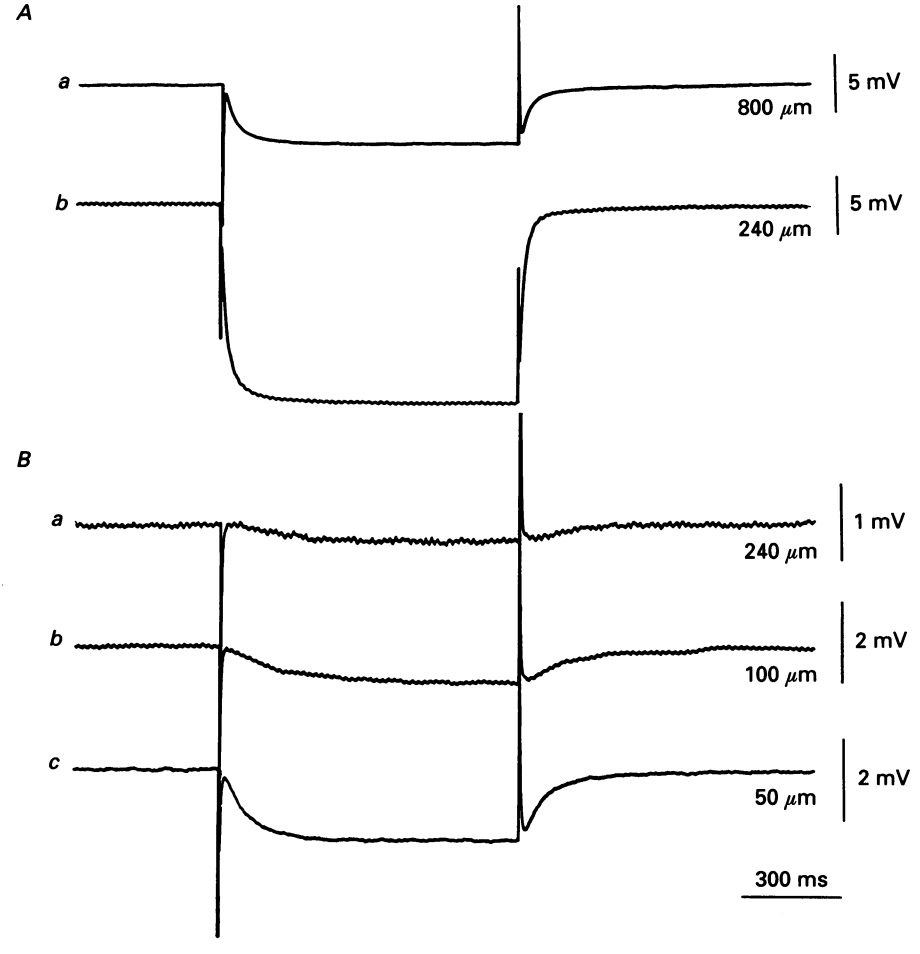

Fig. 7. Electrotonic potentials recorded from a sheet of longitudinal muscle in response to point injection of current. In each trace a current pulse of amplitude 5 nA, duration 1 s, was injected through an intracellular electrode placed in the centre of the muscle sheet. The electrotonic potentials, *Aa* and *Ab*, were recorded in line with the current-passing electrode along the longitudinal axis of the preparation: the separation between electrodes in *Aa* was 800 μm and in *Ab* was 240 μm. The electrotonic potentials, *Ba*, *Bb* and *Bc* were recorded in line with the current-passing electrode at right angles to the longitudinal axis of the preparation: the separation between electrodes in *Ba* was 240 μm, in *Bb* was 100 μm and in *Bc* was 50 μm. It can be seen that electrotonic potentials had larger amplitudes when recorded at small electrode separations and that electrotonic potentials recorded with a transverse orientation (*B*) had smaller amplitudes and slower time courses than those recorded with an axial orientation (*A*). The time calibration bar refers to all records: each trace is the average of 25 successive electrotonic potentials.

Since the longitudinal smooth muscle layer is only some 20 μm thick, it was assumed that an adequate electrical description was that of a two-dimensional infinite plane cell (see p. 84 of Jack, Noble & Tsien, 1975). Transformations were incorporated to allow for the dissimilar axial and transverse length constants. A

