*Br. J. Pharmac.* (1984), **83**, 677–686

# Effects of isoprenaline on the contraction–relaxation cycle in the cat trachea

Yushi Ito & Takeo Itoh

Department of Pharmacology, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

**1** Effects of isoprenaline (Isop) on the contractile properties of the smooth muscle cells of cat trachea were investigated using intact and chemically skinned muscle preparations and an isometric tension recording method.

**2** In the intact muscle preparations, Isop $3\times10^{-10}$ or $3\times10^{-9}$ M significantly suppressed the amplitude of tonic contractions evoked by acetylcholine (ACh) $10^{-7}$ M or $10^{-5}$ M, respectively.

**3** Following treatment of the tissue with $Ca^{2+}$-free 2 mM EGTA-containing solution after depletion of stored $Ca^{2+}$ with caffeine, 2.5 mM $Ca^{2+}$ was applied for 5 min (procedure 1), and subsequently 10 mM caffeine was applied in $Ca^{2+}$-free 2 mM EGTA containing solution. The object was to estimate the amount of stored $Ca^{2+}$ during procedure 1 from the amplitude of the caffeine (10 mM)-induced contraction (procedure 2).

**4** Isop, applied during procedure 1, did not affect the amplitude of the caffeine-induced contraction; however, when applied during procedure 2, this agent ($10^{-8}$M) significantly suppressed the amplitude of the caffeine-induced contraction to about 90% of the control value.

**5** ACh ($10^{-5}$M), applied during procedure 1, evoked phasic and tonic contractions. Isop ($10^{-8}$M), applied simultaneously with ACh ($10^{-5}$M), suppressed the amplitude of the ACh-induced contraction yet increased the amplitude of contraction evoked by the subsequent application of caffeine 10 mM (procedure 2).

**6** Effects of conditioning application of ACh ($10^{-7}$ or $10^{-5}$M) on the caffeine-induced contraction were observed in the presence or absence of Isop during procedure 2. When ACh $10^{-5}$M was used, subsequent application of caffeine 10 mM evoked no mechanical response, in control conditions. However, after the pretreatment of the tissue with Isop during procedure 2, the amplitude of the ACh ($10^{-5}$M)-induced contraction was not affected, yet the subsequent application of caffeine (10 mM) evoked minute but discrete contractions, indicating that Isop did enhance the sequestration of free $Ca^{2+}$ into the storage sites.

**7** In the saponin-treated skinned muscles, the minimum concentration of $Ca^{2+}$ required to produce contraction was $1\times10^{-7}$M, and the maximum contraction was obtained with $1\times10^{-5}$M $Ca^{2+}$. Isop ($10^{-6}$M) had no effect on the relationship between free-$Ca^{2+}$ and the amplitude of the contraction. However, simultaneous application of high concentrations of cyclic AMP ($10^{-4}$M) and cyclic AMP-dependent protein kinase (50 $\mu g\,ml^{-1}$) significantly suppressed contractions evoked by $3\times10^{-7}$ or $10^{-5}$M $Ca^{2+}$.

**8** These results indicate that Isop suppresses the contraction evoked by various agonists in the cat trachea, mainly through sequestration of $Ca^{2+}$ into the intracellular storage sites, rather than by direct or indirect (through cyclic AMP) actions on the contractile proteins.

## Introduction

The tracheobronchial smooth muscle is innervated by the adrenergic inhibitory nervous system, and *in vivo* experiments revealed that stimulation of sympathetic nerves or injection of sympathomimetic agents such as isoprenaline (Isop) dilates airways in the presence of bronchoconstriction (Mills *et al.*, 1969; Cabezas *et al.*, 1971).

Low concentrations of noradrenaline (NA) or Isop *in vitro* raised the mechanical threshold of the dog tracheal smooth muscle to evoke contraction, and suppressed the amplitude of phasic contractions evoked by depolarization of the membrane. These agents had no effect on the electrical membrane properties of the smooth muscle cells. The action

potential evoked by an outward current pulse in the presence of tetraethylammonium (TEA) was not affected by Isop, thus indicating that this agent does not affect the voltage-dependent inward $Ca^{2+}$ currents in the presence of TEA, but does reduce the amplitude of contraction (Ito & Tajima, 1982).

Since contraction of smooth muscle cells is largely dependent on the free $Ca^{2+}$ concentration in the myoplasm, as is the case with skeletal or cardiac muscle (Endo, 1977; Ebashi, 1980; Kuriyama *et al.*, 1982), reduction in the amplitude of the contraction induced by the application of NA or Isop might be induced through reduction in the cytoplasmic free calcium concentration and/or suppression of the $Ca^{2+}$ sensitivity of the contractile proteins (see for example Kuriyama *et al.*, 1982).

The calcium sensitivity of contractile proteins has been studied, using chemically skinned smooth muscles (Filo *et al.*, 1965; Endo *et al.*, 1977; Saida & Nonomura, 1978; Iino, 1981; Itoh *et al.*, 1981). To estimate the amount of $Ca^{2+}$ within intracellular sites, assesments were made of the contractions evoked by caffeine or acetylcholine in $Ca^{2+}$-free EGTA containing solution (Endo, 1977; Itoh *et al.*, 1981; 1982a,b; Ito & Itoh, 1984).

In an attempt to elucidate the cellular and subcellular mechanisms involved in the action of Isop on the airway smooth muscle cells, the effects of Isop on the cat trachea were observed using intact and chemically skinned muscle fibres. This particular preparation was used because the excitation-contraction coupling have been studied in detail (Ito & Itoh, 1984).

## Methods

Adult mongrel cats of either sex weighing 2–3 kg were anaesthetized with pentobarbitone (30 mg $kg^{-1}$ i.v.) and exsanguinated. Segments of cervical trachea were excised, and a dorsal strip of transversely running smooth muscle fibre were separated from the cartilage. The mucosa and adventitial tissue were carefully removed. For tension recordings from intact and chemically skinned muscle cells, the muscle strips were cut to a width of 0.05–0.1 mm and a length of about 0.3 mm, under a binocular microscope. Mechanical response of the muscle strips were measured isometrically using a strain gauge (U-gauge, Shinko Co.). The linearity of the transducer to the load was previously checked. The tissue was superfused in an organ bath (0.9 ml capacity) filled with modified Krebs solution (hereafter referred to as Krebs solution) with the following ionic concentration (mM): $Na^+$ 137.4, $K^+$ 5.9, $Mg^{2+}$ 1.2, $Ca^{2+}$ 2.5, $Cl^-$ 134.0, $H_2PO_4^-$ 1.2, $HCO_3^-$ 15.5 and glucose 11.5. The solution was aerated with 97% $O_2$ and 3% $CO_2$ and the pH was adjusted to 7.3–7.4. The $Ca^{2+}$-free 2 mM EGTA-containing solution was prepared by replacing 2.5 mM $CaCl_2$ with equimolar $MgCl_2$ and adding 2 mM EGTA.

