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CYCLIC AMP-MEDIATED REGULATION OF EXCITATION–CONTRACTION COUPLING IN CANINE GASTRIC SMOOTH MUSCLE

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SUMMARY

1. Agonists known to increase cyclic AMP levels in gastrointestinal smooth muscles were studied in isolated circular muscles of the canine antrum to investigate the mechanisms of the inhibitory effects of these agents.

2. Muscles were electrically active, generating typical slow wave activity. Cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$; measured by Indo-1 fluorescence) and tension increased in response to slow waves.

3. Stimulation by isoprenaline (via β_2-receptors) or forskolin, in the presence or absence of acetylcholine, inhibited the plateau phase and reduced phasic $[Ca^{2+}]_{cyt}$ and contractile responses.

4. Vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP), had similar effects to isoprenaline and forskolin.

5. Increases in the plateau phase of slow waves and the associated increases in $[Ca^{2+}]_{cyt}$ and tension caused by direct activation of voltage-dependent Ca^{2+} channels by Bay K 8644 (0·1 μM) were also reduced by forskolin.

6. Isoprenaline and forskolin induced negative chronotropic effects, but VIP increased frequency.

7. At a given level of $[Ca^{2+}]_{cyt}$, contractions were greater under control conditions than in the presence of isoprenaline, VIP and CGRP, suggesting that part of the inhibition produced by these agents may be due to decreased Ca^{2+} sensitivity of the contractile apparatus.

8. Experiments performed on α-toxin-permeabilized muscles confirmed that cyclic AMP-dependent effects involve reduced Ca^{2+} sensitivity of the contractile apparatus. Addition of cyclic AMP (3–300 μM) caused a reduction in Ca^{2+}-induced contraction at a constant level of Ca^{2+} (pCa 5·5).

9. These results suggest that increased cyclic AMP and probably subsequent activation of protein kinase A: (i) decrease $[Ca^{2+}]_{cyt}$ and contraction by an inhibition of Ca^{2+} influx during slow waves, and (ii) decrease the sensitivity of the contractile apparatus to $[Ca^{2+}]_{cyt}$. The membrane effects might occur directly by inhibition of Ca^{2+} channels or indirectly by increasing the open probability of K^+ channels which would tend to cause premature repolarization of slow waves.

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INTRODUCTION

In some regions of the gastrointestinal tract contraction of smooth muscle cells is controlled by electrical slow waves (for reviews, see Szurszewski, 1987; Sanders & Publicover, 1989; Sanders & Smith, 1989). Slow waves consist of an upstroke depolarization and a plateau phase that is several seconds in duration. It appears that the force of contraction is largely dependent upon the amplitude and duration of the plateau phase (Morgan & Szurszewski, 1980; Szurszewski, 1987), and agonists which alter these parameters affect the amplitude and duration of phasic contractions in a parallel manner. Recent studies on gastric cells have suggested that L-type Ca^{2+} current is activated during the plateau phase of slow waves (Vogalis, Publicover, Hume & Sanders, 1991). The resulting influx of Ca^{2+} leads to a significant rise in $[Ca^{2+}]_{cyt}$ and couples slow waves to contraction (Vogalis *et al.* 1991; Ozaki, Stevens, Blondfield, Publicover & Sanders, 1991*b*).

Although the rise in $[Ca^{2+}]_{cyt}$ is thought to initiate contractions, there is not a simple relationship between $[Ca^{2+}]_{cyt}$ and force in smooth muscles (for reviews, see Karaki, 1989; Somlyo & Himpens, 1989). Several agonists have been shown to alter the sensitivity of contractile apparatus for Ca^{2+}. In gastric muscle there also appears to be a time-dependent (and possibly Ca^{2+}-dependent) decrease in Ca^{2+} sensitivity which is manifest by dephosphorylation of myosin and relaxation that precede the decrease in $[Ca^{2+}]_{cyt}$ during spontaneous phasic contractions (Ozaki *et al.* 1991*b*; Ozaki, Gerthoffer, Publicover, Fusetani & Sanders, 1991*a*).

