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Comparison of contractions produced by carbachol, thapsigargin and cyclopiazonic acid in the guinea-pig tracheal muscle

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1 Thapsigargin (TPG, 3 μM) and cyclopiazonic acid (CPA, 10 μM) slowly increased muscle tone in the guinea-pig isolated tracheal muscle. A large sustained contraction was produced when 2.4 mM Ca^{2+} was readmitted after 10 min exposure to Ca^{2+}-free solution following 30 min treatment with TPG or CPA.

2 The sustained contraction after Ca^{2+} readmission was partially inhibited by nifedipine (3 μM) and highly dependent on external Ca^{2+}. The TPG- and CPA-induced sustained contractions were 75% and 67%, respectively, of the sustained contraction produced by carbachol (Cch, 1 μM, EC_{80}) in the presence of nifedipine.

3 The contractions produced by Cch, TPG and CPA were all inhibited by isoprenaline (ISO) and sodium nitroprusside (SNP). In the presence of nifedipine, the IC_{50} of ISO was 11, 17, and 23 nM and that of SNP was 0.5, 1, 0.8 μM for Cch-, TPG-, and CPA-induced contractions, respectively. The contraction produced by 60 mM K^+ was only weakly inhibited by ISO and SNP. As with ISO and SNP, the Cch-, TPG- and CPA-induced contractions were also similarly inhibited by SKF 96365 (100 μM) and cadmium (Cd^{2+}, 100 μM).

4 It was concluded that TPG and CPA increased Ca^{2+} influx probably *via* a mechanism activated by Ca^{2+} depletion of the sarcoplasmic reticulum. The susceptibility of the contraction produced by TPG, CPA and Cch to inhibition by ISO and SNP and also by SKF-96365 and Cd^{2+} suggests that the contractions use common pathways for increasing intracellular Ca^{2+}, and that the contractions produced by K^+ involve a different mechanism.

Keywords: Airway muscle; carbachol; thapsigargin; cyclopiazonic acid; isoprenaline; sodium nitroprusside

Introduction

In most smooth muscles Cch-induced contraction is generally resistant to agents blocking voltage-gated Ca^{2+} channels. This is thought to be due to the fact that the contraction is produced by Ca^{2+} influx through a special receptor-operated pathway (Bolton, 1979; Murray & Kotlikoff, 1991) and/or by Ca^{2+} released from the sarcoplasmic reticulum (SR) (Coburn & Baron, 1990). Recently another Ca^{2+} influx pathway, the capacitative pathway, has been proposed which is activated by depletion of Ca^{2+} from the SR (Putney, 1986; 1990; Missiaen *et al*., 1990). Thapsigargin (TPG) and cyclopiazonic acid (CPA), inhibitors of the Ca^{2+} pump in the SR, are reported to produce a sustained contraction in some smooth muscles (Gibson *et al*., 1994; Amrani *et al*, 1995; De La Fuente *et al*., 1995). This contraction could be due to Ca^{2+} influx through the capacitative pathway. It is possible that the capacitative Ca^{2+} influx pathway may also be partly involved in the Cch-induced contraction, because inositol,1,3,4-trisphosphate (IP_3) produced by Cch-activation of the muscarinic receptors in airway muscle is known to release Ca^{2+} from the SR (Hashimoto *et al*., 1985; Coburn & Baron, 1990). Therefore, it is interesting to compare the properties of TPG- and CPA-induced contractions with Cch-induced contractions. In the present experiments these contractions were studied using the smooth muscle of the guinea-pig trachea. It was found that in the presence of nifedipine, TPG- and CPA-induced contractions were inhibited by isoprenaline (ISO) and sodium nitroprusside (SNP), cyclic AMP- and cyclic GMP-related relaxants respectively, with similar susceptibility to the contractions produced by 1 μM Cch.