To estimate the amount of stored $Ca^{2+}$, the following procedures were used. The muscle tissue was pretreated with $Ca^{2+}$-free 2 mM EGTA-containing solution for over 10 min, during which time 10 mM caffeine was applied to deplete the stored Ca. The tissues were then loaded with 2.5 mM-$Ca^{2+}$ for 5 min (procedure 1) and caffeine (10 mM) was applied after incubation with $Ca^{2+}$-free 2 mM EGTA-containing solution for 2–5 min (procedure 2).

The experimental procedures for the chemically skinned muscles were the same as described previously (Itoh *et al.*, 1981; 1982). Briefly, to prepare the skinned muscles, saponin (50 $\mu g\,ml^{-1}$) was superfused for 20 min. The relaxing solution for the skinned muscles had the following composition (mM): KCl130, Tris maleate 20, $MgCl_2$ 5, ATP5 (10 mM Na as $Na_2$ ATP) and EGTA 4 at pH 6.8. Various $Ca^{2+}$ concentrations were prepared by adding appropriate amounts of $CaCl_2$ to EGTA. The apparent binding constant of EGTA for $Ca^{2+}$ was considered to be $10^6 M^{-1}$ at pH 6.8 (Itoh *et al.*, 1981).

The following compounds were used; caffeine (Wako), ethyleneglycolbis (β-aminoethylether) – N,N'-tetraacetic acid (EGTA; Dozin), isoprenaline HCl (Tokyo Kasei), acetylcholine Cl (Sigma), adenosine 3',5'cyclic monophosphate (cyclic AMP;

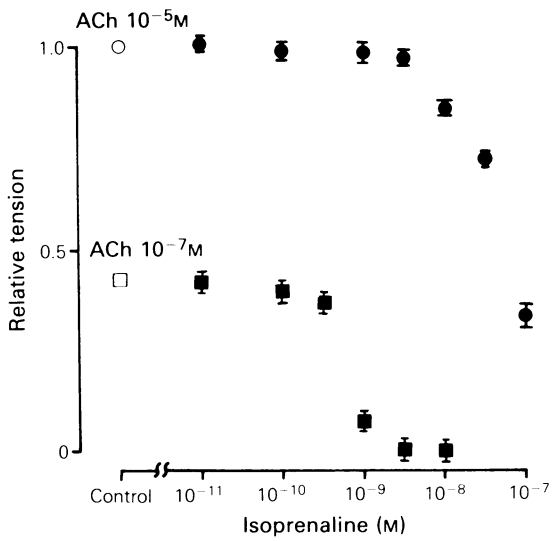


**Figure 1** Effects of isoprenaline ($10^{-11}$–$10^{-7}$M) on the amplitude of tonic contraction evoked by $10^{-7}$M or $10^{-5}$M acetylcholine (ACh). The amplitude of tonic contraction evoked by $10^{-5}$M ACh was defined as a relative amplitude of 1.0. Each point indicates the mean value of five to six experiments and vertical bars indicate 2 × s.d.

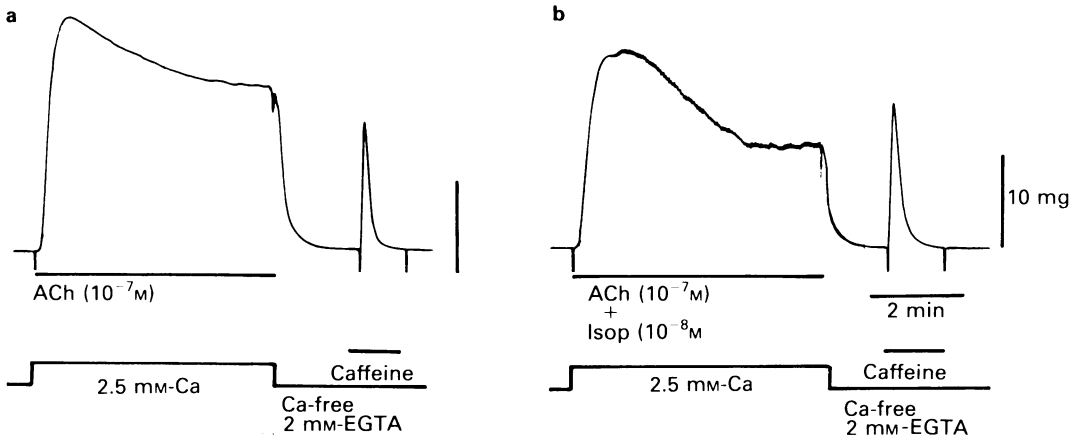


**Figure 2** Effects of isoprenaline (Isop, $10^{-8}$M) on the acetylcholine (ACh, $10^{-7}$M)-induced and on the caffeine-induced contractions. The tissue was pretreated with $Ca^{2+}$-free 2 mM EGTA containing solution after depletion of the stored $Ca^{2+}$ by caffeine (10 mM). The tissue was then loaded with 2.5 mM $Ca^{2+}$ for 5 min in the presence of ACh ($10^{-7}$M), and after rinsing the tissue with $Ca^{2+}$-free EGTA containing solution for 2 min, 10mM caffeine was applied to estimate the amount of stored Ca (a). Isop ($10^{-8}$M) was applied during the loading period of the tissue with 2.5 mM $Ca^{2+}$ (b).

Sigma) and cyclic AMP-dependent protein kinase (protein kinase (A-kinase); Sigma P5511).

Results (amplitude of contractions) were expressed as mean ± s.d., and analysed for significance using Student's *t* test.