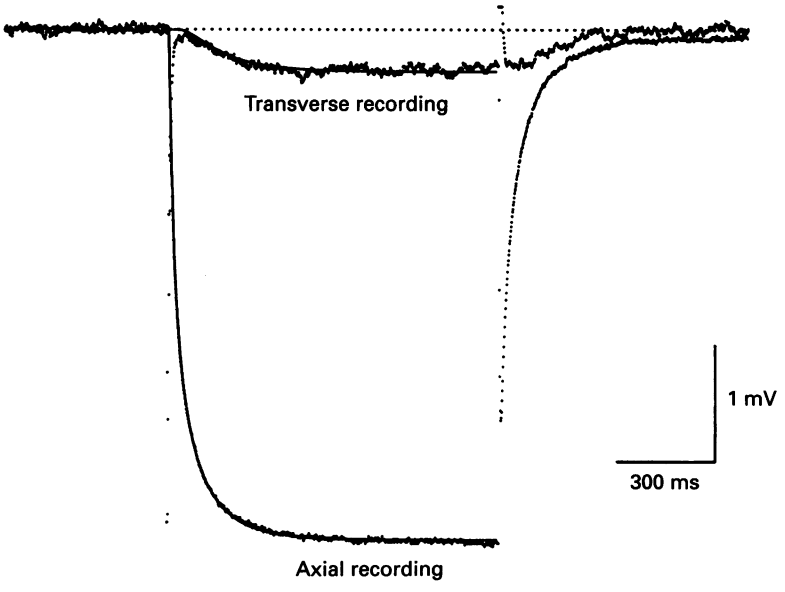

Fig. 8. Cable properties of longitudinal smooth muscle. The figure shows two electrotonic potentials each recorded with the electrode 120 μm distant from the current injection electrode (current amplitude 2 nA). The larger potential was recorded with an axial displacement, the smaller potential with a transverse displacement. The data are shown as dots. The theoretical solutions to describe the two electrotonic potentials are shown with continuous lines. The cable properties from this calculation appear in Table 1, expt 3. The voltage and time calibration bars refer to both electrotonic potentials: each trace is the average of 25 successive electrotonic potentials.

complete description of these transformations is given in an appendix to Bywater *et al.* (1990). Using the time courses and amplitudes of pairs of axial and transverse electrotonic potentials, the measured electrode separations and the known injected current amplitudes, the passive electrical properties were determined using a best-fit procedure (see Methods section of Bywater *et al.* 1990). Examples of experimental data and their fitted solutions are shown in Fig. 8. The cable constants measured in six separate preparations are given in Table 1. It has been pointed out previously that the axial and transverse length constants differ widely (see also Table 1). Any error in electrode positioning particularly along the axial dimension will mean that this method of analysis will underestimate the axial length constant and conversely overestimate the transverse length constant. Thus the values of axial and transverse length constants given in Table 1 should be taken as lower and upper limits respectively. These limitations, however, do not markedly compromise the estimate of the membrane time constant.

From Table 1 it can be seen that the preparations had a mean time constant of 140 ms and an axial length constant of 630 μm. These values are quite similar to those determined by Abe & Tomita (1968) for the longitudinal muscle layer of guinea-pig taenia caecum for current spread in the axial direction. The axial and the transverse length constants differed by about a factor of ten. It is to be stressed that

TABLE 1. Passive electrical properties of ileal longitudinal muscle

Expt no.	Membrane resistance* ($k\Omega$ cm^2)	Membrane capacitance* (μF cm^{-2})	Time constant (ms)	Axial length constant (μm)	Transverse length constant (μm)	Equivalent thickness* (μm)
1	17·6	4·9	86	786	29	1·09
2	15·5	3·3	50	441	46	1·9
3	3·8	37·2	142	310	50	9·6
4	4·1	15·4	63	285	25	0·7
5	9·0	21·3	192	1136	69	1·7
6	12·5	24·7	308	828	133	1·8
Mean	10·4	17·8	140	631	59	2·8
±S.E.M.	2·3	5·1	39·1	135	15·8	1·4

* The values of membrane resistance, membrane capacitance and equivalent thickness are those derived from 2-dimensional planar sheet model (see Jack *et al.* 1975; Bywater *et al.* 1990). A few layers of cells are treated as two lipid sheets separated by a single layer of cytoplasm, consequently the membrane capacitance is an overestimate of the specific membrane capacitance and similar arguments apply to membrane resistance and equivalent thickness. However, the membrane time constant and length constants give accurate estimates of those of the syncytium.

the values given in Table 1 for membrane resistance and membrane capacitance do not imply that the specific membrane resistance is 10·4 $k\Omega$ cm^2 nor that the specific membrane capacitance is 17·8 μF cm^{-2}. These values apply to a planar sheet model. The longitudinal muscle layer is not a planar sheet, rather it is composed of a layer of muscle which is a few cells thick. A better estimate of specific membrane resistance may be obtained by assuming that the specific membrane capacitance was 1 μF cm^{-2} (Cole, 1968), that is a specific resistance of some 140 $k\Omega$ cm^2.

Having determined the passive electrical properties of the longitudinal muscle layer, the time course of the excitatory junctional current, EJC, underlying an EJP can be calculated (see Hirst & Edwards, 1989). The calculation is simplified since it appears that the longitudinal muscle layer is isopotential during each EJP: successive EJPs recorded at one point show little variation and EJPs recorded from several points in the same preparation have similar time courses and amplitudes. The time course of an EJC is then calculated using the relationship:

$$I_t = C(\mathrm{d}V/\mathrm{d}t + V/\tau) \quad \text{(Curtis \& Eccles, 1959)},$$

where I_t is current at time t, C is capacitance, V is change in membrane potential, τ is membrane time constant. (For further details see Hirst & Edwards, 1989.)