There are several inhibitory agonists which have very similar effects on slow wave activity and phasic contractions in the gastric antrum. It is possible that the effects of several of these compounds are mediated by common mechanisms. For example, β-adrenergic stimulation by isoprenaline (ISO) causes relaxation in many gastrointestinal smooth muscles that is accompanied by hyperpolarization (Bulbring & Tomita, 1987). The relaxation caused by β-agonists is thought to be mediated by stimulation of adenylate cyclase and an increase in cyclic AMP formation. In the stomach adrenergic stimulation causes a reduction in the amplitude and duration of slow waves in addition to hyperpolarization (El-Sharkawy & Szurszewski, 1978). Vasoactive intestinal peptide (VIP), which has been considered as a candidate for a non-adrenergic, non-cholinergic (NANC) inhibitory transmitter in gastrointestinal muscles (Fahrenkrug, Haglund, Jodal, Lundgren, Olbe & Shaffalitzky De Muckadell, 1978), is also known to suppress contractile activity by stimulating adenylate cyclase in gastric and other smooth muscles (Bitar & Makhlouf, 1982). VIP has been shown previously to inhibit the contractile activity of gastric muscles (Morgan, Schmalz & Szurszewski, 1978). It has also been reported that calcitonin gene-related peptide (CGRP), another inhibitory agonist associated with changes in cyclic AMP (Maton, Sutliff, Zhao, Collins, Gardner & Jensen, 1988), may be released from NANC nerves in some tissues (Kawasaki, Takasaki, Saito & Goto, 1988). CGRP has been found in the circular layers throughout the gut, with highest concentrations in the stomach and proximal intestine (Sternini, Reeve & Brecha, 1987). The abundance of CGRP-containing neurons suggests that this peptide may play a role in regulating gastric and intestinal motility.

In the present study, we attempted to characterize the mechanisms of inhibition

caused by ISO, VIP and CGRP on gastric smooth muscle. Since the effects of each of these agonists may be mediated by an increase in cyclic AMP, we compared their effects with those of forskolin (FK), an agent known to increase cyclic AMP in a variety of smooth muscles (Lincoln & Fisher-Simpson, 1983; Muller & Bauer, 1983; Ozaki, Kasai, Hori, Sato, Ishihara & Karaki, 1990*a*). We measured electrical activities, $[Ca^{2+}]_{cyt}$ and tension in circular smooth muscles of canine antrum to determine whether common electrical mechanisms exist, and we studied whether an increase in cyclic AMP may also cause inhibition of contractile force by decreasing the Ca^{2+} sensitivity of contractile proteins using α-toxin-permeabilized muscles.

METHODS

Mongrel dogs of both sexes were killed with sodium pentobarbitone (45 mg/kg). After opening the abdomen, the entire stomach was removed and placed in a bath of Krebs–Ringer–bicarbonate solution (KRB). A sheet of muscularis from the ventral surface, 7–9 cm proximal to the pyloric sphincter, was removed from the underlying submucosa. Strips of muscle (1 × 15 mm) were cut parallel to the circular muscle fibres. For contractile studies, muscle strips were pinned out in cross-section and the submucosal half of the circular layer was removed. This left the myenteric region of the circular muscle and the longitudinal layer. Although contractile activity of the myenteric circular muscle was studied, the longitudinal layer was left attached to the circular muscle strip because spontaneous electrical activity originates at the border between the circular and longitudinal fibres (Bauer, Reed & Sanders, 1985). Contractile activity of muscle strips was recorded isometrically with a stain-gauge transducer (Gould, Type UTC2).