## Results

### *Effects of isoprenaline on tonic component of acetylcholine-induced contractions*

Isop, in doses of over $3 \times 10^{-10}$M or $3 \times 10^{-9}$M, produced a dose-dependent reduction of the $10^{-7}$ or $10^{-5}$M ACh-induced tonic contraction, respectively. Isop ($10^{-7}$M) abolished the tonic contraction evoked by ACh $10^{-7}$M, and reduced the tonic contraction evoked by ACh $10^{-5}$M to 33 ± 5.0% of control (Figure 1).

### *Effects of isoprenaline on the acetylcholine or caffeine-induced contraction*

Figure 2 shows the effects of Isop on the ACh-induced, and subsequent caffeine-induced contractions (see methods). With application of ACh ($10^{-7}$M) and 2.5 mM $Ca^{2+}$ during procedure 1, a contraction was evoked. Isop ($10^{-8}$M) reduced the amplitude of contraction evoked by ACh, and in turn led to an increase in the amplitude of caffeine-induced contraction to 1.15 ± 0.08 (± s.d., $n = 4$) times the control value ($P < 0.05$), in $Ca^{2+}$-free solution (procedure 2). This indicates that Isop reduced the amplitude of the ACh-induced contraction and in turn the mechanisms involved in this process enhanced the amount of $Ca^{2+}$ stored in the caffeine-sensitive intracellular store (i.e. the sarcoplasmic reticulum).

To investigate the effects of Isop on the intracellular $Ca^{2+}$ store, the effects of Isop on the caffeine-induced contraction in $Ca^{2+}$-free solution were studied. As shown in Figure 3a, loading the tissue with 2.5 mM $Ca^{2+}$ (procedure 1), following pretreatment of the tissue with $Ca^{2+}$-free EGTA containing solution and caffeine (10 mM), did not evoke a mechanical response. However, application of caffeine (10 mM) in $Ca^{2+}$-free solution (procedure 2) evoked a contraction, thereby indicating that loading of 2.5 mM $Ca^{2+}$ during procedure 1 replenished the $Ca^{2+}$ in storage sites, without activating the contractile proteins. The amplitude of the caffeine-induced contraction was constant, in the same preparations,

**Table 1** Effects of isoprenaline ($10^{-9}$–$10^{-7}$M), added during procedures 1 or 2, on the caffeine-induced contraction

| | *Isoprenaline* | | |
|---|---|---|---|
| | $10^{-9}$M | $10^{-8}$M | $10^{-7}$M |
| 1 | 95.4 ± 5.5 | 98.5 ± 3.2 | 90.7 ± 9.4 |
| 2 | 99.0 ± 2.6 | 87.9 ± 5.3 | 86.2 ± 6.0 |

Mean values of six to eight experiments are presented with s.d. Isoprenaline ($10^{-8}$M) applied during procedure 2 produced significant effects on the amplitude of caffeine-induced contraction ($P < 0.01$).

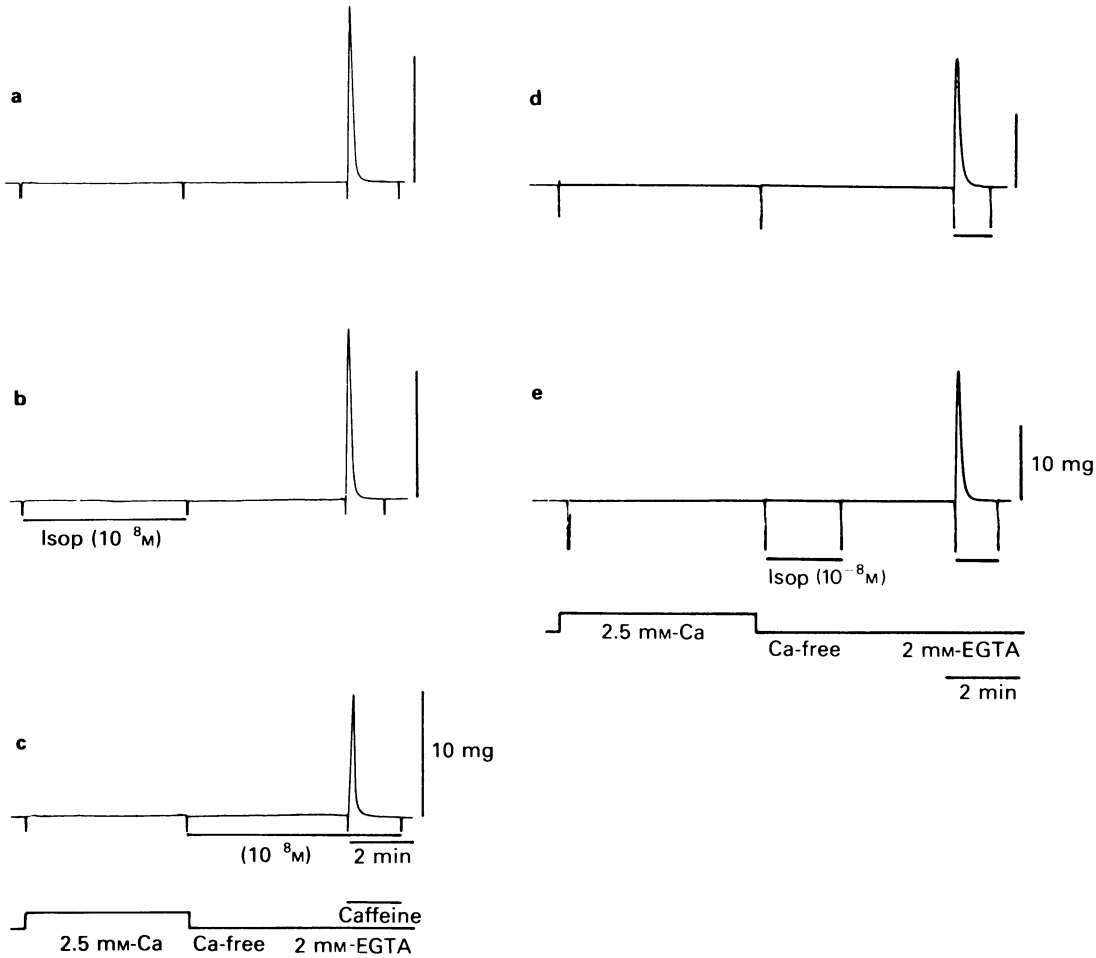


**Figure 3** Effects of isoprenaline (Isop $10^{-8}$M on the caffeine (10 mM)-induced contractions. The tissue was pretreated with $Ca^{2+}$-free 2 mM EGTA containing solution after depletion of the stored Ca by caffeine (10 mM), then the tissue was loaded with 2.5 mM $Ca^{2+}$ for 5 min (procedure 1) and after rinsing the tissue with $Ca^{2+}$-free 2 mM EGTA containing solution 10 mM caffeine was applied (procedure 2). During procedure 1 (b) or 2 (c), Isop ($10^{-8}$M) was applied in a series of experiments (a–c). (d and e). Effects of Isop applied during the first half of procedure 2 on the caffeine-induced contraction. (d and e) are records from the same preparation.

