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SPONTANEOUS TRANSIENT OUTWARD CURRENTS IN SINGLE VISCERAL AND VASCULAR SMOOTH MUSCLE CELLS OF THE RABBIT

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SUMMARY

1. Whole-cell membrane current recordings under voltage clamp were made at room temperature from dispersed single cells of longitudinal smooth muscle of rabbit jejunum and dispersed single smooth muscle cells of rabbit ear artery using patch pipettes containing up to 10 mM-EGTA Ca buffer.

2. Spontaneous transient outward currents (s.t.o.c.s) up to 250 pA in size and about 100 ms in duration were observed in conditions which might lead to an elevated internal Ca concentration. The amplitude distribution in some cells and form of the currents suggested that they were evoked by a quantal stimulus.

3. S.t.o.c. amplitude was voltage dependent and reversed at the K equilibrium potential. S.t.o.c.s were blocked by 1 mM-tetraethylammonium or 10 mM-Ba applied externally or by perfusing Cs inside the cell.

4. Removing external Ca abolished s.t.o.c. activity in the jejunal cells but not in arterial cells. Increasing EGTA buffering within the cells from 1 mM or less to 10 mM abolished activity in both cell types.

5. Caffeine (5 mM) applied to the bathing solution stimulated rapid discharge of transient outward currents and then a prolonged period of inhibition. The stimulated discharge was sensitive to external Ca in jejunal cells but much less so in arterial cells. ACh applied by ionophoresis to jejunal cells or noradrenaline bath-applied onto arterial cells also stimulated discharge of transients followed by a prolonged inhibitory phase.

6. It was suggested that s.t.o.c.s represent the simultaneous opening of up to 75–100 Ca-activated K channels at −40 mV in response to sudden discharge of Ca from internal stores when these became overloaded, and that this process may occur cyclically.

INTRODUCTION

In smooth muscle there are thought to be two main sources of Ca for the initiation of cell contraction. These are extracellular Ca, which may enter the cell through voltage- and/or receptor-operated Ca channels and Ca stores within the cell which may be released into the cytoplasm (for reviews see Bolton, 1979 and Brading, 1981). This general hypothesis was built upon observations of the rate of loss of contractile responses to various agonists upon bathing tissues in solutions with low extracellular

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Ca. The presence of a Ca store in visceral smooth muscle was first recognized by Edman & Schild (1962) and subsequent work with arterial muscles indicated that the stores in these tissues were more resistant to wash-out in low-Ca solutions than the stored Ca in visceral muscle (Deth & Casteels, 1977).

In addition to being releasable by transmitter substances, the intracellular stores of Ca are thought to be releasable by methylxanthines such as caffeine (Endo, 1977) although the actions of caffeine on smooth muscle tissues are complex, producing hyperpolarization, depolarization, relaxation and contraction under various conditions (for review see Bolton, 1979). Single cardiac cells maintained in conditions likely to elevate free ionized Ca concentrations show spontaneous fluctuations in aequorin luminescence which have been interpreted as evidence for spontaneous cyclical release of an internal Ca store (Allen, Eisner & Orchard, 1984). Studies on single skinned cardiac cells have suggested that release of the Ca store may be controlled in a complex manner by the intracellular free Ca concentration and its rate of change (Fabiato, 1983).

If this cyclical release of Ca within the cell occurred in cells that contained a population of Ca-activated K channels within the cell membrane then the rise in free intracellular Ca might be expected to stimulate a transient increase in K conductance. Spontaneous miniature outward currents (s.m.o.c.s) have been recorded in cultured neurones from frog sympathetic ganglia and were thought to be stimulated by the release of packets of intracellular Ca (Brown, Constanti & Adams, 1983). Ca-activated K channels are also known to occur in both vascular and visceral smooth muscle cells (Inoue, Kitamura & Kuriyama, 1985; Benham, Bolton, Lang & Takewaki, 1986). We describe here the characteristics of spontaneous transient outward currents (s.t.o.c.s) in smooth muscle cells of rabbit ear artery and jejunum. The properties of these currents are consistent with the hypothesis that they result from opening of Ca-activated K channels in the cell membrane triggered by the cyclical release of Ca from within the cell.

Some of these results have been reported (Benham & Bolton, 1986). Klockner & Isenberg (1985) have also briefly reported the occurrence of outward currents stimulated by release of intracellular Ca stores in single smooth muscle cells from trachea and bladder.

METHODS

Preparation of cells

Adult rabbits weighing 1–2 kg of either sex were killed by cervical dislocation. Lengths of longitudinal muscle from the jejunum were peeled from the underlying circular muscle and placed in physiological salt solution (composition given below). A 4 cm length of ear artery was dissected free of connective tissue and placed in physiological salt solution. The artery was cut longitudinally and then the resulting sheet cut into six rectangular segments for dispersal.

Jejunal smooth muscle cells were dispersed as previously described (Benham & Bolton, 1983; Bolton, Lang, Takewaki & Benham, 1985) using collagenase (0·5 mg/ml), trypsin inhibitor (1 mg/ml) and bovine serum albumin (2 mg/ml). We modified the 'Ca-free' solution used previously for dispersal by adding 50 μM-Ca to it. Ear artery cells were dispersed using the same technique except that the dispersal solution contained collagenase (0·4 mg/ml), elastase (1·2 u./ml) and bovine serum albumin (2 mg/ml) and three successive incubations of 30 min were used for this tissue. Both types of cell were stored on cover-slips for up to 30 h at 4 °C in a solution containing 0·2 mM-Ca.

Whole-cell recording

Whole-cell membrane-current recordings were made at 22–25 °C using standard patch-clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The current amplifier used was either a List EPC-5 or EPC-7 and current records were stored on FM tape and replayed onto a Gould Brush recorder through a low-pass filter at 0·5 kHz (−3 dB) for illustration and analysis. Patch pipettes had a resistance of 2–5 MΩ when filled with intracellular solution. For intracellular perfusion, the List pipette holder was modified so that the pipette solution could be changed while recording from a cell. This was based on the technique of Soejima & Noma (1984). A 1 mm polyethylene tube was pulled to a fine taper and inserted through the side arm of the pipette holder and then down the pipette to within 1–2 mm of the pipette tip. Negative pressure applied to the usual suction tube on the side arm drew waste solution up this tube to a reservoir and fresh solution down the fine tube to the pipette tip. Tests with dye-containing solution showed that test solutions took 15 s to reach the pipette tip and then a further 30 s to completely exchange the pipette solution (perfusion rate 50 μl/min). Perfusion was usually continued for 2 min. Measurements of loss of K currents following replacement of K in the pipette solution with Cs suggested that equilibration with the cell contents took 2–3 min.

Amplitudes of s.t.o.c.s were measured from paper records by hand. Frequency distributions were measured by hand or by BBC microcomputer in a similar way to that used for measuring open and closed times of single-channel records (Benham *et al.* 1986).

Solutions

The cells were bathed at room temperature (20–23 °C) in physiological saline solution containing (mM): Na, 125; K, 6; Ca, 2·5; Mg, 1·2; Cl, 133; HPO_4, 1·2; HEPES, 10; glucose, 11. The pipette (intracellular solution) contained (mM): Na, 5; K, 126; Mg, 1·2; Cl, 128; HEPES, 10; ATP, 1; glucose, 11. EGTA concentration was 0·077, 0·77 or 10 mM as stated in the text. All solutions were adjusted to pH 7·2 with NaOH. In some experiments no ATP was added to the pipette solution but this had no apparent effect on transient currents.

Drugs and chemicals

The following were used: adenosine 5′ triphosphate (ATP; sodium salt), bovine serum albumin, caffeine, elastase, trypsin inhibitor, acetylcholine chloride, noradrenaline hydrochloride (Sigma); collagenase (Cooper Biomedical); ethylene glycol bis-(2-aminoethyl) tetraacetic acid (EGTA) (Fisons).