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Methods

Guinea-pigs (250–350 g) of either sex were stunned by a blow to the head and killed by exsanguination. The trachea was dissected out, connective tissue was removed from the dorsal surface, and the trachea was opened longitudinally along the ventral surface. Four preparations each containing only one cartilage ring were excised from a central region of the trachea after carefully removing the mucosa with fine forceps under a binocular microscope. The cartilage was cut off leaving about 1.5 mm at each end of the muscle, one side was fixed to the bottom of a chamber (0.2 ml) using a fine pin and the other side was connected to a strain gauge transducer by a fine silk thread. The chamber was perfused with physiological solution, prewarmed at 35°C, at a constant rate of 1 ml min^{-1}. The control solution contained (mM): NaCl 127, $KHCO_3$ 6, $CaCl_2$ 2.4, $MgCl_2$ 1.2, glucose 12, HEPES buffer 10 (pH adjusted to 7.4 at 35°C with NaOH). When Ca^{2+} was removed EGTA (1 mM) was always added, but EGTA was removed 5 min before changing the external Ca^{2+} concentration ($[Ca^+]_o$).

The preparations were stretched by applying tension of about 2 mN and allowed to stabilize at least for 1 h before starting experiments. During the stabilization period, two contractures produced by increasing $[K^+]_o$ to 60 mM, by replacing Na^+ isosmotically, for 10 min were recorded. Indomethacin (1 μM) was added to all solutions to block any contribution of endogenous prostaglandins and all experiments were carried out under a dim light to minimize photolysis of nifedipine.

All drugs used were obtained from Sigma (St Louis, U.S.A.), except for SKF 96365 (Biomol Res. Lab., Plymouth Meeting, PA, U.S.A.). TPG and CPA were dissolved in DMSO to obtain a 5 mM stock solution. Nifedipine was dissolved freshly every week in ethanol to make a 3 mM stock

solution. All numerical data were expressed as means ± s.e.mean, and n is the number of preparations. Significance was tested using Student's t test.

Results

Contractions produced by Cch, TPG and CPA

Cch (1 μM) produced a sustained contraction which was about 80% of the maximum contraction produced by 10 μM Cch. The Cch-induced contraction was inhibited only by 11.4 ± 4.9% (n = 28) by nifedipine (3 μM), but readily blocked by removal of the external Ca^{2+}. The time for 50% relaxation on Ca^{2+} removal was 72 ± 20 s (n = 12). Reapplication of Ca^{2+} (2.4 mM) produced complete recovery to the level before Ca^{2+} removal.

TPG (3 μM) very slowly increased muscle tone, but the rate of increase varied greatly in different preparations. This tension quickly disappeared on Ca^{2+} removal and Ca^{2+} reapplication following 10 min Ca^{2+} removal always produced a tension greater than the level before Ca^{2+} removal (Figure 1a). A large contraction on Ca^{2+} reapplication was also observed even when the muscle tone was not clearly increased by TPG on its own. In four preparations TPG was applied for 90 min until the muscle tone was increased more or less to a steady level and then Ca^{2+} was removed for 10 min. Ca^{2+} reapplication following this treatment produced 3.4 times larger tension than that before Ca^{2+} removal. The tension produced by the Ca^{2+} readmission was also only slightly (12.0 ± 3.3%) inhibited by nifedipine (3 μM) and it was 75.0 ± 6.0% (n = 24) of the contractions produced by 1 μM Cch in the presence of nifedipine. The tension remained high at least 2 h after TPG wash-out. The time for 50% relaxation on Ca^{2+} removal was 52 ± 18 s (n = 12) in the presence of nifedipine (3 μM).

CPA (10 μM) produced contractions similar to TPG (3 μM), but in many preparations (18 out of 24) rhythmic contractions gradually developed which were blocked by nifedipine (3 μM) (Figure 1b). Such rhythmic contraction was never observed with TPG (0.1 – 3 μM). The tension obtained after Ca^{2+} reapplication and in the presence of nifedipine was 67 ± 7% of the contraction produced by Cch (1 mM). In four preparations TPG (3 μM) was added during CPA-induced contraction, but no further increase in tension was observed. The time for 50% relaxation on Ca^{2+} removal was 54 ± 20 s (n = 12). The CPA-induced contractions decreased slowly after wash-out of CPA in contrast to TPG, where they were irreversible.

The tonic contractions produced by TPG and CPA were not affected by atropine (1 μM) which blocked the response to Cch.