To calculate the time courses and amplitudes of EJCs it was assumed that longitudinal muscle cells of the guinea-pig ileum had the same cell dimensions as those of guinea-pig colonic longitudinal muscle, that is a length of 250 μm and a diameter of 5 μm (R. J. Lang, personal communication). Assuming that the cell

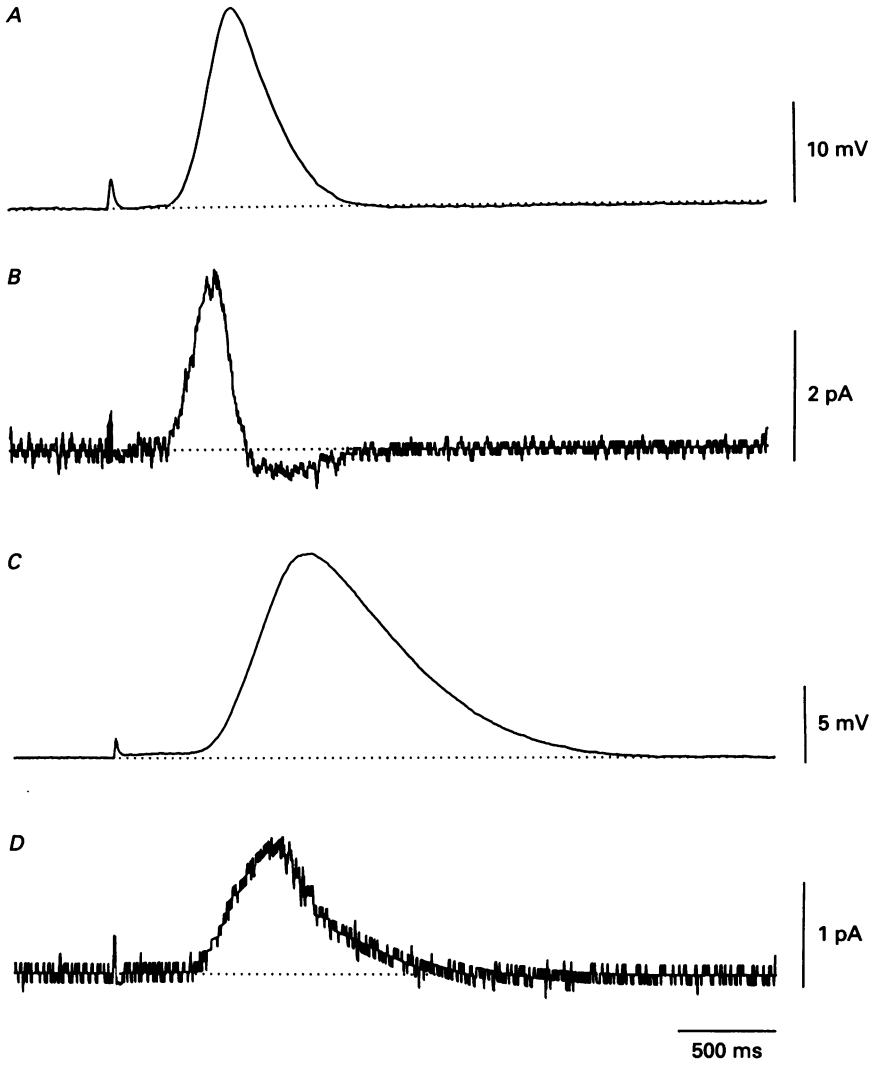

Fig. 9. Calculation of the time course of EJCs which underly EJPs. The time course of current was calculated using the method described by Curtis & Eccles (1959), assuming a membrane time constant of 140 ms. Two examples are shown. The upper pair of traces (*A* and *B*) shows an EJP and the underlying current respectively. On this occasion the current appeared to reverse polarity suggesting that a voltage-dependent conductance might have been activated. The lower pair of traces (*C* and *D*) shows an EJP and the underlying current recorded from a different preparation: in this example no reversal of current was apparent. The time calibration refers to all traces.

surface area could be adequately represented as the surface area of two cones and that the capacitance of biological membranes is about 1 μF cm^{-2} (Cole, 1968), the capacitance of a single cell is about 20 pF. The current underlying EJPs recorded from five different preparations was then calculated using a membrane time constant of 140 ms (see Table 1). The EJPs had a peak amplitude $10{\cdot}8 \pm 2{\cdot}7$ mV ($n = 5$), a

delay of 610 ± 50 ms ($n = 5$), a rise time of 360 ± 50 ms ($n = 5$) and a half-width of 1·09 ± 0·12 s ($n = 5$; Fig. 9*A* and *C*). EJCs had maximum amplitudes of 1·1 ± 0·4 pA, and preceded EJPs (EJC delay = 480 ± 40 ms, $n = 5$). EJCs had shorter rise times than EJPs (rise time = 270 ± 40 ms, $n = 5$) and noticeably shorter time courses (half-