RESULTS

Occurrence of s.t.o.c.s

S.t.o.c.s were observed in ear artery and jejunal smooth muscle cells from rabbits under certain conditions at 21–25 °C. Cells were held under voltage clamp using standard patch-clamp recording in the whole-cell configuration (Hamill *et al.* 1981). A recording of membrane current from an ear artery cell voltage clamped at −40 mV is shown in Fig. 1*C*. The s.t.o.c.s had a duration of about 100 ms; current rose to a peak of 250 pA in the largest transients in less than 30 ms and then decayed more slowly to the basal current level. The decay phase of the currents could be fitted to a single exponential in some cells but the decay phase was usually more complex. S.t.o.c.s were observed if the pipette solution perfusing the cell contained less than 1 mM-EGTA and if the cells were voltage-clamped at potentials more positive than −50 mV or if the cells had been bathed in solutions containing elevated K concentrations (see Fig. 4*B*). These requirements suggest that an elevated concentration of internal Ca was required to stimulate the currents. S.t.o.c.s were not generally seen in recordings from cells in normal physiological salt solution when the membrane potential was −50 mV or more negative, unless the cell had previously been held for long periods at more depolarized potentials.

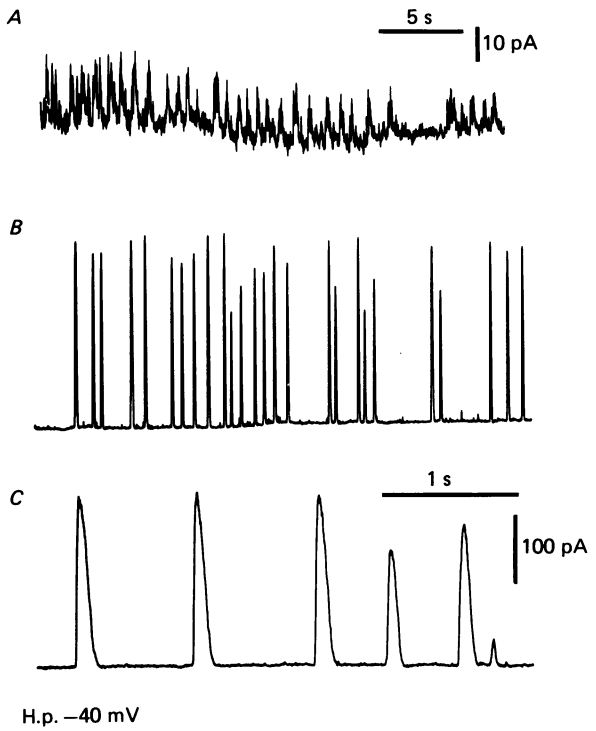

Fig. 1. Cell-attached patch (*A*) and whole-cell (*B* and *C*) membrane-current recordings from the same rabbit ear artery cell. *A*, current recording from cell-attached patch held at +30 mV relative to cell membrane potential. Outward current with respect to pipette is upward in this and all other records. Thus upward oscillations correspond to inward currents across the patch. E_K is probably close to 0 mV across the patch as the pipette contained 126 mM-K. Pipette contained intracellular solution with 0·07 mM-EGTA. *B*, same cell following patch rupture under voltage clamp at holding potential (h.p.) of −40 mV, same time scale as *A*, showing outward currents in the membrane of whole cell. *C*, section of membrane current record on expanded time scale.

Patch-current recordings from cell-attached patches before disrupting the patch to achieve whole-cell recordings were usually devoid of single-channel activity in healthy cells and there were no fluctuations in the basal current level. This suggests that in these cells s.t.o.c.s were not occurring, as they should either be observed as channel openings in the patch or in the rest of the cell, in which case they would be indicated by small currents driven through the patch (Fenwick, Marty & Neher, 1982). When s.t.o.c.s did develop, they did so within a few seconds of disrupting the patch and clamping the cell membrane potential, usually at −40 mV. However, very occasionally it was possible to observe oscillations in current in the cell-attached mode. Fig. 1 *A* shows an example of activity recorded from a cell-attached patch from a rabbit ear artery cell. The patch was held at a potential 30 mV positive to the cell membrane potential. The current record shows upward current oscillations (inward through the cell-attached patch where the K equilibrium potential (E_K) is close to 0 mV and hence arising by channel opening in the patch) with a frequency of 1·15 Hz

and an amplitude of 10–15 pA. Detailed examination of these oscillations suggested that they were composed of a smooth change in the basal current and a change in amplitude of the superimposed single-channel current events. These changes in amplitude from 1–2 to 6–7 pA at the peak of oscillations may be attributed to changes in driving force due to fluctuations in the cell membrane potential which would also generate smooth oscillations in basal current.

Such fluctuations in the cell membrane potential were probably caused by the s.t.o.c.s which could be seen in the whole-cell recording from the same cell immediately after breaking through from the cell-attached mode although the frequency was slightly less (Fig. 1*B*). At a holding potential of −40 mV, the frequency of the s.t.o.c.s was 0·86 Hz, close to that (1·15 Hz) of the oscillations in the cell-attached patch. The events in the whole-cell recording were of shorter duration (Fig. 1*C*) but the fluctuations in the cell-attached patch current would be slowed by the time constant of the cell membrane. Thus, in this cell it would appear that s.t.o.c.s were occurring spontaneously in the intact cell. However the presence of Ca-activated K-channel activity in this cell at the resting membrane potential indicated that it was Ca-loaded.

Current amplitude distribution

As the cells were not receiving any synaptic input, an internal modulator of the membrane conductance would seem to be responsible for generating the s.t.o.c.s. The most obvious candidate for this role is the internal Ca concentration as this is known to modulate the activity of a class of K channels in these cells (Benham *et al.* 1986). Records from a total of 114 jejunal cells were examined for evidence of s.t.o.c.s. The recording pipette either contained 0·077, 0·77 or 10 mM-EGTA and the occurrence of s.t.o.c.s is listed in Table 1. All cells perfused with 10 mM-EGTA-containing solution held at −40 mV had stable membrane currents with no s.t.o.c.s. 61 % of cells perfused with 0·77 mM-EGTA showed s.t.o.c.s when held at −40 mV and this increased to 80 % of cells in which the EGTA added to the pipette solution was only 70 μM. Thus the internal EGTA concentration and hence the internal free Ca concentration seemed to be important in regulating the genesis of these currents.

Measurement of the amplitudes of individual s.t.o.c.s in each cell at −40 mV revealed several patterns which seemed to derive from two basic types of distribution. Some cells showed a repetitive discharge of transients of rather similar large amplitude (> 100 pA) with rather few, if any, small-amplitude transient currents (Figs. 1 and 3). In other cells the amplitudes of the s.t.o.c.s were much more variable and had an exponential distribution of amplitudes (Figs. 6*B* and 7*A*), as seen in frog sympathetic neurones (Brown *et al.* 1983). Patterns of activity were also seen showing a combination of the two types of distribution. An amplitude histogram of a cell that showed this composite activity is shown in Fig. 2. There was a clear population of large-amplitude s.t.o.c.s with a mean of 95 pA and a second component with an exponential distribution (above 5 pA) and mean amplitude of 13 pA. Twenty-two cells perfused with 0·77 mM-EGTA showed a clear population of large-amplitude transients with a mean amplitude of 142 ± 17 pA. Sixteen cells perfused with 0·077 mM-EGTA showed similar distributions with a mean amplitude of the large transients of 117 ± 13 pA. The frequency of discharge of these large

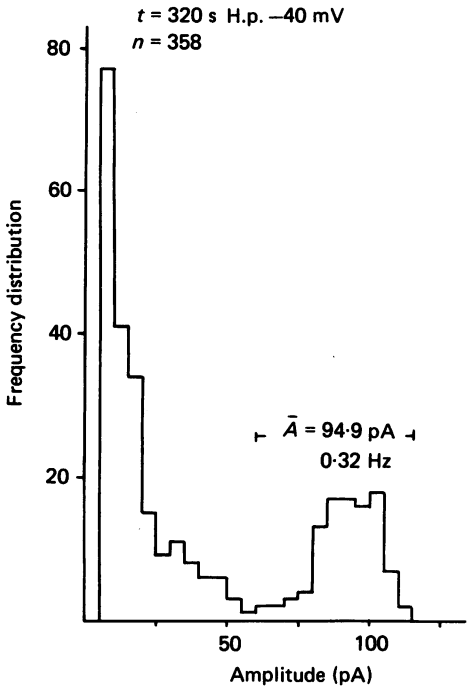

Fig. 2. Frequency distribution of s.t.o.c.s. All outward currents greater than 5 pA in amplitude were measured. The distribution between 5 and 50 pA was exponential with mean amplitude 13 pA. Pipette contained 0·77 mM-EGTA. Jejunal cell.