In other experiments we measured membrane potential (using conventional microelectrode techniques), $[Ca^{2+}]_{cyt}$ (using Indo-1 fluorescence) and muscle tension simultaneously with a specially designed fluorescence chamber as previously described (Ozaki *et al.* 1991*b*). In fluorescence experiments, muscles were treated with 5 μM-acetoxymethyl ester of Indo-1 (Indo-1 AM) and 0·01 % cremophore EL for 2 h at 37 °C. After dye-loading, muscle strips were rinsed with KRB for approximately 30 min before starting experiments. During experiments the chamber was constantly perfused with warmed, oxygenated KRB and the temperature was maintained at 37·5 ± 0·5 °C with a thermistor probe placed near the muscle. Muscles were exposed to 340 nm light (using a 75 W xenon lamp) and fluorescent emissions at 400 nm (F_{400}) and 500 nm (F_{500}) were monitored with paired photomultiplier tubes (CAF 102, Japan Spectroscopic). The fluorescence of pyridine nucleotides increases in relation to $[Ca^{2+}]_{cyt}$ in smooth muscles and this can interfere with Indo-1 measurements of $[Ca^{2+}]_{cyt}$ (Ozaki, Satoh, Karaki & Ishida, 1988). To avoid these artifacts, the F_{400} and F_{500} signals were constantly monitored, and only data (ratio of F_{400}/F_{500}) from experiments in which these signals changed in opposite directions were used (see Fig. 1). Antral muscles loaded with Indo-1 displayed normal spontaneous activity, with resting membrane potentials and electromechanical events similar to non-loaded muscles (see Ozaki *et al.* 1991*b*).

Cells near the myenteric surface of the circular layer were impaled with microelectrodes filled with 3 M-KCl and having resistances ranging from 30 to 50 MΩ. Impalements were accepted based on criteria described previously (Bauer *et al.* 1985). Transmembrane potential was measured by a standard electrometer (WPI-707), and outputs were displayed on an oscilloscope (Tektronix 5111). Electrical, fluorescence and mechanical signals were recorded digitally using customized software and hardware (Sierra Imaging Technologies) interfaced to an AT-style computer.

Permeabilized muscle was prepared with staphylococcal α-toxin as described by Nishimura & Van Breemen (1989). Small strips, 0·1–0·2 mm in diameter and 1·5–2·0 mm in length, were prepared from antral muscles. Permeabilization was accomplished by incubating the muscle strips with staphylococcal α-toxin (30 μg/ml) for 10–15 min in Ca^{2+}-free solution. The solution used in this study contained 130 mM-potassium propionate, 4 mM-$MgCl_2$, 2 mM-Na_2ATP, 5 mM-creatine phosphate, 10 U/ml creatine phosphokinase, 20 mM-Tris-maleate (pH 6·8), 2 mM-EGTA and indicated concentration of free Ca^{2+}. The apparent binding constant of the Ca^{2+}–EGTA complex was 10^6 M^{-1}. Experiments were performed at room temperature (22–24 °C).

The KRB used in this study contained (mM): Na^+, 137·5; K^+, 5·9; Ca^{2+}, 2·5; Mg^{2+}, 1·2; Cl^-, 134;

HCO_3^-, 15·5; $H_2PO_4^-$, 1·2; dextrose, 11·5. When aerated with a 97% O_2–3% CO_2 gas mixture, the pH was 7·4±0·1. Drugs used were Indo-1 AM (Molecular Probes), acetylcholine (ACh), isoprenaline (ISO), vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), prazosin, salbutamol, atenolol, Bay K 8644 (Sigma) and forskolin (FK) (Calbiochem). Staphylococcal α-toxin, purified from culture media of *Staphylococcus aureus* Wood 46 strain, was a generous gift from Dr Iwao Kato (Chiba University, Japan).

Numerical data are expressed as mean ± standard error. Differences were evaluated by paired *t* test, and *P* values less than 0·05 were taken as a statistically significant difference.

