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β-Adrenergic agonists and cyclic AMP decrease intracellular resting free-calcium concentration in ileum smooth muscle

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Intracellular free-calcium levels were measured in strips of longitudinal smooth muscle from guinea-pig ileum; fura-2 was used as a calcium monitor. At rest the calcium concentration was about 180 nM, and this rose to 300–400 nM following electrical stimulation and during spontaneous calcium transients (all measurements at 23–25 °C). Isoprenaline suppressed the spontaneous calcium transients, and reduced the resting calcium level to about 130 nM. This fall in resting calcium concentration was seen even in muscle strips which did not have spontaneous activity. Elevation of intracellular cyclic AMP levels, produced by forskolin or dibutyryl cyclic AMP, mimicked the actions of isoprenaline. We conclude that the relaxant effects of β-adrenergic agonists of visceral smooth muscle may be explained partly by a fall in intracellular resting free-calcium level, mediated via an increase in cyclic AMP.

Introduction

Activation of β-adrenoceptors causes relaxation of vascular and visceral smooth muscles (Kuriyama *et al.* 1982) acting through an increase in cyclic AMP (Bueding *et al.* 1966; Andersson & Nilsson 1977; Itoh *et al.* 1982; Itoh *et al.* 1985). It has long been supposed that this relaxation may be due, at least partly, to a reduction in intracellular free calcium (Schild 1967; Bülbring & Tomita 1969; Bülbring *et al.* 1981). However, previous attempts to detect such a fall in intracellular calcium by using aequorin or quin2 as calcium monitors did not give clear results (Morgan & Morgan 1984; Williams & Fay 1985; Williams *et al.* 1985). The newly developed fluorescent indicator fura-2 provides a more sensitive monitor (Poenie *et al.* 1985; Grynkiewicz *et al.* 1985), which we have used in strips of longitudinal smooth muscle from guinea pig ileum. In this tissue, activation by the β-adrenergic agonist isoprenaline, and elevation of intracellular cyclic AMP, caused a fall in the resting free calcium concentration, from about 180 nM to about 130 nM. In addition, spontaneous calcium transients were suppressed by isoprenaline, at concentrations lower than those required to give a clear fall in resting-calcium level.

Methods

Strips of longitudinal smooth muscle (mass *ca.* 1 mg) were obtained from guinea-pig ileum and loaded with fura-2 by incubation for 1–2 h at room temperature in a solution of 5 μM fura-2 AM (Molecular Probes, Inc.) in Krebs solution (composition in mM: NaCl, 122; KCl, 4.7; $MgCl_2$, 1.2; $CaCl_2$, 2.5; glucose, 11.5; $NaHCO_3$, 15.5; KH_2PO_4, 1.2). After loading, the strips were attached with thread to a force transducer and positioned in a glass cell for fluorescence measurements. Recordings were made at room temperature (23–25 °C) while continuously superfusing with Krebs solution bubbled by 95 % O_2, 5 % CO_2. Fluorescence excitation was made at wavelengths of 350 or 373 nm, using light from a 100 W tungsten–halogen lamp operated from a well-stabilized power supply focused through interference filters (Oriel Corporation; half bandwidth 10 nm). Fura-2 fluorescence emission was monitored at 510 nm, via an interference filter (10 nm half bandwidth) and a photomultiplier (EMI 9524B). Background light readings (due to stray light and fluorescence of chamber components) were recorded after removing each muscle, and all values given here were obtained after subtraction of this background. Control muscle strips, not loaded with fura-2, showed only slight fluorescence (less than 10 % of that of loaded strips) and gave no detectable optical signals on stimulation. Drugs were applied in the superfusate, and the muscle strip was stimulated electrically through Ag–AgCl wires above and below the muscle.