TABLE 1. Occurrence of two types of s.t.o.c. activity in 114 jejunal cells with three different EGTA concentrations in the pipette. Mean amplitudes for large-amplitude distributions were calculated from distributions as shown in Fig. 2. Cells classified under small amplitude showed no separate population of large-amplitude s.t.o.c.s. Peak amplitudes measured for these cells were of the largest events occurring in at least 20 s of recording

	Type of s.t.o.c.s						
	None	Large-amplitude distribution			Small-amplitude exponential distribution		Total
EGTA concn. (mM)	*n*	*n*	Mean amplitude (pA)	Frequency Hz	*n*	Peak amplitude (pA)	*n*
10	12	0	—	—	0	—	12
0·77	26	22	142±17	0·23	19	32·3±7	67
0·077	7	16	117±13	0·22	12	31·0±6	35

transients was similar in the two groups of cells, 0·23 and 0·22 Hz respectively (Table 1). Qualitatively similar results were obtained from thirty-four arterial cells.

The behaviour of the s.t.o.c.s was further analysed in the cells showing a clear population of large-amplitude s.t.o.c.s. The relationship between the amplitude of a s.t.o.c. and the time interval to the previous s.t.o.c. was examined. In most of the

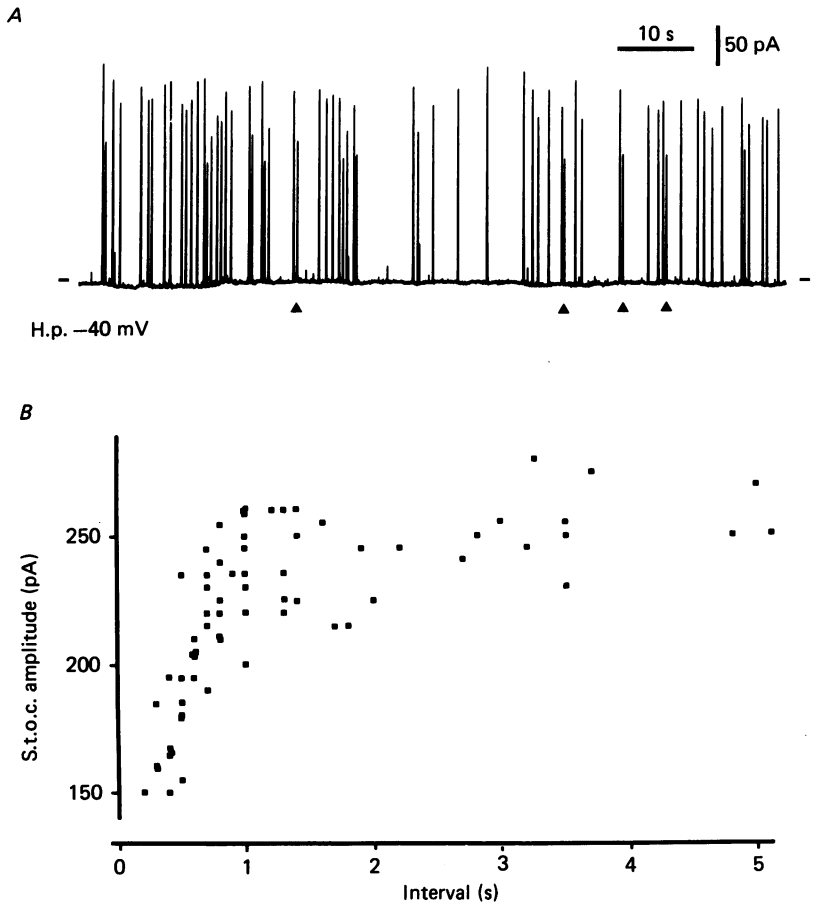

Fig. 3. *A*, membrane current record of ear artery cell and *B*, relationship between s.t.o.c. amplitude and interval since preceding event. *A*, current record indicates examples of lower-amplitude events following less than 1 s after a large-amplitude current. Mean amplitude of sixty-seven events was 225 ± 4 pA and frequency was 0·70 Hz. *B*, distribution was plotted from 95 s of record. Pipette solution contained 0·077 mM-EGTA. Membrane potential −40 mV.

cells this revealed a distribution with no obvious correlation. However, in five cells there seemed to be a predominance of smaller amplitudes following short latencies, and in two cells (one arterial and one jejunal) an even clearer picture emerged. In these two records, all the s.t.o.c.s of amplitude less than 2 S.D. from the mean amplitude were events occurring with latencies of less than 1 s from the previous event. For these short-latency s.t.o.c.s there was a direct correlation between amplitude and latency (Fig. 3).

In most records there were examples of s.t.o.c.s that overlapped one another producing partially summated events with double peaks. No overlapped events were seen in the two cells that showed amplitude–latency correlations and there was a minimum latency of 150 ms between events. These results suggest that the transient currents in most cells are triggered from more than one source while in a few cells,

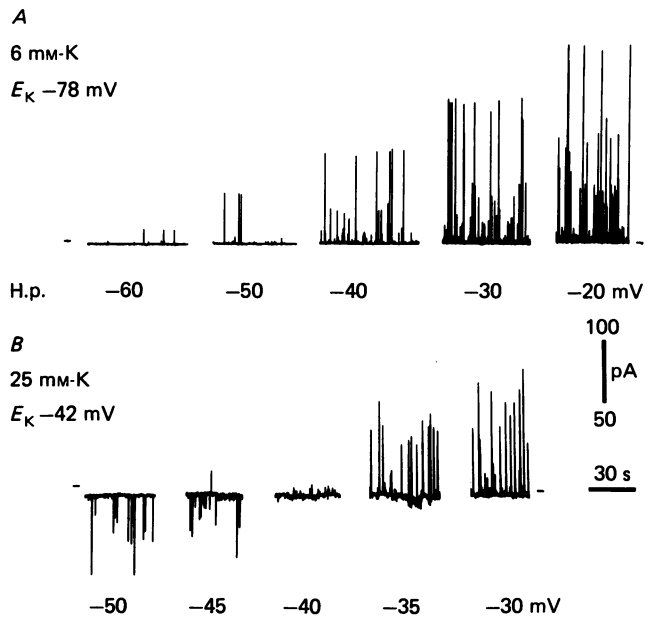

Fig. 4. Membrane potential dependence of current amplitude in jejunal cells. *A*, cell bathed in normal extracellular solution containing 6 mM-K. *B*, another cell bathed in extracellular solution containing 25 mM-K made up by equimolar exchange of KCl for NaCl. Pipette solution contained 0·77 mM-EGTA. Current calibration: *A*, 100 pA, *B*, 50 pA. Calculated E_K is shown.

of which these two are examples, there appeared to be only one source which required recharging before it could trigger a further large-amplitude s.t.o.c.

Ionic selectivity

The amplitude of the s.t.o.c.s was potential dependent and this was particularly clear in cells that showed a population of large-amplitude s.t.o.c.s. Fig. 4*A* shows current records from such a cell held at five different potentials. The amplitude of the largest s.t.o.c.s declined on hyperpolarization as did the frequency of discharge. This reduction in frequency meant that reversal of currents was seen only in one cell of four, and in the others a reversal potential had to be extrapolated from plots of the mean amplitude of the s.t.o.c.s. These gave a mean value of $-69{\cdot}5 \pm 1{\cdot}3$ mV ($n = 4$) and compares with a calculated E_K of -78 mV assuming that the cells were effectively perfused with the pipette solution. In three cells the frequency of discharge of the large transients declined from $0{\cdot}16 \pm 0{\cdot}05$ to $0{\cdot}07 \pm 0{\cdot}05$ Hz between -20 and -60 mV.

The lower record in Fig. 4 shows membrane current activity from another jejunal cell bathed in an elevated external K concentration of 25 mM. Under these conditions clear reversal of the transient currents was seen at -42 mV (interpolated), the calculated E_K. In four cells bathed in a higher concentration of 45 mM-K the reversal potential was shifted to $-24{\cdot}0 \pm 0{\cdot}9$ mV, also very close to the calculated E_K of -25 mV. The currents generated thus appear to be carried by K ions.