RESULTS

Control activity

Ca^{2+} transients in canine gastric antrum are biphasic and induce biphasic contractile responses (Ozaki *et al.* 1991*a*, *b*). The first phase of the Ca^{2+} transient occurs in response to the upstroke depolarization of each electrical slow wave. This phase is relatively constant in amplitude from event to event in a given preparation. The second phase of the Ca^{2+} transient is far more variable; it depends upon the degree of depolarization and duration of the plateau phase of slow waves (Ozaki *et al.* 1991*b*; Vogalis *et al.* 1991). The plateau potential and associated $[Ca^{2+}]_{cty}$ and tension responses are readily blocked by dihydropyridines (Ozaki *et al.* 1991*a*). After the peak of the plateau potential and during repolarization, $[Ca^{2+}]_{cyt}$ and tension decrease. An example of spontaneous slow waves and associated changes in $[Ca^{2+}]_{cyt}$ and tension are shown in Fig. 1 and average values are given in Table 1. In

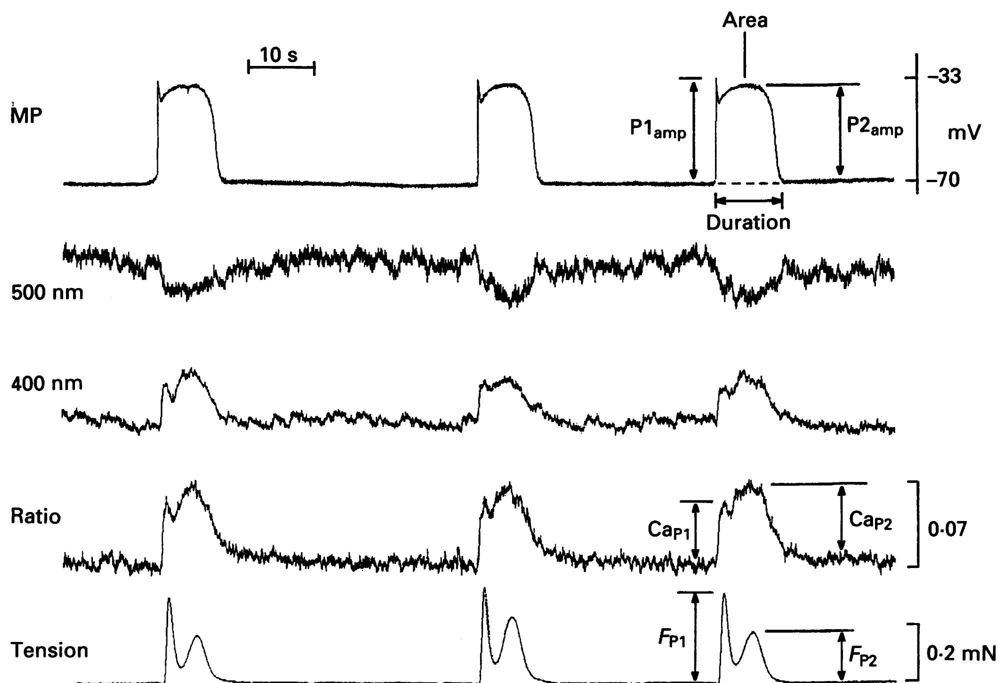


Fig. 1. Spontaneous slow wave activity (MP), 500 nm fluorescence (F_{500}), 400 nm fluorescence (F_{400}), F_{400}/F_{500} ratio, and tension parameters measured. See Table 1 for description of abbreviations. The electrical slow waves are followed by an increase in F_{400}, a decrease in F_{500}, and an increase in the F_{400}/F_{500} ratio, indicating a rise in $[Ca^{2+}]_{cyt}$.

approximately 50% of muscles, the second phase of the Ca^{2+} transient was similar in amplitude to the first phase, but the second phase of contraction was significantly smaller or undetectable. Further, in all cases, tension decreased more rapidly than $[Ca^{2+}]_{cyt}$ during the relaxation phase of phasic contractions, indicating that an inactivation process (perhaps a Ca^{2+}-dependent phosphatase) may participate in the