in a given procedure. Thus, estimation of the amount stored $Ca^{2+}$ from the amplitude of caffeine-induced contraction, before and after treatment of the tissue with Isop at various stages was feasible.

Table 1 summarizes the results obtained with $10^{-9}$, $10^{-8}$, $10^{-7}$M Isop applied during procedure 1 or 2. When Isop was applied during procedure 1, the amplitude of caffeine-induced contractions was not affected (Figure 3b). However, when applied during procedure 2, Isop ($10^{-8}$ and $10^{-7}$M) significantly reduced the amplitude of the caffeine-induced contractions ($P<0.01$) (Figure 3c).

As shown in Figure 3d and e, application of Isop during the first half of procedure 2 did not affect the amplitude of the caffeine-induced contraction ($1.02 \pm 0.01$ times the control value, $n=5$). Therefore, the effects of conditioning application of ACh ($10^{-7}$M or $10^{-5}$M) or caffeine (1 mM) on the caffeine (10 mM)-induced contraction during the first half of procedure 2 were observed in the presence or absence of Isop ($10^{-8}$M).

Figure 4A shows the effects of conditioning application of $10^{-7}$M ACh on the caffeine (10 mM)-induced contraction during procedure 2. Applica-

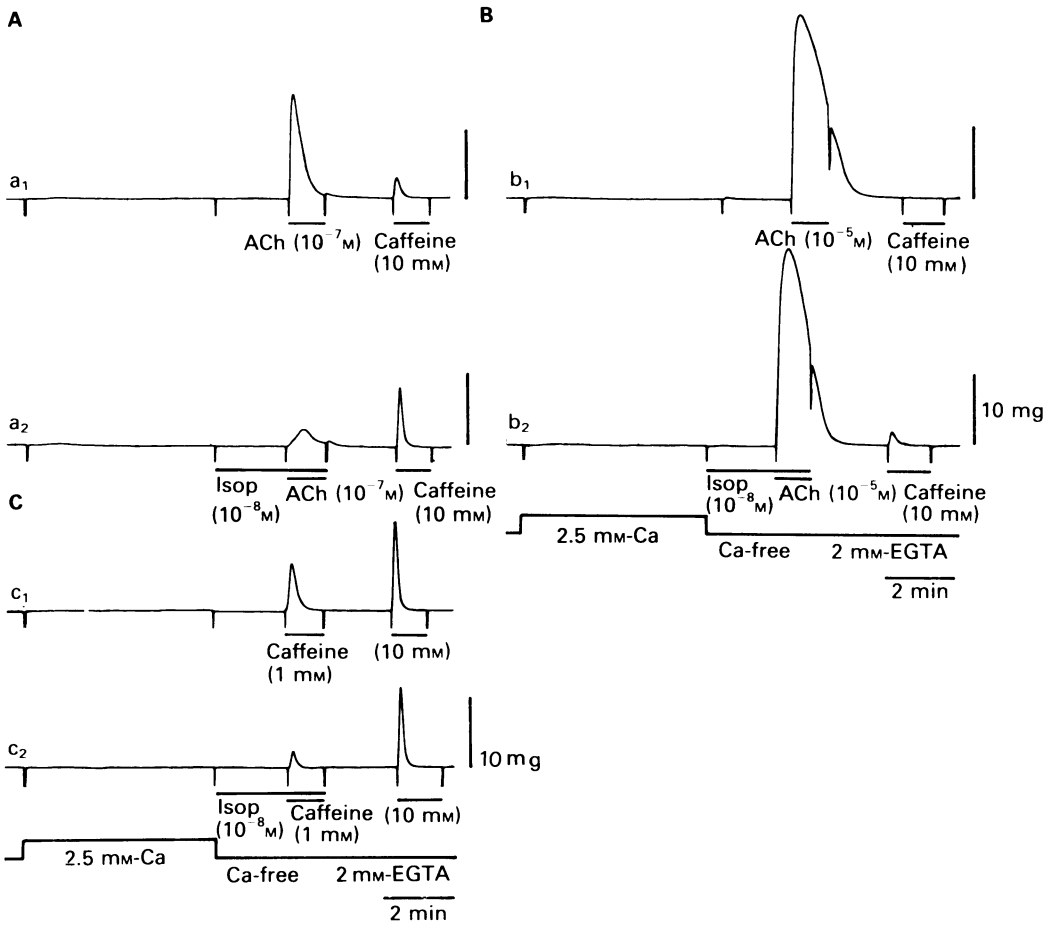


**Figure 4** Effects of isoprenaline (Isop, $10^{-8}$M) on conditioning application of $10^{-7}$M acetylcholine (ACh, A), $10^{-5}$M ACh (B) or 1 mM caffeine (C) during procedure 2, on the caffeine (10 mM)-induced contractions. In (A) application of Isop ($10^{-8}$M) during the first half of procedure 2 reduced ACh ($10^{-7}$M)-induced contraction, but increased the caffeine (10 mM)-induced contraction ($a_1$ vs $a_2$). In (B) after conditioning application of ACh ($10^{-5}$M), caffeine (10 mM) evoked no mechanical response ($b_1$), whereas after application of Isop ($10^{-8}$M) during the first half of procedure 2, caffeine evoked minute but discrete contractions ($b_2$). In C, Isop ($10^{-8}$M) reduced the 1 mM caffeine-induced contraction, but did not affect the amplitude of 10 mM caffeine-induced contraction.

tions of ACh ($10^{-7}$M) evoked a phasic contraction and the subsequently applied caffeine (10 mM) produced only a small contraction. The mean amplitude of the caffeine-induced contraction after the conditioning application of ACh ($10^{-7}$M), was reduced to 25% of the control value (data not shown in Figure 4). Treatment with Isop ($10^{-8}$M) during the first half of procedure 2 reduced the amplitude of ACh ($10^{-7}$M)-induced contraction to one-fifth of the control value, whereas the amplitude of contraction evoked by subsequently applied caffeine (10 mM) was markedly increased.