Pharmacological evidence that the currents were carried through K channels was provided by the effects of K-channel blockers. $BaCl_2$ (1–10 mM) applied in the bathing solution produced a concentration-dependent reduction in s.t.o.c. amplitude which was nearly complete at 10 mM. Application of 0·2 to 1 mM-tetraethylammonium (TEA) also produced a concentration-dependent reduction in s.t.o.c. amplitude. The reduction in amplitude at 0·5 mM-TEA was to about 40 % of control amplitude which was similar to the blocking action of external TEA on single Ca-activated K channels (Inoue *et al.* 1985). Finally, using an internally perfused pipette it was possible to change the contents of the pipette from a solution containing mainly K ions to one in which all K was replaced with Cs ions. When this was done, the amplitude of the s.t.o.c.s steadily declined and they were lost in the base-line noise (Fig. 7*A*). This was consistent with the ionic replacement steadily reducing the driving force for K currents across the cell membrane.

Effect of reduction in the Ca concentration in the bathing solution on s.t.o.c. activity

The dependence of s.t.o.c. activity on the internal concentration of EGTA suggested that internal Ca concentration was important for regulating the activity. Reduction of external Ca should affect its internal concentration by increasing efflux of Ca and reducing influx across the cell membrane. When jejunal cells were bathed in a solution containing no added Ca (free Ca about 10 μM) then the s.t.o.c.s ceased immediately (Fig. 5*A*). In contrast, arterial cells responded differently to low external Ca concentration. Bathing these cells in low external Ca-containing medium only reduced s.t.o.c. activity, and further reducing free Ca by adding 1 mM-EGTA to the bathing medium still did not abolish s.t.o.c. activity. In the example shown in Fig. 5*B* the frequency of s.t.o.c. discharge was reduced by about 50 % and the amplitude of the s.t.o.c.s was unaffected. Thus there was a clear difference in the effect of external Ca on the generation of the s.t.o.c.s in arterial and visceral smooth muscle cells.

If reduction in a steady Ca influx across the cell membrane was the reason for the effect of low external Ca on the jejunal cells, then agents that block Ca entry would be expected to mimic the effect of Ca-removal. We have observed that Cd is an effective blocker of voltage-dependent Ca channels in jejunal cells, 200 μM-Cd producing 90 % block. This concentration of Cd was found not to reduce the amplitude or frequency of the s.t.o.c.s and in the record shown in Fig. 6*A* the amplitude of the transients increased slightly. If the Cd concentration was increased to 2 mM, then the s.t.o.c.s were slowly abolished, the block appearing as a steady reduction in amplitude of the transient currents (Fig. 6*B*). However, we have observed that this higher concentration of Cd has a direct blocking action on Ca-activated K channels, which may be its mechanism of action here. The failure of low concentrations of Cd to block s.t.o.c. activity implies that inward movement of Ca through voltage-dependent Ca channels is not necessary for maintaining the activity of s.t.o.c.s.

Changing internal Ca concentration by intracellular perfusion

Results obtained from different cells with different pipette concentrations suggested that the amount of Ca buffering within the cell regulated s.t.o.c. activity, and so it

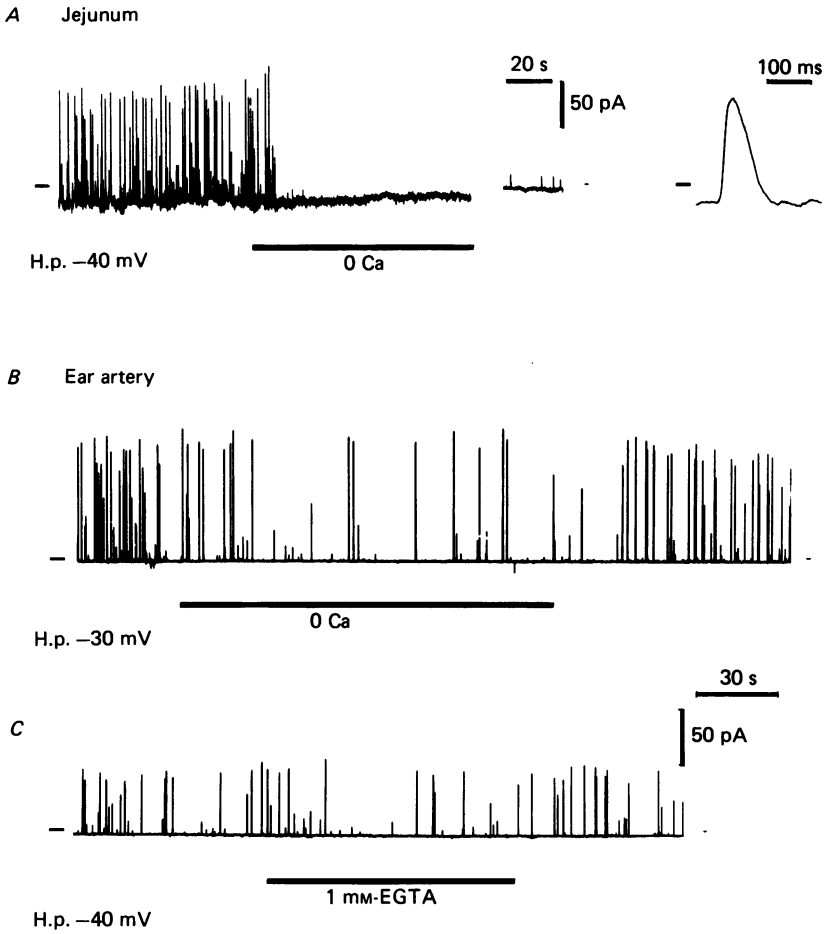

Fig. 5. Effect of reducing extracellular Ca on s.t.o.c.s in jejunal and arterial cells. *A*, jejunal cell. Horizontal bar indicates period during which cell was bathed in extracellular solution containing no added Ca. Small length of trace shows activity after 3 min wash-out. Right-hand trace shows single s.t.o.c. at expanded time scale. *B*, effect of same intervention on arterial cell. *C*, effect of bathing another arterial cell in solution containing no added Ca and 1 mM-EGTA for the period indicated by the horizontal bar. Pipettes contained 0·77 mM-EGTA. Membrane potential −40 mV.

was of obvious interest to investigate the effect of changing the buffering while recording membrane current. This was attempted by perfusing the patch pipette internally through a fine tube inserted close to the tip of the pipette (Soejima & Noma, 1984). Using this technique to replace K in the pipette with Cs produced a reduction in s.t.o.c. activity over 2–3 min (Fig. 7*A*) and indicates that small ions in the pipette solution will equilibrate over this time period with the cytoplasm.

Attempts to produce changes in internal free Ca using constant 0·77 mM-EGTA and varying added Ca gave inconsistent results and usually had little effect if the calculated free Ca concentration in the pipette was set between 10^{-8} and 10^{-6} M. If EGTA was omitted from the perfusing solution and 10 μM-Ca added then cells

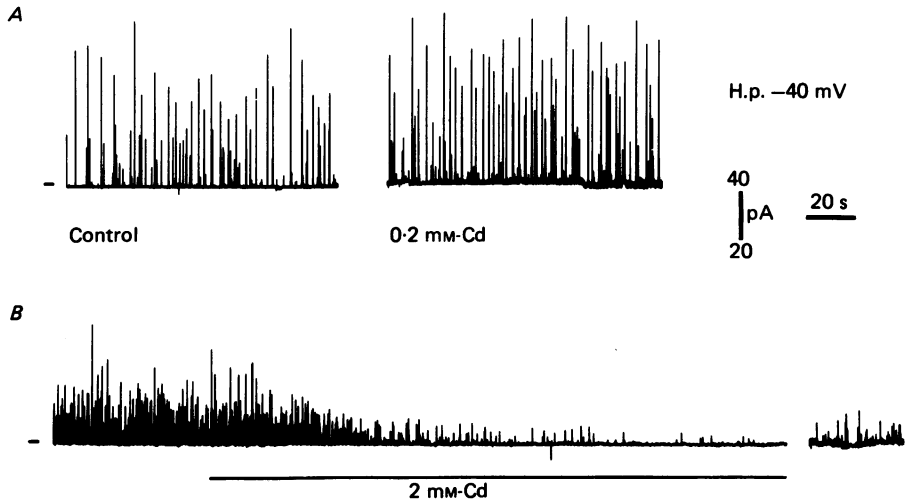

Fig. 6. Effect of applying Cd to jejunal cells. Upper pair of traces show control s.t.o.c. activity (left) and current activity when bathing cell in 0·2 mM-Cd (right). Membrane potential −40 mV. Lower trace shows another cell; continuous record showing effect of applying 2 mM-Cd and recovery 2 min after wash-out of Cd. Membrane potential −40 mV.