Fluorescence signals were calibrated in terms of absolute free-calcium concentration by introducing solutions of fura-2, set to different *p*Ca levels by using a K_2H_2EGTA–$K_2CaEGTA$ buffer system (Grynkiewicz *et al.* 1985), into the empty recording cell. The ratio of fluorescence excitation by 350 or 375 nm light was used to calibrate the resting free-calcium level in the muscle. Changes from this level were estimated from changes in fluorescence intensity (ΔI) with 375 nm excitation. All drugs were tested for intrinsic fluorescence with 373 nm excitation. Except for dibutyryl cyclic AMP (see later) none gave detectable signals at the concentrations used.

Results

Fura-2 calcium signals

Fura-2 shows a small increase in fluorescence emission on binding calcium when excited at 350 nm, and a larger decrease when excited at 373 nm (Poenie *et al.* 1985; Grynkiewicz *et al.* 1985). As expected from this, longitudinal muscle strips from guinea-pig ileum, loaded with fura-2, showed large fluorescence decreases with 373 nm light and smaller increases with 350 nm light when stimulated electrically (see, for example, figure 4*a*) or by exposure to high potassium or acetylcholine concentrations (not shown). Calcium transients also occurred spontaneously in many muscle strips, and were accompanied by increases in tension (figures 1*a* and 2*b*). The mean ratio of fura-2 fluorescence at 350/373 nm was 0.63 ± 0.02 (s.e.m., 14 muscle strips) measured during resting intervals between spontaneous calcium transients. This corresponds to a resting free-calcium level of about 180 nM, in good agreement with previous estimates in other smooth-muscle tissues (DeFeo & Morgan 1985; Morgan & Morgan 1984; Williams & Fay 1985; Williams *et al.* 1985; Pritchard & Ashley 1986).

Isoprenaline and cyclic AMP reduce the calcium level

Bath application of the β-adrenergic agonist isoprenaline rapidly suppressed the spontaneous calcium transients and contractions. In addition, the resting free calcium level declined, as indicated by an increase in fura-2 fluorescence with 373 nm excitation (figure 1*a*, *c*) and a decrease with 350 nm excitation (figure 1*b*).