TABLE 1. Effects of ISO, VIP and CGRP on slow wave parameters, $[Ca^{2+}]_{cyt}$ and contraction

Parameters	Control	Test	Change
	ISO (1 μM) (n = 5 tissues)		
RMP (mV)	−70·8±1·1	−70·3±1·2	+0·5±0·9 (n.s.)
$P1_{amp}$ (mV)	38·0±1·0	40·1±1·4	+2·1±1·2 (n.s.)
$P2_{amp}$ (mV)	36·2±1·3	33·2±1·6	−3·0±0·6**
Duration (s)	9·0±0·9	8·2±0·4	−1·2±0·3 (n.s.†)
Area (mV s)	253±28·9	202±23·8	−51±7·5**
Ca_{P1} (%)	100	85·9±7·9	−14·1±7·9 (n.s.)
Ca_{P2} (%)	144±24·2	28·3±11·5	−79·3±6·1**
F_{P1} (%)	100	46·8±8·5	−53·2±8·5**
F_{P2} (%)	153±54·8	0±0	−100±0*
	VIP (30 nM) (n = 8 tissues)		
RMP (mV)	−70·6±1·1	−71·0±1·0	−0·4±0·6 (n.s.)
$P1_{amp}$ (mV)	38·8±1·8	39·6±1·7	−0·8±0·7 (n.s.)
$P2_{amp}$ (mV)	33·1±1·6	29·5±2·0	−3·6±1·2*
Duration (s)	8·8±0·5	7·3±0·4	−1·5±0·2**
Area (mV s)	236±25·0	165±22·2	−71±14*
Ca_{P1} (%)	100	91·6±6·1	−8·4±6·1 (n.s.)
Ca_{P2} (%)	160·0±30·6	40·9±8·3	−69·3±3·3**
F_{P1} (%)	100	60·1±6·8	−39·9±6·8**
F_{P2} (%)	86·9±22·7	2·2±1·1	−96·4±2·0**
	CGRP (30 nM) (n = 6 tissues)		
RMP (mV)	−71·3±0·8	−71·8±0·9	−0·5±0·2 (n.s.)
$P1_{amp}$ (mV)	39·3±0·8	39·8±1·0	+0·5±0·9 (n.s.)
$P2_{amp}$ (mV)	36·3±1·2	34·1±1·4	−2·2±0·6*
Duration (s)	8·9±0·7	8·0±0·7	−0·9±0·1**
Area (mV s)	247±27·5	214±30·0	−33±9*
Ca_{P1} (%)	100	93·2±5·7	−6·8±5·7 (n.s.)
Ca_{P2} (%)	147·0±31·6	59·4±19·9	−65·1±9·9**
F_{P1} (%)	100	53·6±6·9	−46·4±6·9**
F_{P2} (%)	232·0±114	12·4±9·1	−94·7±4·4 (n.s.†)

RMP, resting membrane potential. $P1_{amp}$, amplitude of first upstroke depolarization phase. $P2_{amp}$, amplitude of second plateau depolarization phase. Duration, from upstroke depolarization to repolarization. Area, time integral of slow wave amplitude. Ca_{P1}, $[Ca^{2+}]_{cyt}$ related to P1 (considered as 100%). Ca_{P2}, relative $[Ca^{2+}]_{cyt}$ related to P2. F_{P1}, relative tension related to P1 (considered as 100%). F_{P2}, relative tension related to P2 (see Fig. 1). * and **, significantly different between control and test groups with $P < 0·05$ and $P < 0·01$, respectively. n.s., not significantly different from control. n.s.†, although there was no statistical significance between control and test in which parameters are tabulated, the agents decreased parameters in all cases.