usually developed a large outward current at −40 mV and rapidly deteriorated. This suggests that when using solutions with low buffering capacity, the endogenous buffering capacity of the cell may have been able to set the ionized intracellular Ca concentration to that required by the cell and only gross attempts to alter it had any effect. This effect has been confirmed in perfused snail neurones by direct measurement of internal Ca (Byerly & Moody, 1984). More consistent results were achieved by increasing the EGTA concentration from 0·77 to 10 mM with no added Ca in the perfusing system. Using this protocol it was possible to consistently modulate s.t.o.c. activity. Fig. 7*B* shows records from one cell perfused first with solution containing 0·77 mm-EGTA and then with solution containing 10 mM-EGTA. After 3 min perfusion with the higher EGTA concentration activity had virtually ceased, as shown in the second record from this cell, and this change occurred through a reduction in frequency of discharge rather than a decrease in amplitude of individual events. This is clearly shown in Fig. 7*C* which shows a record from another cell held at −30 mV subjected to the same intervention. The initial frequency of discharge was higher because of the more depolarized holding potential. Increasing the EGTA concentration reduced the frequency of transients from 1·35 to 0·29 Hz in the last 60 s of this record before the cell was lost. The amplitude of the s.t.o.c.s was not reduced.

Action of caffeine

Caffeine is known to cause contraction by releasing internal Ca stores in smooth muscle. Caffeine was applied in the bathing solution to both arterial and jejunal smooth muscle cells in the presence and absence of Ca in the external solution.

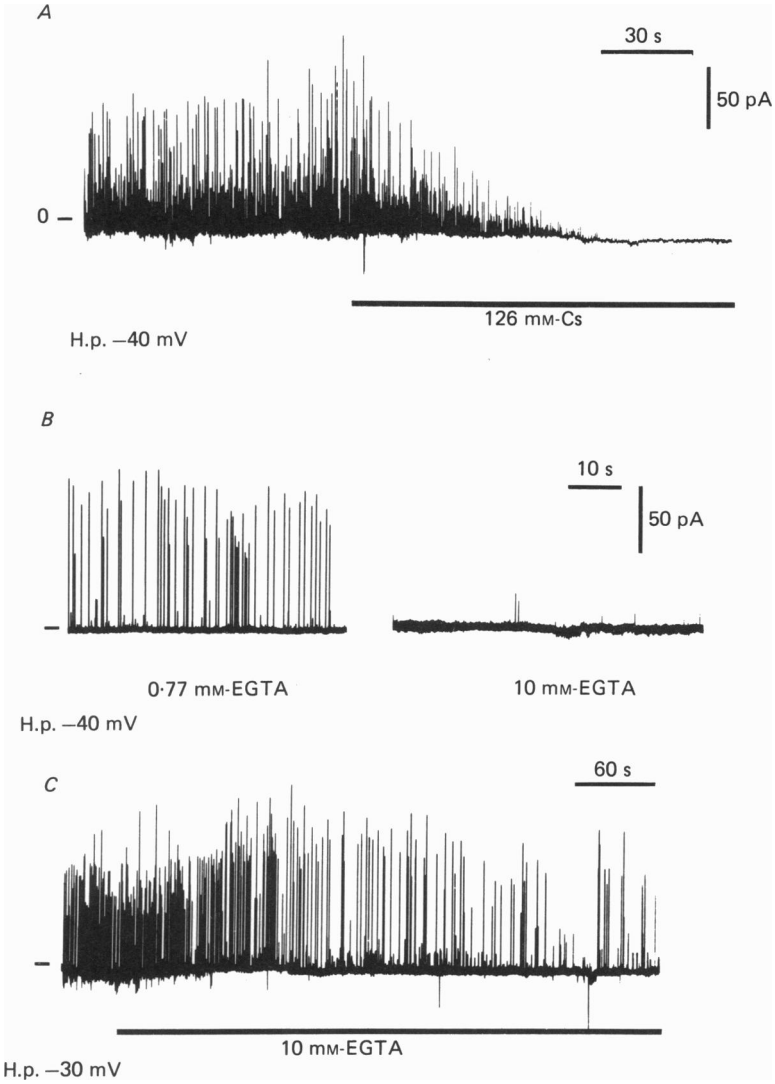

Fig. 7. Intracellular perfusion of jejunal cells. *A*, membrane current recorded first with pipette containing 126 mM-K which was then changed to 126 mM-Cs at start of horizontal bar. Transient currents disappear within 2 min. *B*, membrane currents with pipette containing 0·77 mM-EGTA (left). Same cell 3 min after perfusing pipette with 10 mM-EGTA (right). *C*, another cell showing continuous record of membrane currents in response to increasing pipette solution from 0·77 to 10 mM-EGTA.

Typical responses obtained from two jejunal cells are shown in Fig. 8. Caffeine (1 mM) produced a small increase in the frequency of s.t.o.c.s and also in their amplitude. Wash-out of this low concentration caused a depression of the discharge rate. Application of a higher concentration of caffeine (5 mM) induced a rapid burst of transient currents followed by a prolonged quiet period with no transient currents. Following wash-out of the caffeine, s.t.o.c.s took some minutes to reappear and were of very small amplitude at first, slowly increasing towards control values (Fig. 8*A*).

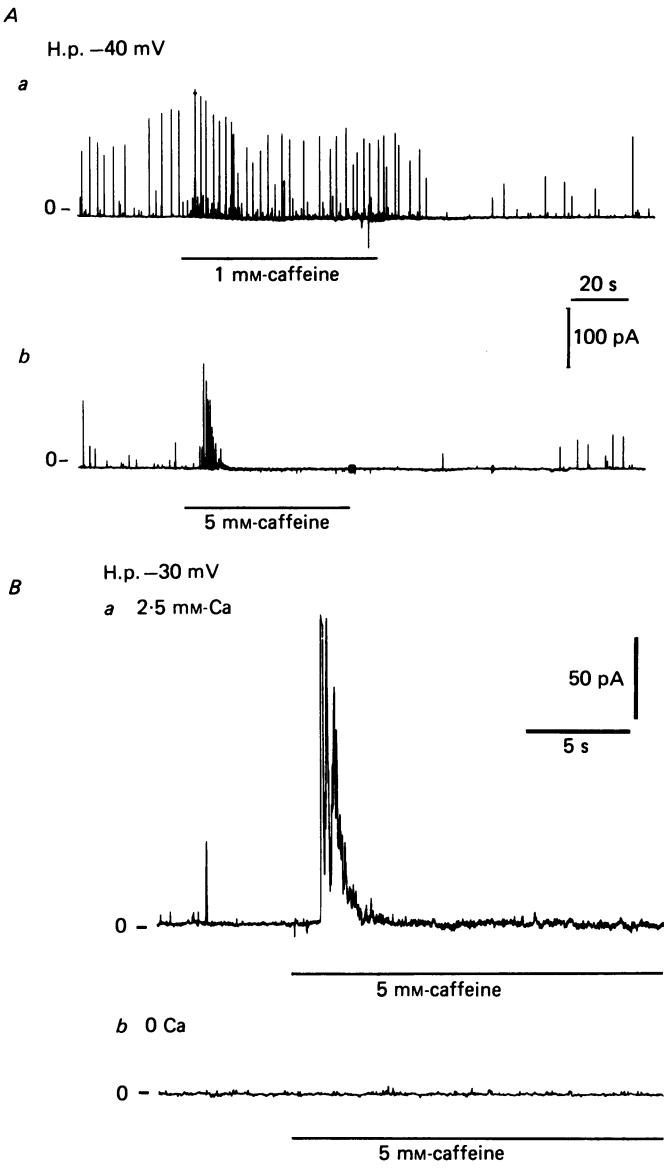

Fig. 8. Responses of jejunal cells to caffeine application. *A*, response of cell to 1 mM-caffeine (*a*) and same cell to 5 mM-caffeine (*b*). Interval of about 3 min between traces. *B*, responses of another cell to 5 mM-caffeine in normal extracellular solution containing 2·5 mM-Ca (*a*); after wash-out of caffeine and recovery of s.t.o.c. activity the cell was bathed in extracellular solution containing no added Ca (*b*) and 5 mM-caffeine reapplied. Cells held at −40 mV.