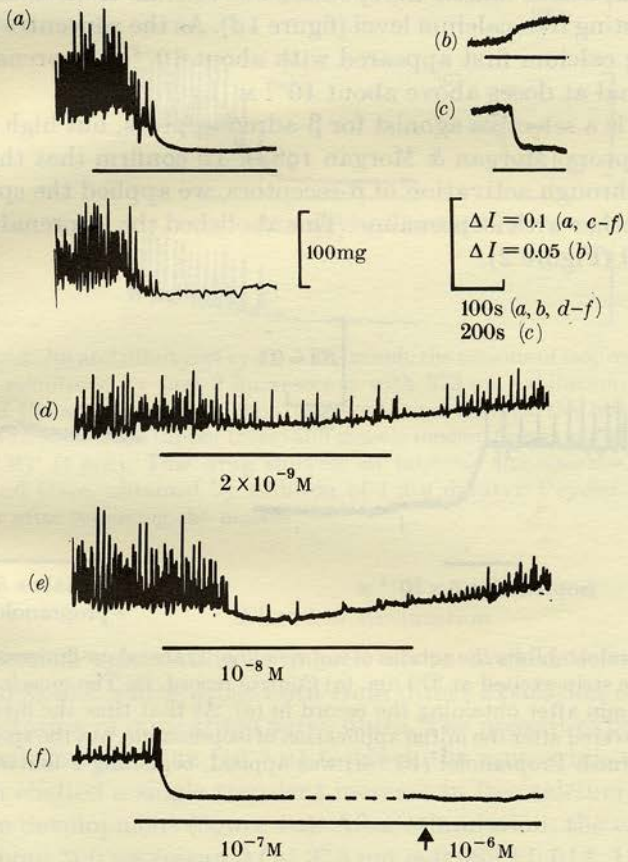

FIGURE 1. Isoprenaline lowers the resting free calcium concentration and inhibits spontaneous activity in strips of longitudinal muscle from guinea pig ileum. All records of fura-2 fluorescence, except for those in (*b*), were monitored with an excitation wavelength of 373 nm. In this, and other figures, a decrease in fluorescence is shown as an upward deflection, as this corresponds to an increase in calcium with 373 nm excitation. Isoprenaline was applied in the superfusion solution when indicated by the bars. Calibration of the fluorescence records is in terms of the fractional change in intensity (ΔI) from the resting level. (*a*) Blocking of spontaneous contractions and lowering of intracellular free-calcium level by isoprenaline (5×10^{-7} M). Upper trace, fura-2 fluorescence with 373 nm excitation; lower trace, muscle tension. Small downward deflections in the tension record are artefactual. (*b*) With 350 nm excitation, isoprenaline (5×10^{-7} M) gives an increase in fura-2 fluorescence, consistent with a lowering of free calcium. (*c*) Lowering of resting free calcium in a muscle strip which showed no spontaneous contractions. (*d*)–(*f*) Dose-dependence of action of isoprenaline, applied at the concentrations indicated. In (*f*) the concentration was raised from 10^{-7} M to 10^{-6} M at the arrow. Records are from one muscle strip, which was washed for 10–20 min between traces.

The mean fluorescence increase at 373 nm produced by maximal doses (more than 10^{-7} M) of isoprenaline was $\Delta I = 0.043 \pm 0.006$ (s.e.m., 8 muscle strips), corresponding to lessening of free calcium by about 50 nM. One explanation for the fall in calcium might be that the spontaneous transients were inhibited, and that these normally acted to elevate the resting level. However, this is not the case, because a similar fall in calcium level was seen also in muscle strips that had no spontaneous activity (figure 1*c*). Furthermore, the predominant effect of isoprenaline at low doses (*ca.* 10^{-9} M) was to inhibit the spontaneous calcium transients, without much effect on the resting free-calcium level (figure 1*d*). As the concentration was raised, a fall in resting calcium first appeared with about 10^{-8} M isoprenaline (figure 1*e*) and was maximal at doses above about 10^{-7} M (figure 1*f*).

Isoprenaline is a selective agonist for β-adrenoceptors, but high doses may also activate α-receptors (Morgan & Morgan 1984). To confirm that the fall in resting calcium arose through activation of β-receptors, we applied the specific β-blocker propanolol together with isoprenaline. This abolished the isoprenaline-induced fall in calcium level (Figure 2).

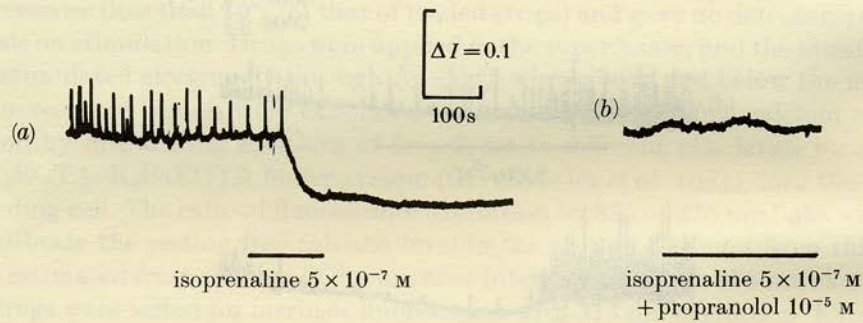

FIGURE 2. Propranolol inhibits the actions of isoprenaline. Traces show fluorescence from a fura-2 loaded muscle strip excited at 373 nm. (*a*) Control record. (*b*) The muscle strip was washed for about 30 min after obtaining the record in (*a*). At that time the intracellular calcium level had recovered after the initial application of isoprenaline, but the spontaneous activity had not returned. Propranolol (10^{-5} M) was applied, beginning a few minutes before the trace shown.