Next, $10^{-5}$M ACh and 10 mM caffeine were applied successively during procedure 2 (Figure 4 B). Application of caffeine (10 mM) following pretreatment with ACh ($10^{-5}$M) evoked no mechanical response (Figure 4 $b_1$), thereby indicating that ACh $10^{-5}$M depleted the stored $Ca^{2+}$ in the cell (Figure 4$b_1$). The amplitude of the ACh ($10^{-5}$M)-induced contraction was not affected by pretreatment of the tissue with Isop ($10^{-8}$M), but the subsequently applied caffeine evoked a small but discrete contraction, as shown in Figure 4$b_2$. This means that Isop does not suppress release of $Ca^{2+}$ from the storage

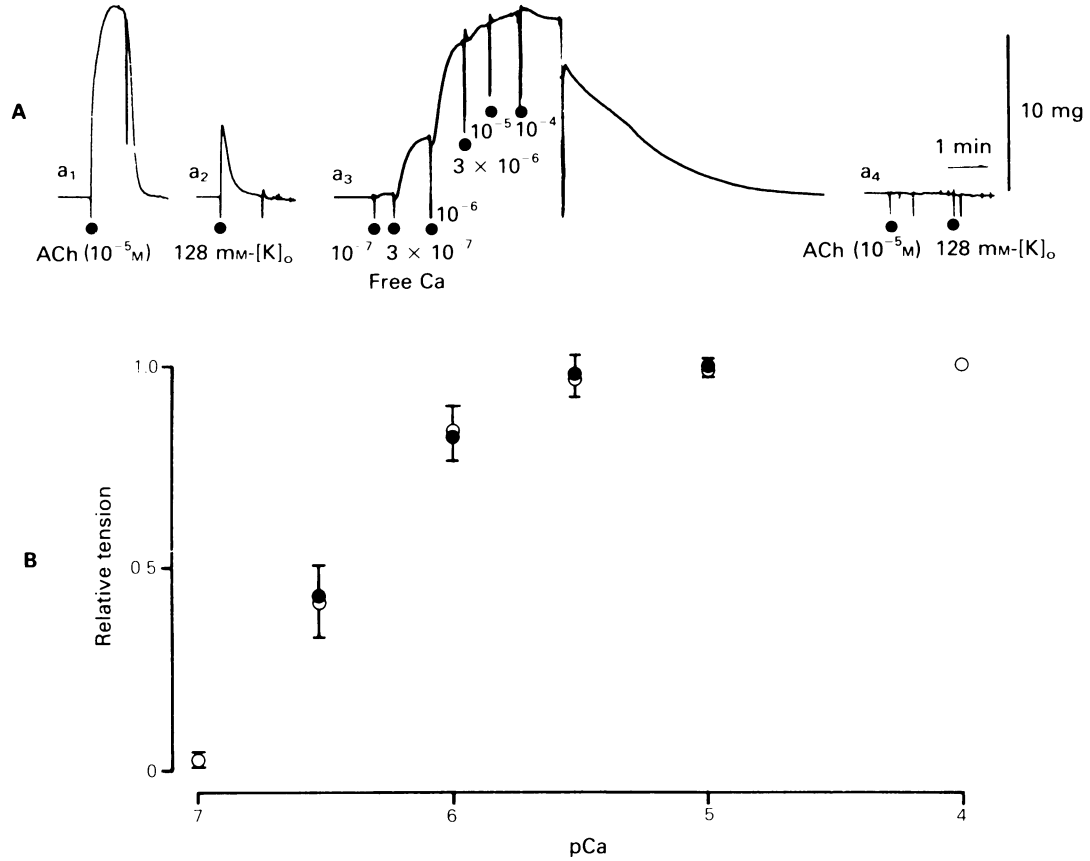


**Figure 5** Effects of isoprenaline (Isop, $10^{-6}$M) on the $Ca^{2+}$-induced contractions of skinned muscle preparations. (A) Before skinning the tissue, ACh ($10^{-5}$M) or 128 mM-$[K]_o$ was applied to the tissue as a control ($a_1$ and $a_2$), and after skinning by saponin, various concentrations of $Ca^{2+}$ were applied cumulatively ($a_3$). After skinning the tissue, ACh ($10^{-5}$M) or 128 mM-$[K]_o$ evoked no contraction ($a_4$). (B) pCa-tension relationship observed in the presence (●) or absence (○) of Isop ($10^{-6}$M). Each point indicates mean value of three to four experiments, and vertical bars indicate 2 × s.d.

sites during ACh ($10^{-5}$M) action, but does enhance the re-uptake process of $Ca^{2+}$ into the store sites.

Figure 4C, shows the conditioning application of caffeine (1 mM) during procedure 2. Caffeine 1mM released some of the stored $Ca^{2+}$ and subsequent application of caffeine (10 mM) released the stored $Ca^{2+}$, thereby evoking relatively large contractions. Treatment of the tissue with Isop ($10^{-8}$M) during procedure 2 reduced the amplitude of contraction evoked by caffeine 1 mM, but did not affect the amplitude of contraction evoked by subsequent application of 10 mM caffeine.

*Effects of isoprenaline on the pCa-tension relationship*

To investigate the effects of Isop on the $Ca^{2+}$-sensitivity of the $Ca^{2+}$-receptive protein (calmodulin or leiotonin C) in the contractile machinery, the effects of this agent on the pCa-tension relationship were observed using chemically skinned muscle cells. Before skinning the muscles, the amplitudes of contractions evoked by ACh ($10^{-5}$M) or 128 mM-$[K]_o$ were recorded (Figure 5A). The tissue was then treated with saponin (50 $\mu g\,ml^{-1}$) containing relaxing solution for about 20 min (see methods). One criterion for the completion of skinning was that the maximum amplitude of the contraction produced by $Ca^{2+}$ was as large as that of the ACh ($10^{-5}$M)-induced contraction of the intact tissue; since in the intact tissue, ACh ($10^{-5}$M), but not excess-$[K]_o$ (128 mM) evoked the maximal contraction. Furthermore, another criterion was that ACh ($10^{-5}$M) or 128 mM $[K]_o$ evoked no mechanical response of the tissue (Figure 5A).