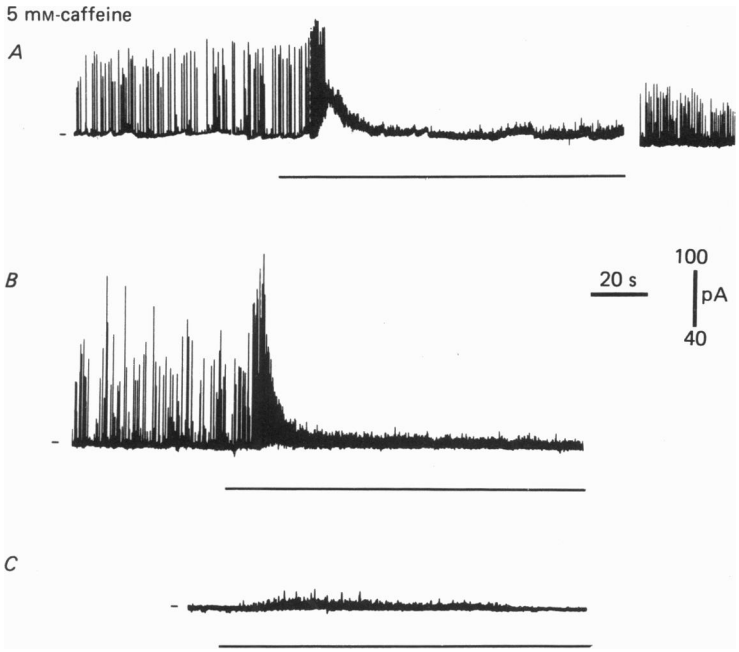

Fig. 9. Membrane current responses to caffeine of arterial cells voltage-clamped at −40 mV. *A*, effect of caffeine (5 mM) when the cell was bathed in normal solution (2·5 mM-Ca) and using a pipette solution containing 0·77 mM-EGTA. *B*, response of another cell to caffeine (5 mM) bathed in a solution with no added Ca. Pipette contained 0·77 mM-EGTA. *C*, response of a third cell to caffeine (5 mM) bathed in a normal solution with 2·5 mM-Ca and using a recording pipette containing 10 mM-EGTA. Calibration 100 pA in *A*, 40 pA in *B* and *C*.

Fig. 8*B* shows current responses from another cell bathed in normal extracellular Ca-containing solution in which 5 mM-caffeine produced a burst of outward currents that overlapped and summated to give a peak outward current of over 200 pA. In the continued presence of the caffeine there was no s.t.o.c. activity but the basal current record was noisier than that in the control condition prior to caffeine application. Following wash-out of the caffeine and recovery of some s.t.o.c. activity the cell was bathed in a solution containing no added Ca for 2 min before repeat of the 5 mM-caffeine. Under these conditions caffeine failed to evoke any outward current in four jejunal cells. Thus the jejunal cells lose both the s.t.o.c. activity and the transients stimulated by caffeine in the absence of normal extracellular Ca. These results are consistent with experiments we have performed measuring contraction of the strips of jejunal smooth muscle tissues where caffeine contractile responses are lost within a minute of removing Ca from the bathing solution.

Similar experiments were performed with ear artery cells. In normal solution containing Ca the response of the arterial cells to caffeine was similar to that of jejunal cells. Caffeine (5 mM) stimulated a burst of transient outward currents and then a prolonged period followed when no large s.t.o.c.s were generated. Following the burst of transient currents there was a period of sustained outward current

lasting about 10 s and then during the period of inhibition of the s.t.o.c.s, the basal current noise was increased (Fig. 9*A*). Wash-out of the caffeine restored the activity of the s.t.o.c.s although their amplitude was reduced. Fig. 9*B* shows a record from another cell bathed in solution with no added Ca and, in contrast to the jejunal cells, the s.t.o.c.s remained and caffeine stimulated a qualitatively similar, although smaller, response to that obtained in normal bathing solution (note the increased current sensitivity of the recording). Thus the caffeine-stimulated transient outward currents and s.t.o.c.s in the arterial cells were little affected by reduction of extracellular Ca to micromolar levels.

Further experiments were performed to see whether increasing the internal buffering of the arterial cells affected the caffeine response. Membrane current recordings were made from arterial cells with pipettes containing 10 mM-EGTA. These cells did not show any s.t.o.c.s, and of four cells to which caffeine was applied two cells showed no response and two cells produced a very small response, consisting of an increase in current noise and a few discernible transient outward currents of less than 10 pA amplitude (Fig. 9*C*). Thus, high intracellular buffering inhibits both s.t.o.c.s and the caffeine-stimulated currents. This might be due to the EGTA preventing Ca uptake into the storage sites or to its binding released Ca before it could reach the membrane ion channels.

Effect of acetylcholine on jejunal cells

Acetylcholine (ACh) is thought to cause contraction of intestinal muscle by stimulating entry of Ca through membrane Ca channels and by releasing intracellular Ca stores (Brading & Sneddon, 1980). Thus if the s.t.o.c.s were due to stored Ca release then ACh would be expected to affect the pattern of discharge.

Fig. 10 shows membrane current records of responses to ionophoretic application of ACh onto two cells at different holding potentials, which exemplify the effect of ACh on the s.t.o.c.s. Application of acetylcholine to a cell at -40 mV is shown in Fig. 10*A*; ACh stimulated a transient outward current and this discharge always preceded the development of the inward current that appears to be a non-selective cation conductance (Benham, Bolton & Lang, 1985). After the initial discharge of a transient outward current, s.t.o.c. discharge was abolished and a period of inhibition occurred which lasted up to a minute. There was then a slow recovery of s.t.o.c. discharge which started at low amplitude and increased back towards control values (Fig. 10*A* and *B*). Thus the effect of ACh on the s.t.o.c.s was similar to that of 5 mM-caffeine. The number of transient outward currents stimulated by ACh varied from one to several. It may be that the cell response in Fig. 10*A* was from a cell with only a single discharging store site like that in Fig. 3.

At more depolarized potentials the inward current was partly obscured by a more sustained outward current that developed with a time course similar to the caffeine-stimulated current (Fig. 10*B*). This record clearly shows the long period of inhibition of the s.t.o.c. activity following ACh ionophoresis. If cells were pre-treated with 5 mM caffeine to abolish the s.t.o.c.s then ACh did not stimulate outward current(s) although a slow inward current was still activated. Thus these results are consistent with ACh releasing a Ca store which is then unable to store Ca for a period of minutes before it regains its ability to refill and discharge at its control level. The