regulation of contractions in gastric muscles (Ozaki *et al.* 1991*b*). Similar Ca^{2+} desensitization has been reported in other smooth muscles (Yagi, Becker & Fay, 1988; Somlyo, Kitazawa, Himpens, Matthijs, Horiuchi, Kobayashi, Goldman & Somlyo, 1989). In the present study we compare the effects of various stimuli on the

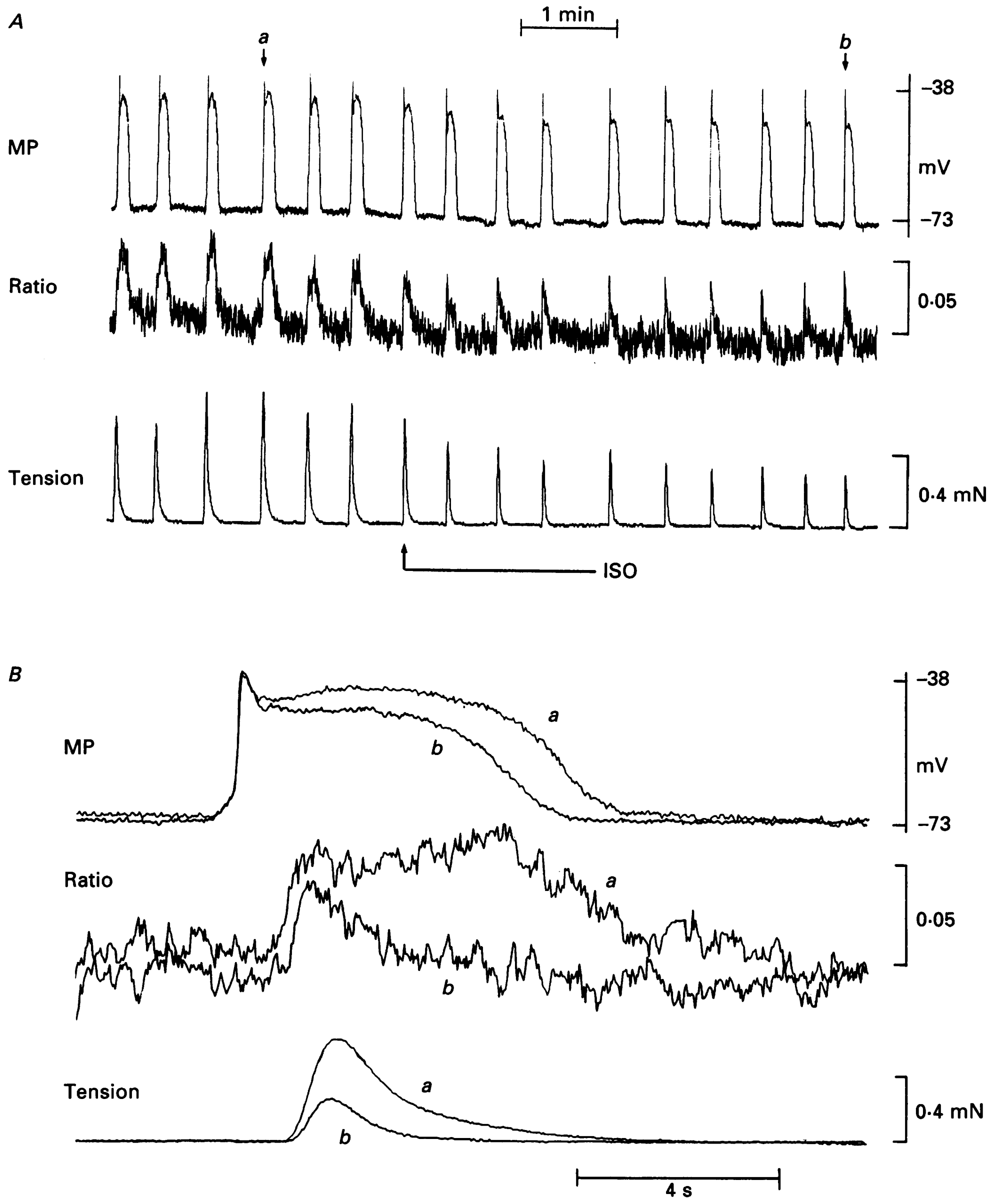
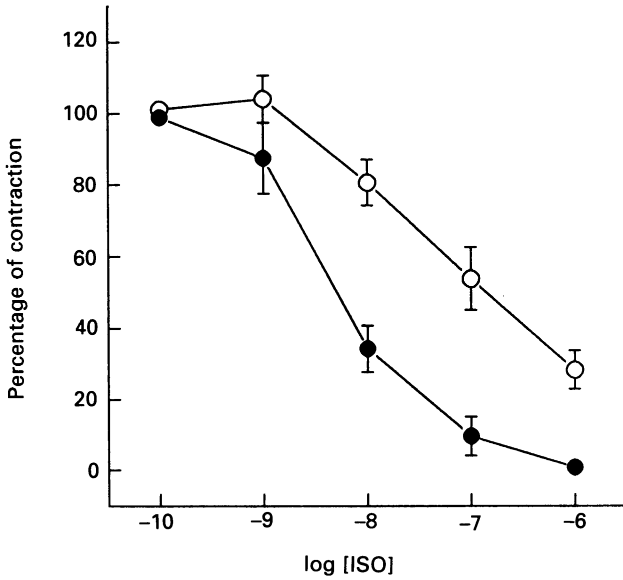