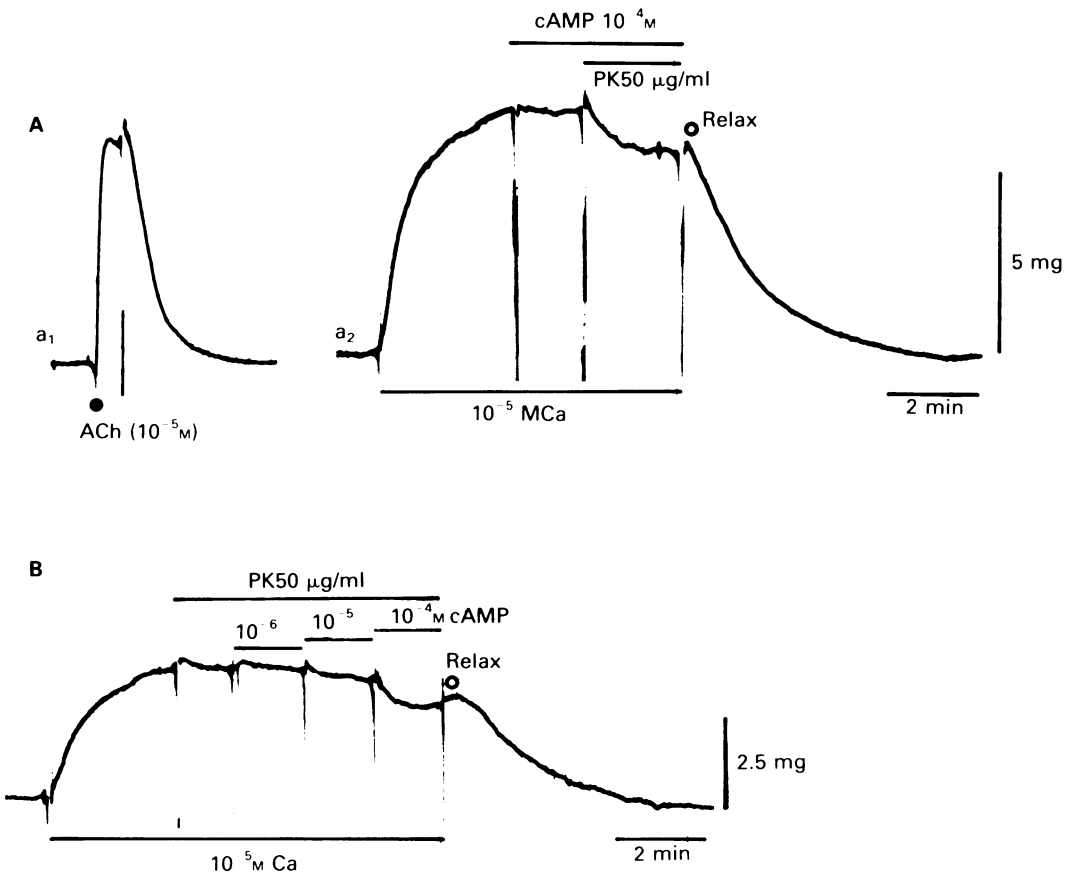


**Figure 6** (A) Effects of cyclic AMP (c AMP, $10^{-4}$M) with or without protein kinase (PK, 50 μg ml$^{-1}$) on $3 \times 10^{-7}$M or $10^{-5}$M $Ca^{2+}$-induced contraction in skinned muscle fibres. (B) Effects of protein kinase (50 μg ml$^{-1}$) in the absence or presence of cyclic AMP (cAMP, $10^{-6}$–$10^{-4}$M).

Cumulative applications of free-$Ca^{2+}$ were made. The minimum pCa required to produce a detectable contraction was above 7, and at 5, the maximum contraction was observed.

Figure 5 B shows the relationship between pCa (free-Ca ion) and the tension produced by skinned muscle cells, in the presence or absence of Isop ($10^{-6}$M). Up to $10^{-6}$M, Isop had no effect on the minimum concentration of $Ca^{2+}$ required to evoke the contraction and no influence on the relationship between pCa and amplitude of the $Ca^{2+}$-induced contraction.

*Effects of cyclic AMP and cyclic AMP-dependent protein kinase (A-kinase) on the $Ca^{2+}$ contraction*

Activation of β-adrenoceptors in smooth muscle cells results in increases in the concentration of cyclic AMP (see for example Hirata & Kuriyama, 1980 Itoh *et al.*, 1982). Therefore, the effects of cyclic AMP and protein kinase on the contraction evoked by free $Ca^{2+}$ were observed using the skinned muscle fibres.

Cyclic AMP ($10^{-4}$M) alone had no effect on contractions induced by $3 \times 10^{-7}$M or $10^{-5}$M $Ca^{2+}$ (Figure 6 $a_2$). However, when protein kinase (50 μg ml$^{-1}$) was applied, cyclic AMP ($10^{-4}$M) reduced the amplitude of the contractions evoked by $3 \times 10^{-7}$M or $10^{-5}$M $Ca^{2+}$ to $60.5 \pm 10.2$ ($n = 3$) or $80.5 \pm 10.2$ ($n = 3$) % of the control value, respectively. As shown in Figure 6 B, A-kinase (50 μg ml$^{-1}$) in itself did not affect the amplitude of the $Ca^{2+}$-induced contractions in the absence of cyclic AMP, while, in the presence of cyclic AMP in doses over $10^{-4}$M, A-kinase (50 μg ml$^{-1}$) significantly reduced the amplitude of these contractions.