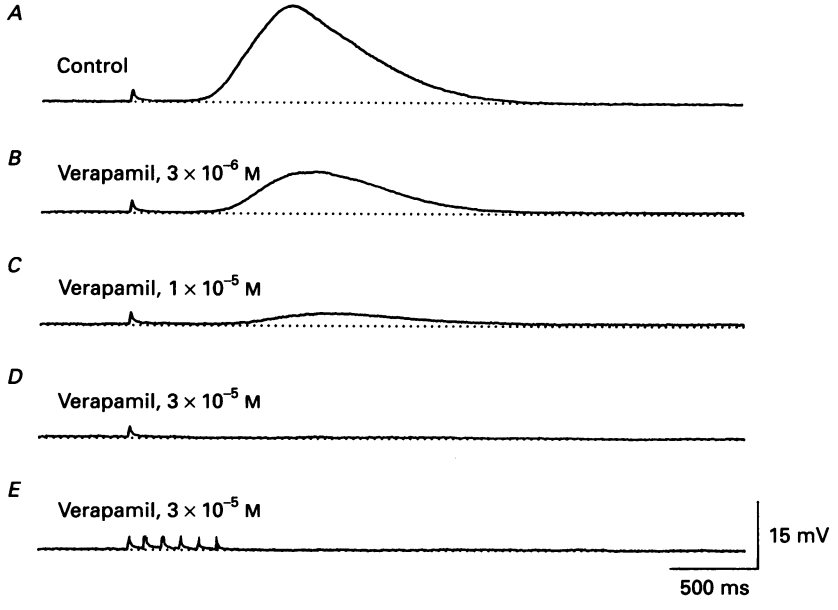

Fig. 10. Effects of high concentrations of verapamil on EJPs recorded from ileal smooth muscle cells. The control EJP was recorded in a nifedipine containing solution (1×10^{-5} M; *A*). The EJP was reduced in amplitude and abolished when verapamil, 3×10^{-6} M (*B*); 1×10^{-5} M (*C*) and 3×10^{-5} M (*D*), was added to the physiological saline. Even when a train of stimuli, 5 at 10 Hz, was given (*E*) blockade was not overcome. The calibration bars refer to each recording.

width = 400 ± 50 ms, $n = 5$; Fig. 9). In three of the five preparations, the calculated junctional current underlying an evoked EJP consisted of a large initial EJC which preceded a smaller outward current (Fig. 9*B*). This reversal of current polarity suggests that a voltage-dependent conductance might be activated. However, since the mean time constant of 140 ms was used to derive the underlying current, the reversal of current polarity may equally be explained by an overestimation of membrane time constant.

Assuming that these currents result from an increase in cation conductance, the change in the internal concentration of cations can be calculated. The volume of a single cell, represented by the volume of two cones, was calculated to be 1·6 pl; the charge entering the cell, obtained by integrating the EJCs over time, was estimated to be 0·46 ± 0·12 pC per cell. Thus during an EJC the internal concentration of cations, calculated as a monovalent ion, is between 1·4 and 5·6 μM (mean 3·08 ± 0·74 μM, $n = 5$).

Effect of nicardipine, verapamil and diltiazem on EJPs

It has been pointed out that the contractile responses to transmural stimulation are reduced by moderate concentrations of either nicardipine, verapamil or diltiazem but are abolished by high concentrations. The effect of these three Ca^{2+} antagonists on EJPs was examined. When intracellular recordings were made from tissues

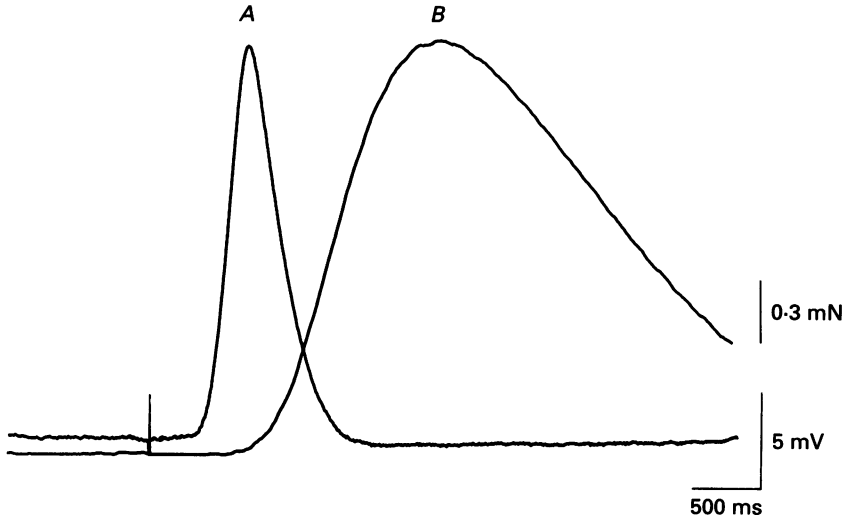

Fig. 11. Comparison between time courses of EJPs and contractions recorded from guinea-pig longitudinal muscle strips in which voltage-dependent Ca^{2+} channels have been blocked with nifedipine. It can be seen that the start of the EJP (*A*) precedes the start of contraction (*B*) by about 400 ms. Similarly the EJP is complete before the peak contractile response occurs. Each trace is the average of ten successive responses. Time calibration refers to both traces.