Fig. 2. Effect of ISO on spontaneous electrical activity, $[Ca^{2+}]_{cyt}$ and phasic contractions. Panel *A* shows a continuous recording of events on a compressed time scale. Panel *B* shows examples, denoted by *a* and *b* in panel *A* (i.e. *a*, control trace; *b*, ISO effects), of activity on an expanded time scale. Electrical slow waves and $[Ca^{2+}]_{cyt}$ consist of two phases, although phasic contractions in this example were monophasic (see Results for details). ISO (1 μM; at arrow) decreased the amplitude and duration of the plateau potential of slow waves, decreased the first and second phases of Ca^{2+} transients, and decreased the amplitude of phasic contractions.



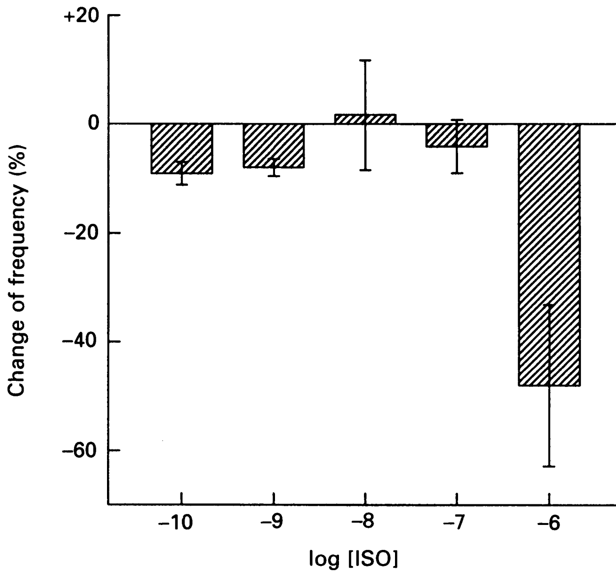


Fig. 3. Concentration–response relationship for ISO of the amplitude (panel *A*) and frequency (panel *B*) of spontaneous phasic contractions. Frequency effects measured as percentage of control (average $2{\cdot}3 \pm 0{\cdot}3$ contractions/min). ISO was more potent in inhibiting the second phase of contraction (P2, ●) than the first phase of contraction (P1, ○). At a concentration of 1 μM, ISO decreased contractile frequency. Data are averages $\pm$ S.E.M. from six experiments.