In the following experiments TPG- and CPA-induced contractions were studied always after reapplication of Ca^{2+} and in the presence of nifedipine and these were compared with the contractions produced by 1 μM Cch also in the presence of nifedipine. Figure 2 shows $[Ca^{2+}]_o$-tension curves for Cch-, TPG- and CPA-induced contractions. These contractions were all $[Ca^{2+}]_o$-dependent. The curves were in parallel and the ED_{50} of Ca^{2+} for Cch-, TPG- and CPA-induced contractions was 0.43 ± 0.06, 0.66 ± 0.08 and 0.67 ± 0.07 mM (n = 8), respectively. The ED_{50} for Cch-induced contraction was slightly smaller, but there was no difference between ED_{50} for TPG- and CPA-induced contractions. The ED_{50} for TPG- and CPA-induced contraction was slightly smaller than the value (0.85 mM) reported for the rat pulmonary artery (De La Fuente *et al*., 1995). The higher Ca^{2+} sensitivity of Cch-induced contractions is probably related to a larger maximum tension compared with TPG- or CPA-induced contractions, but this problem was not further investigated.

Relaxation produced by isoprenaline and sodium nitroprusside

Contractions produced by 1 μM Cch were concentration-dependently inhibited by isoprenaline (ISO) and sodium nitroprusside (SNP) and neither of the concentration-relaxation curves was significantly affected by nifedipine (3 μM) (Figure 3), the IC_{50} of ISO and SNP being 13 ± 2 nM and

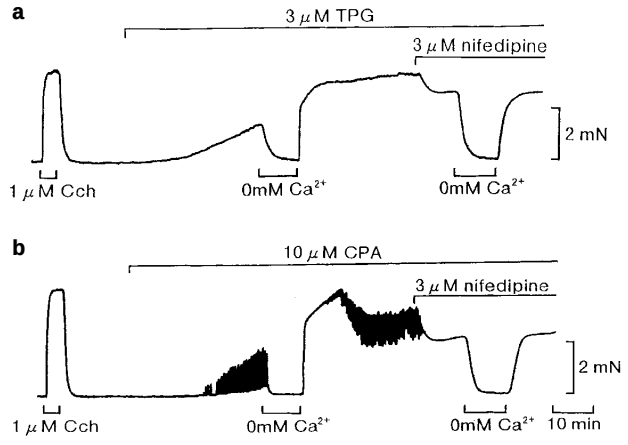

Figure 1 (a) Typical mechanical responses to carbachol (Cch, 1 μM), thapsigargin (TPG, 3 μM). (b) Cyclopiazonic acid (CPA, 10 μM) in the guinea-pig tracheal muscle. In the presence of indomethacin (1 μM) Cch was applied three times at 30 min intervals and 15 min after the third application of Cch TPG or CPA was applied continuously. Ca^{2+} was removed for 10 min after 35 min and 90 min exposure to TPG or CPA. Between Ca^{2+} removals nifedipine (3 μM) was applied as indicated above the recording. Ca^{2+} readmission following the first Ca^{2+} removal produced a large sustained contraction which was partially inhibited by nifedipine. Rhythmic contractions produced by CPA were abolished by nifedipine. For further explanation see text.

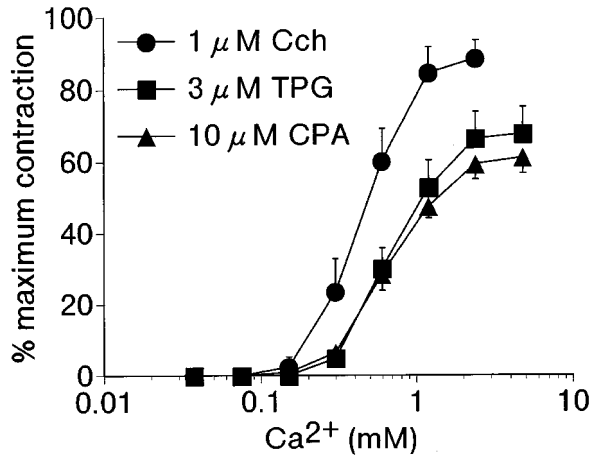

Figure 2 Ca^{2+} concentration-tension curve in the presence of carbachol (Cch), thapsigargin (TPG) and cyclopiazonic acid (CPA). For the TPG and CPA Ca^{2+} was removed for 10 min after 30 min treatment with TPG or CPA, as shown in Figure 1, before Ca^{2+} was cumulatively applied (n = 12). All experiments were carried out in the presence of nifedipine (3 μM). The maximum contraction produced by Cch was taken as 100%. Values are mean ± s.e.