containing sufficient antagonist to abolish the contractile responses to brief transmural stimuli, that is either nicardipine, 5×10^{-5} M; verapamil, 3×10^{-5} M or diltiazem, 3×10^{-4} M, brief stimuli failed to evoke a detectable EJP. When recordings were made in solutions containing sufficient nicardipine, i.e. 1×10^{-7} M, to abolish the contractile responses produced by increasing $[K^+]_o$, EJPs with associated movements were still recorded. As the concentration of nicardipine was increased in the range 1×10^{-7} to 3×10^{-5} M, the amplitude of the EJPs was reduced until, with the highest concentrations, they were abolished. We could not find a concentration of nicardipine in which we were able to record an EJP without an associated muscle movement or one that allowed us to observe a movement without an EJP. Similar observations were made with verapamil and diltiazem. Thus in concentrations of verapamil and diltiazem that abolished the contractile response produced by increasing $[K^+]_o$, EJPs with associated movements were recorded. Both antagonists, in high concentrations, abolished EJPs and the associated contractions of the muscle layer.

In a further set of experiments, EJPs were recorded in solutions containing a high concentration of nifedipine, 1×10^{-5} M, so that all muscle Ca^{2+} channels were blocked.

The additive effects of nicardipine, verapamil or diltiazem, uncontaminated by their effects on muscle voltage-dependent Ca^{2+} channels, were examined. Each produced a dose-dependent decrease in the amplitude of the EJP. The effects of increasing concentrations of verapamil are shown in Fig. 10. It can be seen that as the concentration of verapamil was increased to very high levels, the amplitude of the

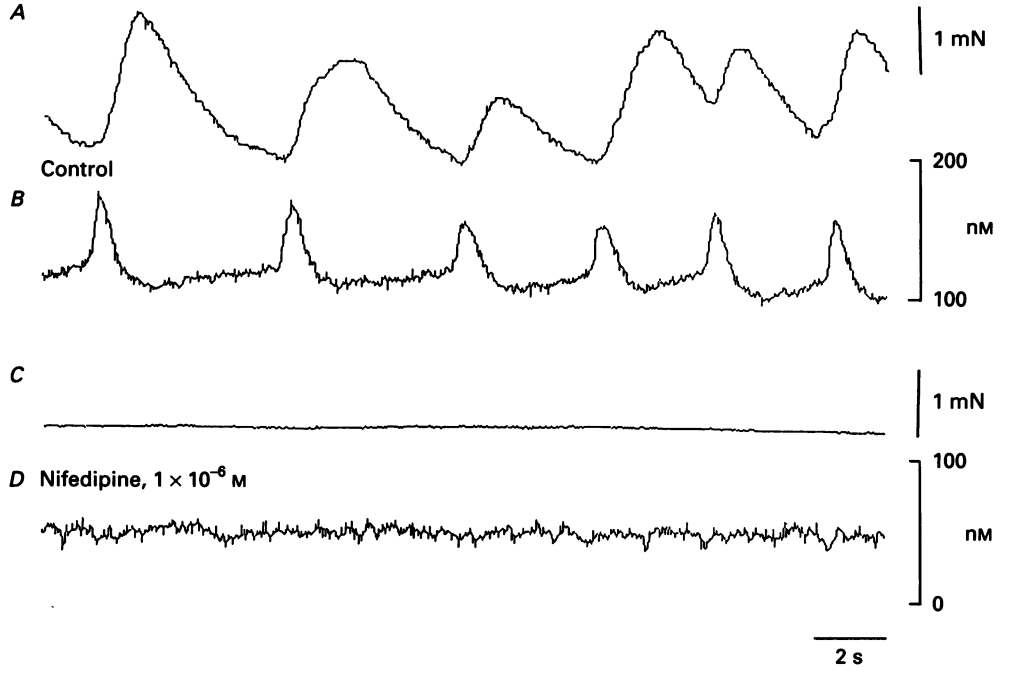

Fig. 12. Changes in $[Ca^{2+}]_i$, determined from tissues loaded with fura-2 AM during spontaneous myogenic activity. Trace *A* shows a series of spontaneous myogenic contractions which are each preceded by an increase in $[Ca^{2+}]_i$ (*B*). Both the contractile responses (*C*) and the increases in $[Ca^{2+}]_i$ (*D*) were abolished by adding nifedipine, 1×10^{-6} M, to the physiological saline. The time calibration refers to all traces.

EJP was reduced. Similar observations were made using either diltiazem, concentration range 1×10^{-5} M to 3×10^{-4} M, or nicardipine, concentration range 3×10^{-7} to 1×10^{-5} M. In two of the experiments involving nicardipine, the preparations were taken from the intestinal muscle close to the ileo-caecal junction. Although the EJPs evoked by transmural stimulation in these preparations were abolished by nicardipine, the small inhibitory junction potential recorded in preparations from the terminal ileum (see also Bywater & Taylor, 1986), persisted. These observations indicate that high concentrations of verapamil, nicardipine or diltiazem each abolish cholinergic EJPs and the associated contractions.