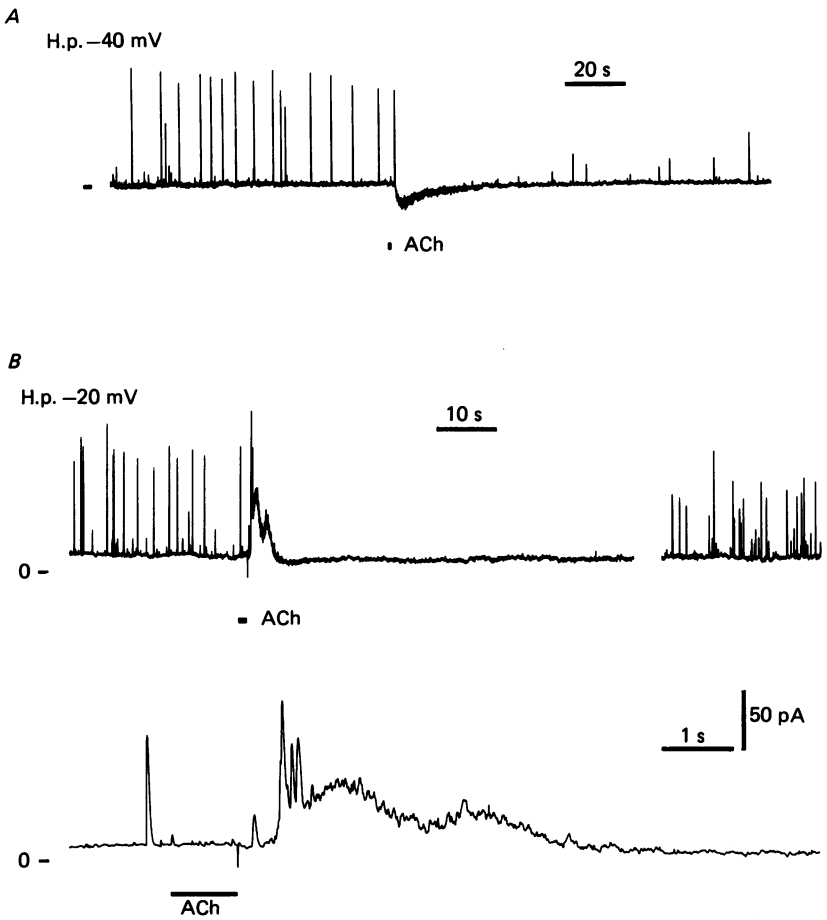

Fig. 10. Responses of jejunal cells to ACh ionophoresis. Ionophoretic pipette was positioned about 20 μm from mid-point of the cell. Ionophoretic current 50 nA, ACh 10^{-1} M, pulse duration 1 s. *A*, cell held at −40 mV membrane potential using a 0·77 mM-EGTA-containing pipette. *B*, another cell held at −20 mV membrane potential using a 0.77 mM-EGTA containing pipette. Lower trace is same response at greater time resolution.

release of Ca stores triggers either a burst of s.t.o.c.s (or a single s.t.o.c.) or sometimes an outward current that may represent summated s.t.o.c.s. An inward current then occurs apparently unrelated to the discharge of Ca from stores.

Depolarizing voltage jumps from −50 mV evoked an inward Ca current followed by an outward K current in jejunal cells (Bolton *et al.* 1985). We examined the effect of different amounts of Ca-buffering capacity in the patch pipette on the form of the outward currents. If the pipette contained 0·07 mM-EGTA, then depolarizing jumps from −50 mV to near zero potential evoked a transient outward current which developed immediately following the inward current (Fig. 11).

The amplitude of this current was 289±27 pA (±S.E. of mean, $n = 10$) at 0 mV and the current decayed rapidly within 100 ms to leave a sustained outward current.

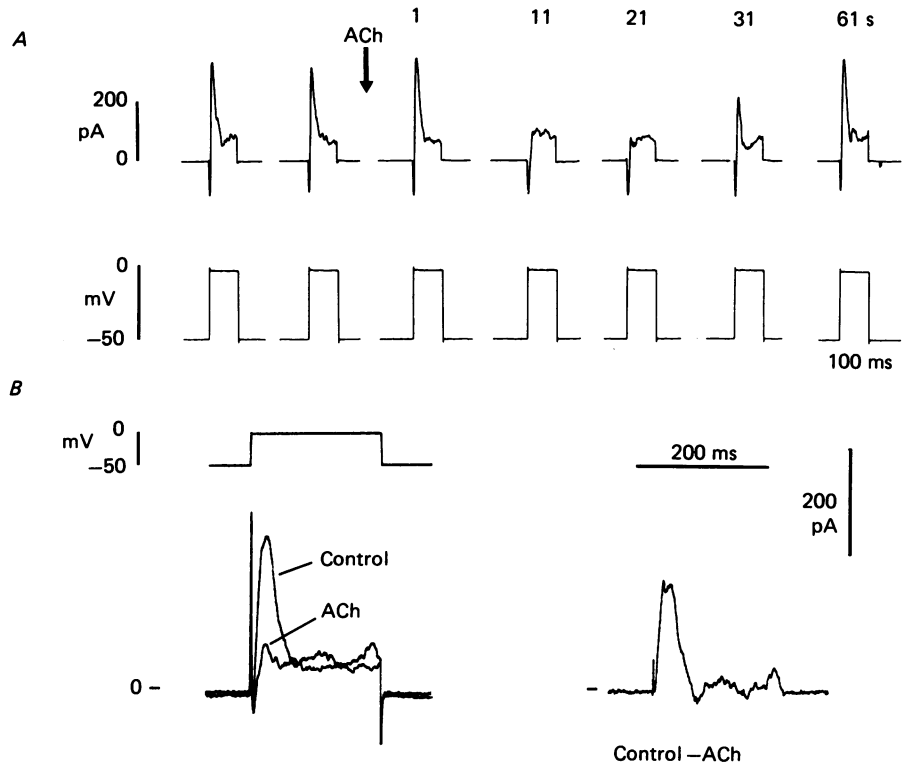

Fig. 11. Effect of ACh on evoked transient outward currents in jejunal cells. Membrane current and potential records from two cells held at −50 mV with pipettes containing 0·077 mM-EGTA. *A*, depolarizing jumps of 100 ms duration evoked every 10s. Numbers above records indicate time after onset of ACh ionophoresis (1 s, 50 nA). The first six jumps are consecutive records, two jumps not shown were made between the sixth and last records shown. Capacitative transient currents have been removed for clarity. *B*, left panel: average of three control current responses (control) to depolarizing steps 24, 14, and 4 s before 1 s ACh ionophoresis superimposed on average of two current responses evoked 5 and 15 s after ACh ionophoresis (ACh); right panel: difference current, the averaged control current minus the current following ACh.

If the pipette contained 10 mM-EGTA then this transient outward current was absent ($n = 7$).

Application of ACh by ionophoresis suppressed this transient outward current. Fig. 11*A* shows membrane current responses of a cell to which voltage jumps of 100 ms were applied every 10 s. ACh was applied ionophoretically for 1 s preceding the third voltage jump. The jump immediately following the ACh application evoked a current response similar to the control response. However, 11 s after the ACh pulse the transient outward current component of the evoked response was completely suppressed. The transient current showed some recovery at 31 s and complete recovery after 61 s. Note that throughout the response the inward current was unaffected. In Fig. 11*B*, current responses to similar voltage jumps before and after ACh ionophoresis have been superimposed and the difference between these two current traces (right panel) shows the current suppressed by ACh. This current

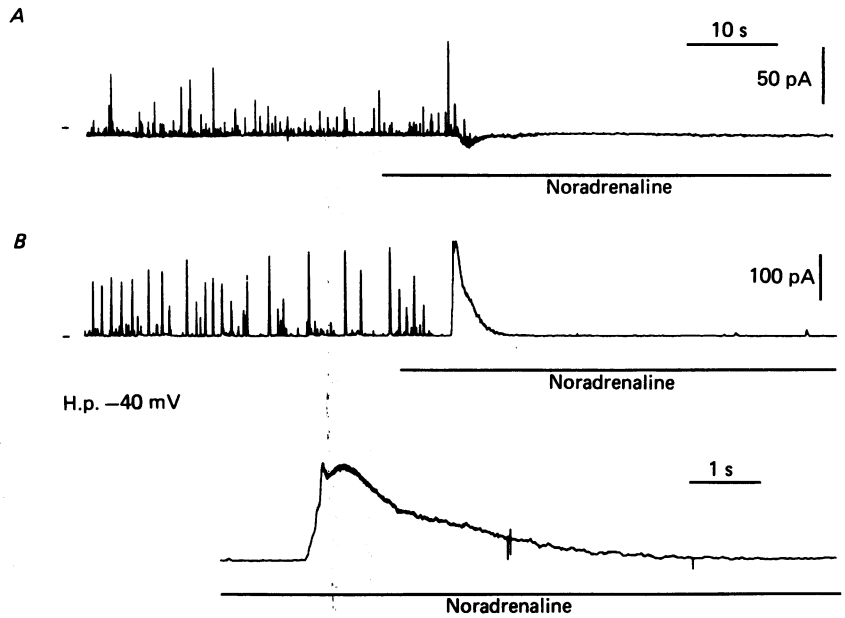

Fig. 12. Responses of ear artery cells to bath application of noradrenaline (10^{-5} M). Cells held at −40 mV using 0·1 mM-EGTA-containing pipettes. *A*, stimulation of transient outward currents and small inward current by noradrenaline. *B*, another cell responding to noradrenaline with a more sustained outward current. Lower record shows same response on expanded time scale.

clearly has similarities to the form of the s.t.o.c.s (Figs. 1*A* and 5*A*). The time course of the inhibition by ACh is similar to the inhibition of the s.t.o.c.s (Fig. 10*A*). Thus it seems possible that these evoked currents are due to release of Ca stores stimulated by depolarization and Ca entry. Application of 10 mM-caffeine also abolished evoked transient current, as would be expected if they resulted from Ca store release. However, caffeine also reduced the amplitude of the inward Ca current, providing an alternative explanation for the reduction of the outward current by caffeine.