543±38 nM ($n=12$) in the absence and 11±2 nM and 486±38 nM ($n=12$) in the presence of nifedipine, respectively.

Contractions produced by TPG and CPA were similarly susceptible to ISO and SNP as shown in Figures 4 and 5. The IC_{50} of ISO and SNP was 17±4 nM and 1030±143 nM in TPG-induced contractions and 23±2 nM and 783±97 nM for CPA-induced contractions, respectively ($n=12$). No significant difference in these IC_{50} values was found between TPG- and CPA-induced contractions and also between these values and those for Cch-induced contractions.

Contractions produced by 60 mM K^+ were much less sensitive to ISO and SNP (Figure 6). The maximum relaxation produced with 3 μM ISO was 40±4% and with 10 μM SNP was 14%±2% ($n=12$).

Effects of SKF 96365 and cadmium

Since the susceptibility to ISO and SNP was very similar between Cch-induced contractions and TPG- and CPA-induced contractions, we have also compared inhibitory actions of the general Ca^{2+} entry blockers, SKF 96365 and cadmium (Cd^{2+}) in the presence of 3 μM nifedipine. In the mouse anococcygeus non-selective cation currents activated by CPA are inhibited by SKF 96365 and Cd^{2+} (Wayman *et al.*, 1996a) and also CPA- and Cch-induced contractions are shown to be inhibited by SKF 96365 (Gibson *et al.*, 1994). Both SKF 96365 and Cd^{2+} at 10–100 μM slowly relaxed the tracheal preparations precontracted by Cch (1 μM) and TPG (3 μM), as shown in Figures 7 and 8, and also by CPA (10 μM). There was no clear difference in the susceptibility to these blockers, as found for ISO and SNP. The IC_{50} values of SKF 96365 were 31.9±8.3, 23.2±4.5 and 24.1±5.2 μM and those of Cd^{2+} were 52.1±6.4, 42.8±4.7, and 46.5±3.6 μM for Cch-, TPG- and CPA-induced contractions, respectively ($n=4$).

Discussion

In the guinea-pig tracheal muscle, the sustained contractions produced by Cch, and also by TPG and CPA, are highly dependent on the $[Ca^{2+}]_o$, suggesting that Ca^{2+} influx is mainly responsible for the contractions. The Cch-induced contraction is only weakly susceptible to nifedipine as reported previously (see Coburn & Baron, 1990). This indicates that Ca^{2+} influx is

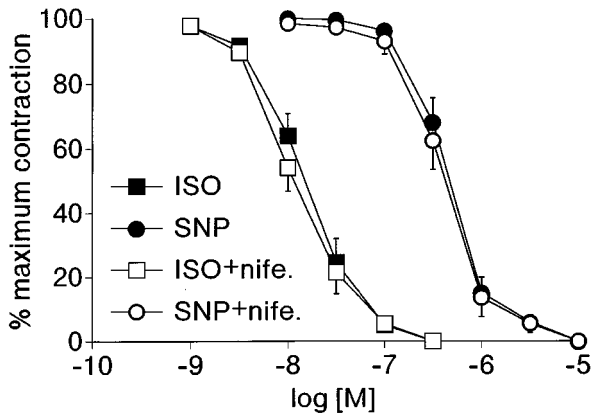

Figure 3 Concentration-relaxation curves of isoprenaline (ISO) and sodium nitroprusside (SNP) against the contraction produced by carbachol (Cch; 1 μM) in the absence and the presence of nifedipine (nife; 3 μM) ($n=12$). Values are mean±s.e.

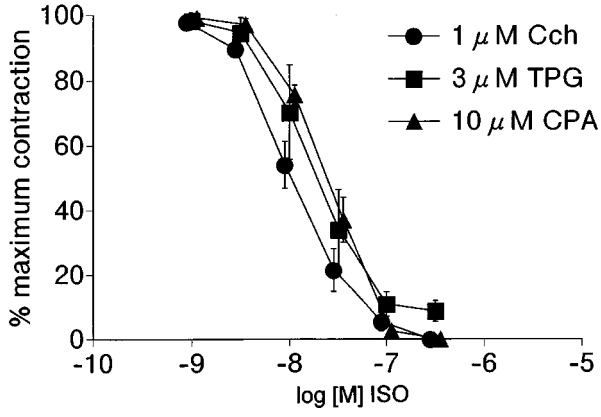

Figure 4 Concentration-relaxation curves of isoprenaline (ISO) in the presence of nifedipine (3 μM), against the contractions produced by carbachol (Cch) and also by Ca^{2+} readmission following 10 min Ca^{2+} removal after 30 min treatment with thapsigargin (TPG) or cyclopiazonic acid (CPA) ($n=12$). The curve for Cch is the same as shown in Figure 3. Values are mean±s.e.