Comparison between the time courses of EJPs and contractile responses

The previous sets of observations have suggested that an EJP triggers a contraction of the longitudinal muscle layer even when muscle voltage-dependent Ca^{2+} channels are blocked. If an EJP was linked to an increase in $[Ca^{2+}]_i$ then the

temporal relationship between an EJP and a contraction would be very similar to that between an increase in $[Ca^{2+}]_i$ and a contraction. This relationship has been described for the same tissue, recorded at the same temperature by Ito *et al.* (1988).

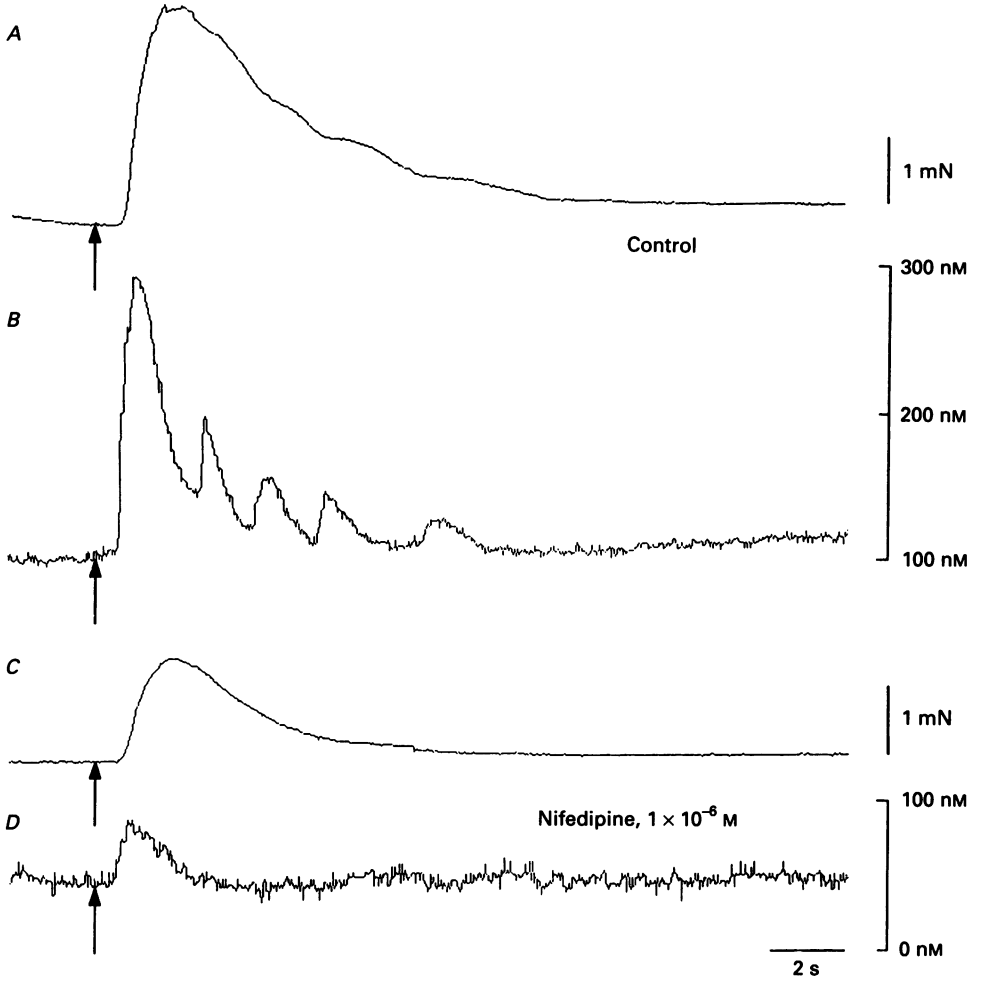

Fig. 13. Changes in $[Ca^{2+}]_i$ produced by brief transmural stimuli. The upper pair of traces shows an evoked contraction recorded in control solution (*A*) which is preceded by an increase in $[Ca^{2+}]_i$ (*B*). After the addition of nifedipine, 1×10^{-6} M, to the physiological saline, a transmural stimulus continues to initiate a contraction (*C*) which is preceded by a transient increase in $[Ca^{2+}]_i$ (*D*). Time calibration refers to each recording.

When the time courses of the change in $[Ca^{2+}]_i$ and of the muscle contraction, produced by direct electrical stimulation were determined, it was found that the increase in $[Ca^{2+}]_i$ preceded the contraction by a few hundred milliseconds (Ito *et al.* 1988). A comparison between the time course of an EJP and the time course of a contraction is shown in Fig. 11, both of which were recorded in the presence of

nifedipine, 1×10^{-5} M. It can be seen that the EJP precedes the contraction by about 400 ms. From this series of experiments, the mean delay before the start of an EJP, again measured as the separation between the stimulus and 10% of the peak response, was 568 ± 31 ms; $n = 10$. For the contractions the delay was 951 ± 21 ms; $n = 10$. Thus the delay between an EJP and its associated contraction is very similar to the delay between an increase in $[Ca^{2+}]_i$ and an associated contraction. This might suggest that the EJP is associated directly with an increase in $[Ca^{2+}]_i$.