## Discussion

Activation of β-adrenoceptors produces a relaxation of smooth muscle tissue or a reduction in the amplitude of contractions evoked by various stimulants, and according to current views, relaxation of smooth muscle occurs when there is a significant reduction in the cytoplasmic free $Ca^{2+}$ concentration (see for example Kuriyama *et al.*, 1982) and/or when there is an inhibition of actin-myosin interaction due to phosphorylation of myosin light chain kinase (Adelstein *et al.*, 1978).

Isop may reduce the free $Ca^{2+}$ concentration in the cytoplasma of smooth muscle cells by modification of influx, intracellular sequestration or extrusion of $Ca^{2+}$. The present experiments were carried out in a $Ca^{2+}$-free EGTA containing solution after a short period of $Ca^{2+}$ loading in order to avoid the influx of $Ca^{2+}$ during the drug action. The effects of Isop on the amount of stored $Ca^{2+}$ in the cell were also estimated from the amplitude of caffeine-induced contraction in $Ca^{2+}$-free containing solution, since in the cat trachea, stored $Ca^{2+}$ seems to play a more important role than does the influx of $Ca^{2+}$ in evocation of the contraction produced by endogenous or exogenous acetylcholine (Ito & Itoh, 1984).

Isop significantly inhibited the amplitude of ACh or caffeine-induced contractions, when applied during the treatment of the tissue with $Ca^{2+}$-free EGTA containing solution after loading 2.5 mM $Ca^{2+}$ for a short period. The reduction in the amplitude of the ACh- or caffeine-induced contractions in $Ca^{2+}$-free EGTA containing solution, induced by Isop, may relate to one or any combination of the following mechanisms, i.e.: (1) Isop may suppress the $Ca^{2+}$ release from the intracellular stores; (2) Isop may enhance the extrusion of free-$Ca^{2+}$ into the extracellular space; (3) Isop may stimulate the sequesteration of $Ca^{2+}$ into storage sites, under the assumption that the $Ca^{2+}$ sensitivity of the contractile proteins is not affected by the direct or indirect action (via a second messenger) of Isop.

The present results obtained with repetitive application of $10^{-5}$M ACh and 10 mM caffeine in the presence or absence of $10^{-8}$M Isop indicates that Isop does not inhibit the $Ca^{2+}$-releasing mechanism, but enhances the sequestration of free $Ca^{2+}$ into the intracellular store sites.

Furthermore, when 1 mM and 10 mM caffeine were repetitively applied after loading the tissue with $Ca^{2+}$, and Isop ($10^{-8}$M) was pretreated during the action of 1 mM caffeine (Figure 5 C), Isop suppressed the 1 mM caffeine-induced contraction, but did not increase the amplitude of contraction evoked by subsequently applied 10 mM caffeine. If Isop enhances the sequestration of free $Ca^{2+}$ into the intracellular storage site, an increase in the amplitude of 10 mM caffeine-induced contraction should be observed, under the assumption that the total amount of $Ca^{2+}$ released by repetitive application of 1 and 10 mM caffeine remains constant. Therefore, the enhancement of the extrusion of cytoplasmic free $Ca^{2+}$ into the extracellular space by Isop had to be considered, or it might be that Isop increases $Ca^{2+}$ sequestration into intracellular sites other than the sarcoplasmic reticulum.

These results indicate that in the cat trachea, Isop mainly enhances sequestration of free cytoplasmic $Ca^{2+}$ into the intracellular store sites and/or extrusion into the extracellular space, but does not suppress the $Ca^{2+}$ releasing mechanisms induced by caffeine or ACh.

Similar conclusions were reached using various smooth muscles and different experimental procedures. For example, in the guinea-pig taenia coli, enhanced efflux of $^{45}Ca^{2+}$ (Bülbring & den Hertog 1980) or an increased sequestration of cytoplasmic free $Ca^{2+}$ into the store sites (Casteels & Raeymaekers, 1979; Meisheri & van Breemen, 1982) was demonstrated during the relaxation caused by Isop. Furthermore, in the guinea-pig mesenteric artery, Itoh *et al.* (1982) concluded that activation of β-adrenoceptors by Isop increased the amount of cyclic AMP and led to an increase in $Ca^{2+}$ accumulation into the storage sites. However, in the rabbit aorta, Isop caused a significant inhibition of $Ca^{2+}$ influx stimulated by excess-$[K]_o$ and Na-free solution, and the time courses of $Ca^{2+}$ influx inhibition and relaxation produced by Isop were in parallel (Meisheri & Van Breemen, 1982). The β-adrenoceptor-induced relaxation was attributed primarily to the inhibition of the stimulated $Ca^{2+}$-influx in the dog saphenous vein (Collis & Shepherd, 1979).

Thus, there are multiple mechanisms by which Isop can alter $Ca^{2+}$ distribution to produce relaxation, and tissue specificities should be considered. For example in the rabbit mesenteric artery, application of Isop during loading of 2.5 mM $Ca^{2+}$ significantly increased the amount of stored Ca, in the presence or absence of procaine (Itoh *et al.*, 1983). On the other hand, in the cat trachea, Isop applied with 2.5 mM $Ca^{2+}$ did not increase the amount of stored Ca (Figure 3). These results indicate that caffeine-sensitive $Ca^{2+}$ stores in the cat trachealis muscle are almost saturated with $Ca^{2+}$, under the physiological conditions.

It is generally accepted that Isop activates adenyl cyclase and increases intracellular concentrations of cyclic AMP. This in turn activates A-kinase and the catalytic subunit of this kinase phosphorylates myosin light chain kinase, thereby inhibiting its interaction with calmodulin, a $Ca^{2+}$ binding regulatory protein (see for example Adelstein *et al.*, 1978). In the guinea-pig taenia coli, application of the catalytic subunit of A-kinase shifted the pCa-tension relation-

ship to the right at relatively low concentrations of $Ca^{2+}$ (Sparrow *et al.*, 1981). However, application of cyclic AMP with A-kinase had a minor effect on the pCa-tension relationship in the skinned muscle preparation of the guinea-pig mesenteric artery (Itoh *et al.*, 1982). In the present experiments simultaneous application of cyclic AMP ($10^{-4}$M) and A-kinase ($50\ \mu g\ ml^{-1}$) inhibited the $Ca^{2+}$-induced contraction, indicating that phosphorylation of myosin by these substances may inhibit the actin-myosin interaction, as previously reported (Adelstein *et al.*, 1978; Itoh *et al.*, 1982). However, high concentrations of cyclic AMP and A-kinase were used in the present experiments, and the amount of catalytic subunit required to regulate the physiological function in the tracheal tissue is unknown. Therefore, it should be clarified whether or not phosphorylation of myosin light chain kinase, evoked by the action of cyclic AMP, does play a role in the inhibitory action of Isop on muscle contraction, under physiological conditions. It was also reported that the relaxation following β-adrenoceptor activation is more likely to involve $Ca^{2+}$ extrusion from the cell and accumulation of $Ca^{2+}$ in internal storage sites than suppression of the binding of calmodulin with the myosin light chain kinase in the guinea-pig mesenteric artery (Itoh *et al.*, 1982).