Effect of noradrenaline on arterial cells

In vascular smooth muscle noradrenaline is thought to release Ca stores through activation of α-receptors. Thus noradrenaline might be expected to have a stimulant action on transient outward currents in the single arterial cells. Typical cell membrane current responses to bath application of noradrenaline (10^{-5} M) are shown in Fig. 12. Cells either showed a small stimulation of transient outward currents (Fig. 12*A*) or a more sustained outward current which lasted for several seconds (Fig. 12*B*). Following the outward currents, a small, slower, inward current of about 20 pA amplitude was sometimes evoked at this relatively depolarized potential of −40 mV (Fig. 12*A*). Similar responses were evoked by 10^{-6} M-noradrenaline. If arterial cells were held at more negative membrane potentials of −50 to −70 mV which spans the likely resting potentials of these cells, then stimulation of transient outward currents was not always seen. Instead, large inward currents of 200–400 pA

amplitude were evoked. At −50 mV where both types of response were sometimes seen in the same cell, the outward currents appeared before the inward current. A detailed description of the inward currents is beyond the scope of this paper.

DISCUSSION

These results suggest that smooth muscle cells contain stores of intracellular Ca that under certain conditions may be cyclically filled and released. This interpretation can be justified on the following grounds. The evidence that the s.t.o.c.s are K currents was clear: the measurement of reversal potential in elevated external K concentration indicated the currents were highly K selective and the currents were blocked by known K-channel blockers. Numerous Ca-activated K channels of large conductance have been identified and characterized in these cells (Bolton *et al.* 1985; Benham *et al.* 1986) and these channels are likely to be responsible for s.t.o.c.s. Another type of K channel has been identified in these cells using patch techniques but its insensitivity to Ca makes it unlikely that it is involved with these currents (Benham & Bolton, 1983). The effects of modifying internal Ca buffering strongly suggested that the currents were Ca-activated.

It was suggested that the s.t.o.c.s were stimulated by release of Ca from internal stores. In the absence of any neuronal input in these single cells, the most likely origin of s.t.o.c.s is on the internal surface of the membrane and one obvious possible cause would be a sudden rise in Ca concentration at the inner membrane surface caused by a sudden discharge of the Ca contained in a store close to the membrane. Strong internal Ca buffering would be expected to prevent a rise in perimembrane Ca concentration and thus abolish s.t.o.c.s. That the source of the Ca was intracellular stores was suggested by the action of caffeine, which is thought to release intracellular Ca stores (Endo, 1977), and by the actions of ACh and noradrenaline, which are also thought to release stores of Ca in visceral and vascular smooth muscle cells respectively (Brading & Sneddon, 1980; Casteels & Droogmans, 1981). Finally, the sensitivity of the s.t.o.c.s to external Ca removal was similar to the sensitivity of the stores in the two tissues as measured by contractile responses (Deth & Casteels, 1977).

Although most cells showed complex patterns of s.t.o.c. activity, the clear relationship between latency and amplitude of s.t.o.c.s in a few cells (Fig. 3) supports the idea that in some cells the s.t.o.c.s were triggered by the discharge of a single store of Ca that then had to be refilled at a fixed rate. The relationship between latency and amplitude of the s.t.o.c.s was obscured in most cells, presumably because they contained two or more stores discharging independently.

The marked difference in the response of s.t.o.c.s in the two cell types to removal of extracellular Ca suggested that there was a difference in the arrangement of the stores. Results of studies using whole tissues could have been explained by differing rates of loss of extracellular Ca from the extracellular matrix but this possibility would seem to be excluded by using single cells. Our results support the idea that stored Ca in arterial cells under appropriate conditions may be released within the cell and then preferentially sequestered to the stores rather than lost from the cell, this would allow repetitive contractions in Ca-free solution in response to caffeine or noradrenaline (see also Bond, Kitazawa, Somlyo & Somlyo, 1984).

In jejunal cells s.t.o.c.s were rapidly lost following a lowering of extracellular Ca, suggesting that a steady Ca influx across the cell membrane was important for refilling the stores for repetitive discharges. However, concentrations of Cd that blocked the voltage-dependent Ca current in these cells were ineffective in abolishing the s.t.o.c.s. This could be explained if there were Cd-insensitive mechanisms of Ca entry operating, either Ca leaking into the cell through channels or entering by a Ca exchange process. Alternatively, Cd may block efflux as well as influx of Ca across the membrane, allowing released stores to be recycled rather than extruded; however, Ca removal in the presence of Cd still abolished the transients, arguing against this second explanation.

About half the jejunal cells studied generated populations of large amplitude s.t.o.c.s, with mean amplitudes of 117 and 142 pA depending on the pipette EGTA concentration (Table 1). When clamped at −40 mV and in a 6–126 mM-K gradient, the unit current amplitude for a Ca-activated K channel would be 1·5 pA (Benham *et al.* 1986). Thus, assuming that the s.t.o.c.s represent the opening of this type of channel, about 75–100 channels would be open at the peak of such a transient current. This number of channels could be opened either by a small rise in intracellular Ca throughout the cell or by a greater rise over a small area of the cell membrane. A rough calculation reveals that this could be a very small area. In isolated patch recordings we found that up to twenty Ca-activated K channels could be active in each patch (Benham *et al.* 1986). If we assume a mean value of ten channels/patch and a patch area of 5 μm^2 (Sakmann & Neher, 1983) then the channel density is two/μm^2. If we further assume that the rise in Ca over the localized membrane area is to 10^{-6} M, then the probability of any channel being open at this potential will be about 0·5 (Benham *et al.* 1986). With these figures the local area of elevated Ca need only be about 100 μm^2 to generate a s.t.o.c. of average amplitude. This represents only about 3% of the membrane area of a typical cell (Bolton *et al.* 1985). This may explain why these s.t.o.c.s were not obviously associated with contractions of the whole cell. It would be interesting to observe if there were localized contractions of the cell associated with localized rises in internal Ca as has been observed in single cardiac cells (Berlin, Cannell, Goldman, Lederer, Marban & Wier, 1986).

These transient currents lead to strong hyperpolarizations in single cells, but membrane potentials recorded in whole smooth muscle tissues do not show such hyperpolarizing events even in depolarised tissues. Thus the question arises as to whether these events have a role in the whole tissue. In whole tissues the cells are electrically coupled so that individual s.t.o.c.s will generate membrane potential perturbations that decay very quickly with distance from the cell of origin as they spread out through the tissue. It might be expected that an effect would only be seen when there was a more synchronized discharge such as when caffeine was applied. Caffeine has been shown to induce hyperpolarization at low concentrations (Osa, 1973). The only smooth muscle tissue in which records of rapid hyperpolarizing events have been reported was tracheal smooth muscle which had been depolarized by ACh (Ahmed, Foster, Small & Weston, 1984). Following ACh application this tissue depolarized and the membrane potential records became noisy, with large hyperpolarizing transients punctuating the record. These could be due to s.t.o.c.s occurring close to the recording electrode.

The response of the jejunal cells to ACh application and of arterial cells to noradrenaline were both consistent with these agonists releasing Ca stores within the cells. However, in the whole tissue these two agonists both give rise to membrane depolarization. This may be explained by the additional actions of these agonists in the respective tissues. Both agonists also evoke inward currents in the single cells which are particularly pronounced compared to the outward currents at holding potentials close to the resting potentials of these two types of cell. Thus these inward currents may dominate the membrane responses of the whole tissues.

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