Effect of brief transmural stimuli on the internal concentration of calcium ions in longitudinal muscle

To examine more directly the possibility that EJPs were associated with an increase in $[Ca^{2+}]_i$ simultaneous recordings of force and fluorescence were made from preparations of longitudinal muscle loaded with the fluorescent Ca^{2+} indicator, fura-2. As has been reported previously (Himpens & Somlyo, 1988; Ito *et al.* 1988) many preparations gave an on-going discharge of mechanical activity which was preceded by an increase in $[Ca^{2+}]_i$ (Fig. 12*A* and *B*). Both the spontaneous mechanical activity and the periodic increases in $[Ca^{2+}]_i$ were rapidly abolished by adding nifedipine, 1×10^{-6} M, to the physiological saline (Fig. 12*C* and *D*).

In control solutions a brief transmural stimulus caused an increase in $[Ca^{2+}]_i$ that was followed by a contraction (Fig. 13*A* and *B*). This illustration was taken from a preparation that was spontaneously active. The transmural stimulus initiated a contraction with a number of tension oscillations superimposed on its falling phase. Each oscillation was preceded by an increase in $[Ca^{2+}]_i$. Even after increasing the concentration of nifedipine to 1×10^{-5} M, transmural stimuli continued to evoke contractile responses and transient increases in $[Ca^{2+}]_i$ (Fig. 13*C* and *D*). These responses to brief transmural stimuli were abolished by adding hyoscine, 1×10^{-6} M, to physiological saline. These experiments were repeated on six preparations ($n = 5$). In control solution, the peak contractile response initiated by single supramaximal stimuli, was $6{\cdot}26 \pm 0{\cdot}42$ mN; the resting $[Ca^{2+}]_i$ was $0{\cdot}11 \pm 0{\cdot}01$ μM and the peak increase in $[Ca^{2+}]_i$ during an evoked response was 145 ± 16 nM. After the addition of nifedipine, 1×10^{-5} M, the peak contractile response was $2{\cdot}36 \pm 0{\cdot}25$ mN, the resting $[Ca^{2+}]_i$ fell to 53 ± 3 nM and the peak increase in $[Ca^{2+}]_i$ during an evoked contraction was 39 ± 5 nM. These observations indicate that cholinergic nerve stimulation causes an increase in $[Ca^{2+}]_i$ in the presence of concentrations of nifedipine which block voltage-dependent Ca^{2+} channels. The increases in $[Ca^{2+}]_i$ were similar in amplitude to those that occurred during a myogenic contraction (compare Figs 12 and 13).

DISCUSSION

In preparations of the longitudinal smooth muscle layer of guinea-pig ileum brief transmural stimuli excite cholinergic nerves and initiate EJPs. These depolarizations cause voltage-dependent Ca^{2+} channels to be activated and Ca^{2+} enters. The EJPs are preceded by a latency of about 500 ms, when recorded at 25 °C (see also Ito *et al.* 1988). The latency was reduced and the time course made more rapid when the temperature was increased. This is consistent with the view that muscarinic responses in intestinal muscle involve a second messenger and indirectly gated channels (Inoue

& Isenberg, 1990*a*; Komori & Bolton, 1990; Komori, Kawai, Takewaki & Ohashi, 1992).

EJPs are associated with an increase in $[Ca^{2+}]_i$ (see Fig. 13). A similar observation on this preparation was attributed to the activation of voltage-dependent Ca^{2+} channels (Ito *et al.* 1988), the response being abolished by verapamil. However, a very high concentration of verapamil, i.e. 1×10^{-4} M, was required (Ito *et al.* 1988). In contrast, we suggest that neuronally released ACh directly increases $[Ca^{2+}]_i$. This idea relies on the assumption that low concentrations of organic Ca^{2+} antagonists are able to block voltage-dependent Ca^{2+} channels. Since the block produced by a number of organic Ca^{2+} antagonists is voltage dependent (Lang & Paul, 1991), it could be argued that the entire inward Ca^{2+} current is not totally abolished. This seems unlikely as a range of Ca^{2+} antagonists, from different chemical families, was used. The possibility was tested directly by examining the effects of the Ca^{2+} antagonists on the responses produced by $[K^+]_o$. Increases in $[K^+]_o$ produced graded contractions which were abolished by low concentrations of each of the Ca^{2+} antagonists (see Fig. 4). Since the changes in membrane potential caused by these changes in $[K^+]_o$ encompass the range of membrane potentials covered during an EJP, each of the organic Ca^{2+} antagonists used must block voltage-dependent Ca^{2+} entry in concentrations where neuronally released ACh continues to cause a contraction.