To observe the effects of raised intracellular cyclic AMP on the calcium level we used a membrane-permeable analogue, dibutyryl cyclic AMP and forskolin, a stimulator of adenylate cyclase (Seamon *et al.* 1981). Forskolin (5×10^{-6} M) mimicked the actions of isoprenaline in suppressing the spontaneous calcium transients and lowering the resting free calcium level (figure 3*a*). The mean fluorescence increase, ΔI, at 373 nm was 0.052 ± 0.02 (s.e.m., 4 muscle strips), similar to that seen with high concentrations of isoprenaline. Dibutyryl cyclic AMP acted similarly (figure 3*b*), but interpretation of the fura-2 signal was complicated by the intrinsic fluorescence of the drug. However, the fluorescence increase in the absence of the muscle (figure 3*c*) was smaller than with the muscle (figure 3*b*); this result suggested that dibutyryl cyclic AMP also lowered the calcium level.

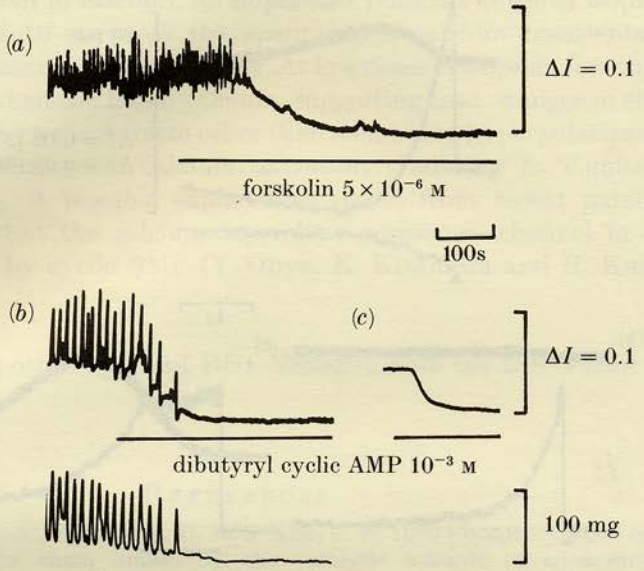

FIGURE 3. Forskolin and dibutyryl cyclic AMP mimic the actions of isoprenaline. (*a*) Intracellular calcium monitored by fura-2 fluorescence with 373 nm excitation. Forskolin (5×10^{-6} M) inhibited the spontaneous calcium transients and lowered the resting free calcium level. (*b*) Fura-2 fluorescence (upper trace) and muscle tension (lower trace) in reponse to dibutyryl cyclic AMP (1 mM). This drug showed an intrinsic fluorescence, as illustrated in the right-hand trace, obtained by addition of 1 mM dibutyryl cyclic AMP to the recording chamber after removing the muscle.

Electrical stimuation

Field electrical stimulation of muscle strips evoked a complex sequence of calcium transients, which arose both from direct excitation of the muscle fibres, and via excitation of muscarinic nerve fibres. The present experiments were made in the presence of atropine (10^{-6} M) to block the muscarinic component, so that stimulation elicited a single transient increase in free calcium, accompanied by a slower force development (figure 4*a*). After stimulation, the calcium level rose to a peak of about 300 nM (mean ΔI at 373 nm = 0.089 ± 0.012; 13 preparations) and then declined to fall transiently below the initial baseline. After treatment with isoprenaline the change in free calcium evoked by stimulation was reduced slightly (figure 4*b*) but the reduction in absolute level at the peak was greater, because of the decrease in resting calcium level. Together with the reduction in the calcium transient, the tension evoked by a single pulse was also reduced. However, tetanic stimulation still evoked large contractions, which were not much smaller than control responses.

Interestingly, the 'undershoot' of the calcium transient was abolished by isoprenaline (figure 4*b*). The mechanism of this effect is not yet clear, but it seems not to be due simply to the lowering of the resting calcium by isoprenaline, since the undershoot was still abolished at low doses (less than 10^{-8} M) which gave little decrease in resting calcium.