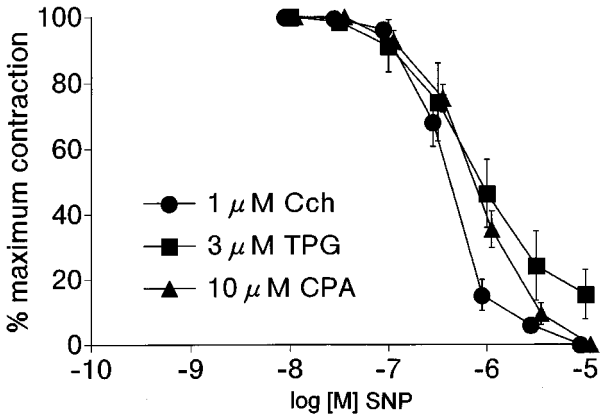

Figure 5 Similar concentration-relaxation curves to Figure 4, but of sodium nitroprusside (SNP; $n=12$). The curve for Cch is the same as shown in Figure 3. Values are mean±s.e.

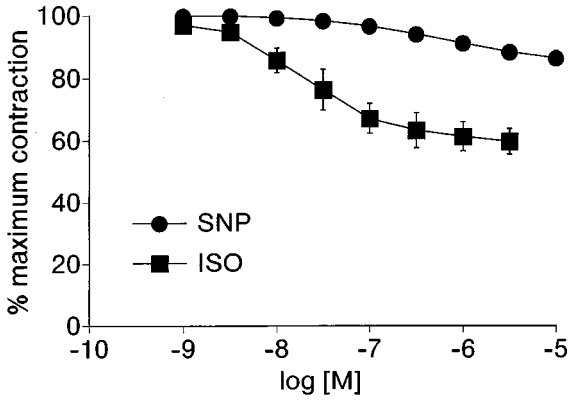

Figure 6 Concentration-relaxation curves of isoprenaline (ISO) and sodium nitroprusside (SNP) against contractions produced by 60 mM K^+ ($n=12$). Values are mean±s.e.

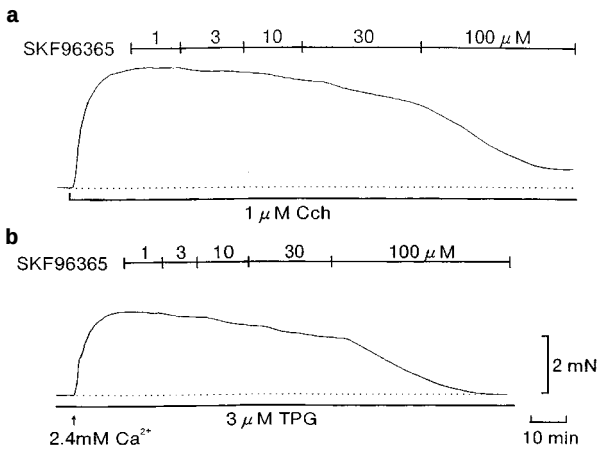

Figure 7 (a) Effects of SKF 96365 on contractions produced by carbachol (Cch; 1 μM). (b) Thapsigargin (TPG; 3 μM) in different preparations. TPG-induced contraction was obtained by applying 2.4 mM Ca^{2+} after removal of Ca^{2+} for 10 min following 30 min treatment with 3 μM TPG. SKF 96365 was applied by increasing concentrations cumulatively as indicated above.