Our present results show that Isop mainly enhances the sequestration and/or extrusion of cytoplasmic $Ca^{2+}$, thereby inducing relaxation of the smooth muscle of the cat trachea. Furthermore, from the actions of Isop or procaine (Ito & Itoh, 1984), it seems likely that the caffeine-sensitive $Ca^{2+}$ store in the muscle cell are almost saturated with $Ca^{2+}$, under physiological conditions.

We are grateful to Prof. H. Kuriyama for his continuous encouragement, and to M. Ohara for readings of the manuscript.

## References


ADELSTEIN, R.S., CONTI, M.A., HATHAWAY, D.R. & KLEE, C.B. (1978). Phosphorylation of smooth muscle myosin light cahain kinase by the catalytic subunit of adenosine 3',5'-monophosphate-dependent protein kinase. *J. biol. Chem.*, **253,** 8347–8350.

BÜLBRING, E. & DEN HERTOG, A. (1980). The action of isoprenaline on the smooth muscle of the guinea-pig taenia coli. *J. Physiol.*, **304,** 277–296

CABEZAS, G.A., GREF, P.D. & NADEL, J.A. (1971). Sympathetic versus para-sympathetic nervous regulation of airways in dogs. *J. appl. Physiol.*, **31,** 651–655.

CASTEELS, R. & RAEYMAEKERS, L. (1979). The action of acetylcholine and catecholamine on an intracellular calcium store in the smooth muscle cells of the guinea-pig taenia coli. *J. Physiol.* **294,** 51–68.

COLLIS, M.G. & SHEPHERD, J.T. (1979). Isoproterenol-induced relaxation of venous smooth muscle contracted by agents which mobilize different calcium pools. *J. Pharmac. exp. Ther.*, **209,** 359–365.

EBASHI, S. (1980). Regulation of muscle contraction. *Proc. R. Soc. Ser. b.*, **207,** 259–286.

ENDO, M. (1977). Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.*, **57,** 71–108.

ENDO, M., KITAZAWA, T., YAGI, S., IINO, M. & KAKUTA, Y. (1977). Some properties of chemically skinned smooth muscle fibres. In *Excitation-Contraction Coupling in Smooth Muscle.*, ed. Casteels, R. *et al.*, pp. 199–209. Amsterdam: Elsevier, North-Holland Biochemical Press.

FILO, R.S., BOHR, D.F. & RUEGG, J.C. (1965). Glycerinated skeletal and smooth muscle; calcium and magnesium dependent. *Science, N.Y.*, **147,** 1581–1583.

HIRATA, M. & KURIYAMA, H. (1980). Does activation of cyclic AMP dependent phosphorylation induced by β-adrenergic agent control the tone of vascular muscle. *J. Physiol.*, **307,** 143–161.

IINO, M. (1981). Tension responses of chemically skinned fibre bundles of the guinea-pig taenia coli under varied ionic environments. *J. Physiol.* **320,** 449–467.

ITO, Y. & ITOH, T. (1984). The roles of stored calcium in contractions of cat tracheal smooth muscle produced by electrical stimulation, actylchlorine and high $k^+$ *Br. J. Pharmac.*, **83,** 667–676.

ITO, Y. & TAJIMA, K. (1982). Dual effects of catecholamines on pre- and post-junctional membranes in the dog trachea. *Br. J. Pharmac.*, **75,** 433–440.

ITOH, T., IZUMI, H. & KURIYAMA, H. (1982a). Mechanisms of relaxation induced by activation of β-adrenoceptors in smooth muscle cells of the guinea-pig mesenteric artery. *J. Physiol.*, **326,** 475–493.

ITOH, T., KAJIWARA, M., KITAMURA, K. & KURIYAMA, H. (1982b). Roles of stored calcium on the mechanical response evoked in smooth muscle cells of the porcine coronary artery. *J. Physiol.*, **322,** 107–125.

ITOH, T., KURIYAMA, H. & SUZUKI, H. (1981). Excitation-contraction coupling in smooth muscle cells of the guinea-pig mesenteric artery. *J. Phyisol.*, **321,** 513–535.

ITOH, T., KURIYAMA, H. & SUZUKI, H. (1983). Differences and similarities in the noradrenaline- and caffeine-induced mechanical responses in the rabbit mesenteric artery. *J. Physiol.*, **337,** 609–629.

KURIYAMA, H., ITO, Y., SUZUKI, H., KITAMURA, K. & ITOH, T. (1982). Factors modifying contraction-relaxation cycle in vascular smooth muscles. *Am. J. Physiol.*, **243,** H641–H662.

MEISHERI, K.D. & VAN BREEMEN, C. (1982). Effects of β-adrenergic stimulation on calcium movements in rabbit aortic smooth muscle relationship with cyclic AMP. *J. Physiol.*, **331,** 429–441.

MILLS, J.E., SELLICK, H. & WIDDICOMBE, J.G. (1969). Activity of lung irritant receptors in pulmonary microembolism, anaphylaxis drug-induced bronchoconstrictions. *J. Physiol.*, **203,** 337–357.

SAIDA, K. & NONOMURA, Y. (1978). Characteristics of

$Ca^{2+}$-and $Mg^{2+}$-induced tension developed in chemically skinned smooth muscle fibres. *J. gen. Physiol.*, **72,** 1–14.

SPARROW, M.P., MRWA, U., HOFMANN, F. & RUEGG, J.C. (1981). Calmodulin is essential for smooth muscle contraction. *FEBS Lett.*, **125,** 141–145.

(*Received February 28, 1984*
*Revised June 21, 1984.*)