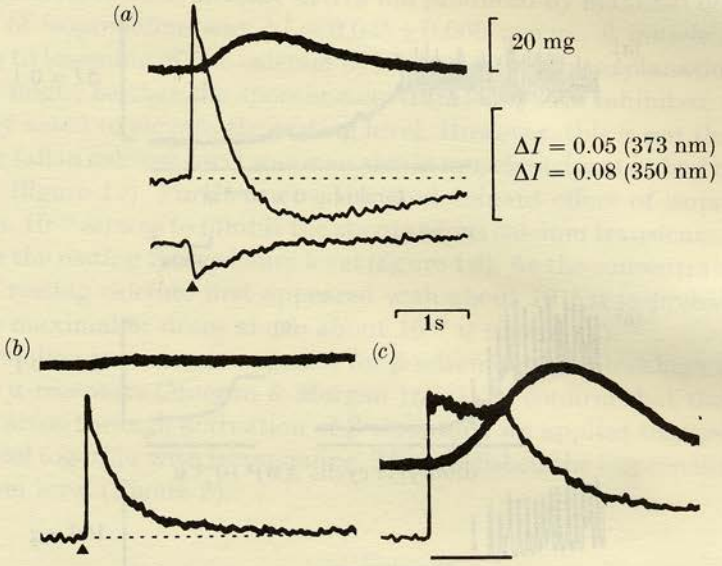

FIGURE 4. Calcium transients and associated contractions evoked by electrical field stimulation. Atropine (10^{-6} M) was added to all solutions to block muscle excitation via muscarinic nerves. (*a*) Control responses to a single pulse (100 V, 10 ms) applied to a fura-2-loaded muscle strip in normal Krebs solution. Upper trace shows muscle tension, middle trance fluorescence with 373 nm excitation, and lower trace fluorescence with 350 nm excitation. Dotted lines in this frame and in (*b*) indicate resting fluorescence levels. (*b*) Record after application of isoprenaline (5×10^{-7} M) to the muscle strip. In this frame, and in (*c*) the upper traces are muscle tension and the lower traces fluorescence with 373 nm excitation. Stimulus pulse was the same as in (*a*). (*c*) Response in the same muscle strip to tetanic stimulation (10 Hz for 1s; each pulse 100 V and 10 ms).

DISCUSSION

The mechanisms underlying the relaxation of smooth muscle by β-adrenergic agonists and intracellular cyclic AMP are still controversial. Two distinct processes have been proposed: (i) an inhibition of myosin phosphorylation through phosphorylation of myosin light chain kinase (Aldstein *et al.* 1978; Mrwa *et al.* 1979; Silver *et al.* 1981; Ruegg *et al.* 1981); and, (ii) activation of calcium-ATPases (calcium pumps) in the sarcoplasmic reticulum and surface membranes (Bahlla *et al.* 1978; Itoh *et al.* 1982; Suematsu *et al.* 1984). The former directly inhibits the contractile proteins; the latter reduces contractions by reducing the free cytoplasmic calcium concentration. Activation of calcium pumps is thought to be the most important effect physiologically (Itoh *et al.* 1982; Miller *et al.* 1983; Sparrow *et al.* 1983); our results give support for this mechanism by showing that isoprenaline and cyclic AMP cause a fall in resting free-calcium levels. However, we do not know yet whether increased calcium extrusion and sequestration are the only factors responsible for the fall in calcium. A reduction in influx of extracellular calcium would give the same result (Meisheri & Van Breemen 1982) and we have found that the organic calcium-channel blockers verapamil and diltiazem, or removal of extracellular calcium, also produce a fall in intracellular free calcium (I.P. & Y.I., unpublished data).

In addition to the fall in calcium, an important relaxant effect of isoprenaline and cyclic AMP was to suppress the spontaneous calcium transients which probably arise from electrical spike activity. At low doses of isoprenaline this effect was more prominent than the fall in calcium, suggesting that changes in electrical excitability occurred by a mechanism other than membrane hyperpolarization due to stimulation of electrogenic calcium extrusion (Bulbring & Tomita 1969; Bulbring *et al.* 1981). A possible explanation comes from recent patch-clamp recordings showing that the calcium-dependent potassium channel in smooth muscle is modulated by cyclic AMP (Y. Ohya, K. Kitamura and H. Kuriyama, unpublished data).

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