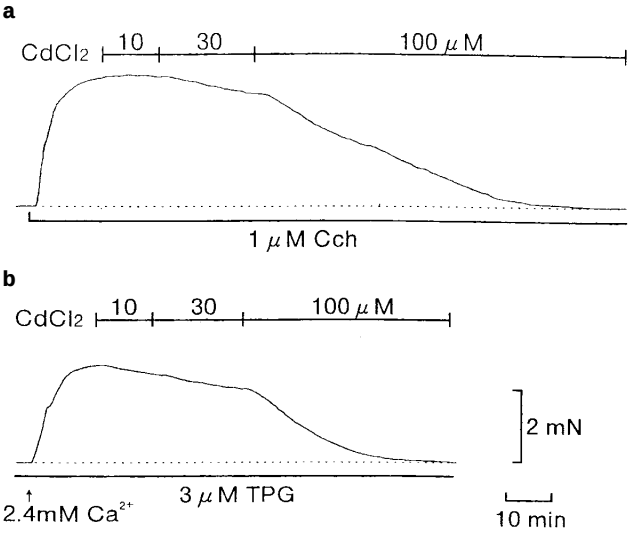

Figure 8 (a) Effects of Cd^{2+} on contractions produced by carbachol (Cch; 1 μM). (b) Thapsigargin (TPG; 3 μM) in different preparations. Similar experiments to those shown in Figure 7.

mainly through a separate pathway from the L-type Ca^{2+} channel, generally assumed to be a receptor-operated channel (Bolton, 1979). TPG- and CPA-induced contractions are also only partially susceptible to nifedipine. The susceptibility to L-type Ca^{2+} channel blockers of the TPG- or CPA-induced contraction varies in different types of smooth muscle. In the longitudinal muscle of the guinea-pig ileum a contraction produced by TPG (1 μM) is blocked by nimodipine (1 μM), a L-type Ca^{2+} channel blocker (Dessy & Godfraind, 1996). On the other hand, the tonic contractions produced by CPA or TPG in the mouse anococcygeus muscle (Gibson *et al*., 1994) and in the circular muscle of the cat gastric fundus (Petkov & Boev, 1996) are partially inhibited by nifedipine, as in the guinea-pig tracheal muscle. In the longitudinal muscle of the rat ileum a sustained contraction produced by Ca^{2+} reapplication following a treatment with CPA has been observed in the presence of nifedipine or methoxyverapamil, but a component susceptible to the Ca^{2+} channel blockers including the rhythmic activity observed in the present experiments has not been mentioned (Ohta *et al*., 1995).

The rhythmic contractions might be due to an inhibitory effect of CPA on the K^+ conductance, since Ca^{2+}-dependent K^+ currents have been shown to be inhibited by CPA in the guinea-pig smooth muscle cells (Suzuki *et al*., 1992). It is rather doubtful that the lack of the rhythmic activity in the TPG-induced contraction is due to its blocking agent of L-type Ca^{2+} channels at a concentration higher than 1 μM (Nelson *et al*., 1994; Buryi *et al*., 1995), since TPG failed to produce the rhythmic activity even at 0.1–1 μM.

TPG- and CPA-induced contractions are markedly potentiated by Ca^{2+} readmission following exposure to Ca^{2+}-free solution and these contractions are largely insensitive to nifedipine, as found in the rat pulmonary artery (De La Fuente *et al*., 1995), strongly suggesting that the contraction is due to a Ca^{2+} influx through a pathway operated by depletion of the Ca^{2+} store (Putney, 1986, 1990). Since the experiments were carried out in the presence of indomethacin and since atropine had no effect, involvement of prostaglandins and cholinergic transmitters in this contraction is unlikely, although a possibility of liberation of other spasmogenic substances from non-muscle cells cannot be neglected.

ISO and SNP relax the guinea-pig tracheal muscle contracted by Cch (1 μM) as well as by TPG (3 μM) or CPA (10 μM). Although SNP is about 30–50 times less potent than ISO, these relaxants are equally effective in producing relaxation in Cch- and TPG- or CPA-induced contractions (the IC_{50} values being about 20 nM for ISO and 0.5–1 μM for SNP). A similar potency of SNP (IC_{40}: about 0.2 μM) on contractions produced by carbachol (5 μM) and CPA (5 μM) has been reported for the mouse anococcygeus muscle (Gibson *et al*., 1994). In the circular muscle of the cat gastric fundus the CPA-induced contraction has been shown to be inhibited by SNP with an IC_{50} of around 1 μM (Petkov & Boev, 1996).