There are a number of possible explanations for why an EJP might be associated with an increase in $[Ca^{2+}]_i$. One possibility is that sufficient Ca^{2+} flows through intestinal muscarinic non-selective cation channels to cause the increase in $[Ca^{2+}]_i$ directly, or to trigger additional Ca^{2+} release from intracellular stores. This seems unlikely. Although these channels have been shown by ion substitution experiments to have a high permeability to Ca^{2+} relative to Na^+ (Inoue & Isenberg, 1990*a*), in solutions of normal ionic composition, the amount of Ca^{2+} entering by this pathway in small (Inoue *et al.* 1987; Pacaud & Bolton, 1991). Secondly, a number of organic Ca^{2+} antagonists, used in the concentrations required to abolish EJPs, do not block muscarinic non-selective cation channels (Inoue & Isenberg, 1990*a*). It could be argued that the block of EJPs and the associated contractions by high concentrations of Ca^{2+} antagonists resulted from a prejunctional rather than a postjunctional action. However this seems unlikely since the concentrations of nicardipine, verapamil or diltiazem required to block the responses of the tissue to cholinergic nerve stimulation have no effect on ACh release from nerve terminals in this tissue (Kaplita & Triggle, 1983).

A second explanation could be that although the EJP and the increase in $[Ca^{2+}]_i$ occur together, the two events are causally unrelated. For example, muscarinic receptor stimulation might activate two separate pathways, one which opens non-selective cation channels and the other which releases Ca^{2+} from an internal store (Inoue & Isenberg, 1990*c*; Komori & Bolton, 1990; Komori *et al.* 1992). The kinetics of both pathways would have to be similar (compare Figs 11 and 13). This suggestion might imply that high concentrations of nicardipine, verapamil and diltiazem interfere with the internal pathways and block both events with similar effectiveness.

Alternatively, EJPs could result from the opening of non-selective cation channels as a result of the release of Ca^{2+} from intracellular stores. The depolarization of rabbit

intestinal muscle by added ACh has been shown to be facilitated by such a mechanism (Pacaud & Bolton, 1991). There, the release of Ca^{2+} from internal stores is mediated by IP_3 production (Komori & Bolton, 1991). If EJPs result from this mechanism, two assumptions must be made. Firstly, since the latency of the EJP is similar to the latency of the nerve-mediated increase in $[Ca^{2+}]_i$, the time between IP_3-induced Ca^{2+} release and the opening of non-selective cation channels must be small. This seems possible: Komori & Bolton (1991) have shown that calcium-activated potassium channels open within 30 ms of the release of Ca^{2+} from internal stores in response to the photolysis of caged IP_3. The second assumption is again that high concentrations of Ca^{2+} antagonists interfere with some part of the messenger pathway.

A fourth possibility is that neuronally released ACh activates a set of receptors which are coupled to a set of channels with high Ca^{2+} selectivity. This is consistent with the observed temporal relationship between EJPs and contractions (Fig. 11) and with the finding that similar levels of $[Ca^{2+}]_i$ occur during muscle action potentials and EJPs (Figs 12 and 13, see also Ito *et al.* 1988). During an EJP the internal concentration of monovalent cations would increase by 3 μM. Alternatively, if carried by Ca^{2+}, the concentration would increase by 1·5 μM. This represents 40 times the increase in $[Ca^{2+}]_i$ of 40 nM measured during an EJP. If the charge flow during an EJP is carried entirely by Ca^{2+}, the implication is that 97·5% of Ca^{2+} entering the cell is rapidly sequestered. This may well be the case: Isenberg & Went-Gallitelli (1989) have shown that nearly 95% of Ca^{2+} entering a cell is rapidly bound or sequestered. Alternatively, only 2·5% of the current is carried by Ca^{2+}, the remainder being carried by other cations. This explanation might suggest that nicardipine, verapamil and diltiazem block cholinergic EJPs in high concentrations by blocking a novel type of Ca^{2+} channel which is opened by neuronally released ACh. This idea suggests that specialized neuroeffector junctions must be present in this tissue. At this stage there is no evidence that this is the case: structural studies suggest that cholinergic varicosities do not form discrete neuroeffector contacts with intestinal muscle (Gabella, 1972). Moreover there are no suggestions that muscarinic receptors are concentrated near cholinergic varicosities in the intestine. However, at the cholinergic neuroeffector junctions formed between vagal postganglionic fibres and cardiac pacemaker cells, transmission occurs at organized neuroeffector contacts (Bywater *et al.* 1990; Klemm, Hirst & Campbell, 1992; Hirst, Bramich, Edwards & Klemm, 1992).

In summary, contractions of the longitudinal muscle layer of the guinea-pig ileum, produced by cholinergic nerve stimulation, are triggered by an increase in $[Ca^{2+}]_i$. In control solutions a part of this increase results from Ca^{2+} entry via voltage-dependent Ca^{2+} channels. However, a substantial increase in $[Ca^{2+}]_i$ accompanies the activation of muscarinic receptors by neuronally released ACh. The most likely explanation is either that ACh triggers the release of Ca^{2+} from an internal store or that neuronally released ACh causes the opening of a set of voltage-independent Ca^{2+}-selective channels.

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