Relaxation produced by ISO and SNP is not significantly affected by nifedipine, so that inhibition of Ca^{2+} influx through L-type channels as a result of membrane hyperpolarization is unlikely to be involved in the relaxation.

Significant correlation is observed between ISO-induced relaxation and a decrease in $[Ca^{2+}]_i$ measured with fura-2 in the guinea-pig tracheal muscle precontracted by prostaglandin E_2 (Ito *et al*., 1995) and the bovine tracheal muscle cells precontracted by methacholine (Hoiting *et al*., 1996). In the guinea-pig tracheal muscle, a similar correlation was also found when SNP was used as a relaxant (Takemoto & Tomita, unpublished observations). In the swine carotid arterial muscle, histamine-induced contraction, Mn^{2+} influx and $[Ca^{2+}]_i$ increase are inhibited by forskolin and nitroglycerin, suggesting that elevation in either cyclic AMP or cyclic GMP concentration decreases Ca^{2+} influx (Chen & Rembold, 1992). Relaxations produced by forskolin and SNP in the rat anococcygeus muscle precontracted by phenylephrine are reduced by CPA, but the degree of reduction is stronger with SNP than forskolin (Raymond & Wendt, 1996). Since the relaxation by SNP is accompanied by a smaller decrease in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) than that by forskolin and since in the presence of CPA, SNP can produce relaxation with almost no detectable decrease in $[Ca^{2+}]_i$, it has been considered that Ca^{2+} sequestration into the SR plays a more important role in the forskolin-induced than SNP-induced relaxation.

In the guinea-pig tracheal muscle, precontracted by prostaglandin E_2, ISO-induced relaxation and $[Ca^{2+}]_i$ decrease are not significantly affected by CPA, suggesting the Ca^{2+} uptake into the SR is not involved in the relaxation (Ito *et al*.,

1995). In canine tracheal muscle, although Ca^{2+} uptake into the SR plays a minor role in cyclic AMP-induced relaxation, this mechanism is considered to be important in cyclic GMP-induced relaxation when L-type Ca^{2+} channels are activated (McGrogan *et al*., 1995). However, when precontracted by Cch as well as by TPG or CPA, both ISO and SNP are equipotent in relaxing the mouse anococcygeus in the absence of nifedipine (Gibson *et al*., 1994) and the guinea-pig tracheal muscle in the presence of nifedipine (the present experiments). Therefore, it is rather unlikely that an increase in the Ca^{2+} uptake into the SR is playing an important role in producing the relaxation in these tissues, although there are possibilities that TPG-resistant Ca^{2+} uptake may be involved or that the relaxants may overcome Ca^{2+} uptake inhibition by TPG and CPA, as considered for the inhibition by SNP or CPA-induced non-selective cation currents in the smooth muscle cells of mouse anococcygeus (Wayman *et al*., 1996b). Contractions produced by 60 mM K^+ are only very weakly inhibited by ISO and SNP, so that a decrease in the Ca^{2+} sensitivity of the contractile protein is probably also not a major mechanism for relaxation, as considered for ISO-induced relaxation in the canine tracheal smooth muscle (Gunst & Bandyopadhyay, 1989). It is more likely that ISO and SNP either inhibit Ca^{2+} influx and/or facilitate Ca^{2+} efflux at the plasma membrane. In the guinea-pig tracheal muscle there is no significant difference in the potency of ISO (cyclic AMP-related) and SNP (cyclic GMP-related) in producing relaxation between Cch- and TPG or CPA-induced contractions. It might be that the same mechanism is involved in the process of relaxations discussed above, for example, such as phosphorylation of key proteins through cross-activation by cyclic AMP- and cyclic GMP-dependent kinases (Schmidt *et al*., 1993).

Since Cch- and TPG- or CPA-induced contractions are inhibited by the relaxants (ISO and SNP) as well as the channel blockers (SKF 96365 and Cd^{2+}) with a similar potency, there is a possibility that Ca^{2+} is mainly supplied through the capacitative entry pathway not only for TPG- or CPA-induced contraction but also for the Cch-induced sustained contraction, as proposed for the noradrenaline-induced contraction in the rat portal vein smooth muscle (Pacaud *et al*., 1993) and Cch-induced contraction of mouse anococcygeus (Wayman *et al*., 1998).

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