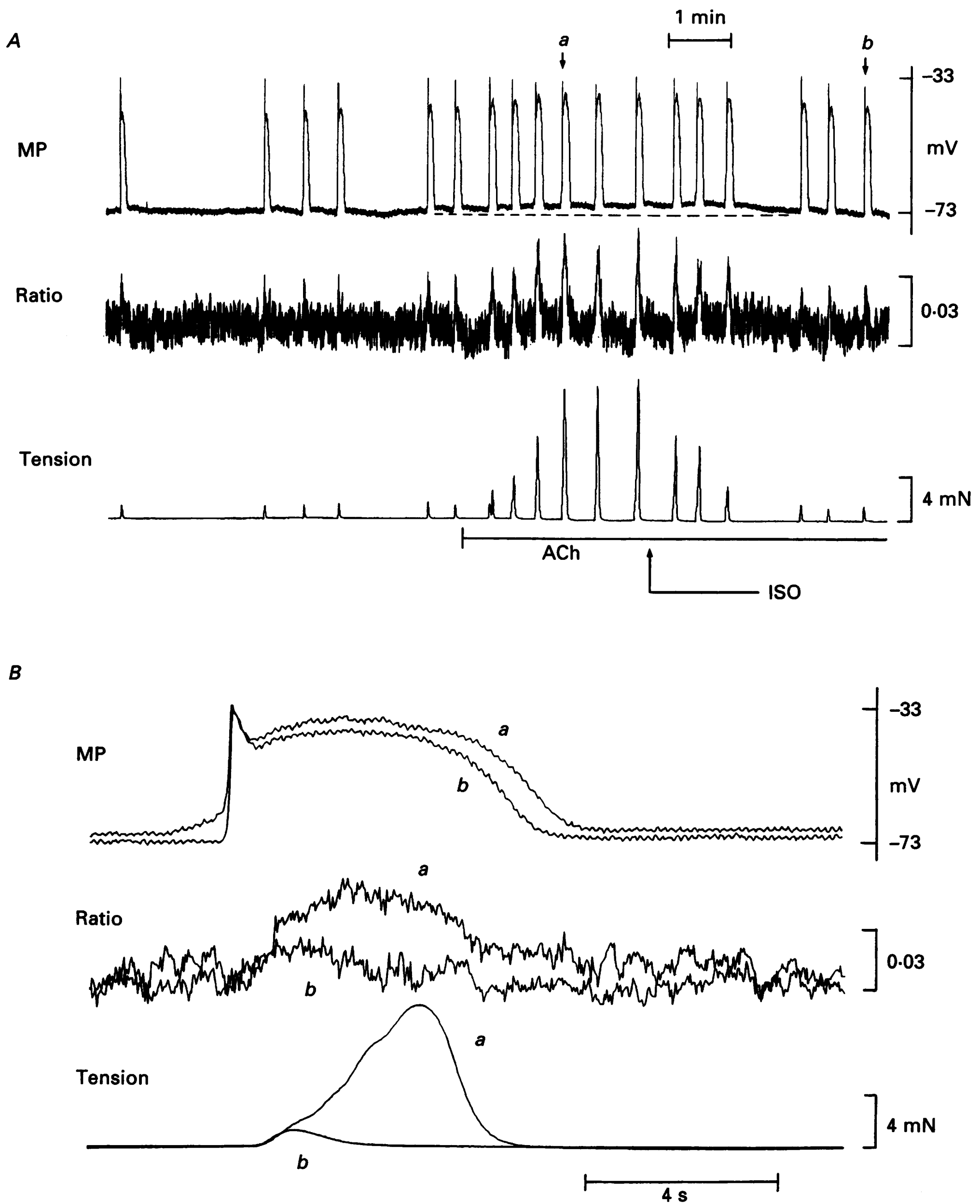


Fig. 4. Effect of ISO on membrane potential, $[Ca^{2+}]_{cyt}$ and tension in muscle stimulated by ACh. Panel *A* shows a record of a series of events. ACh (0·3 μM) depolarized the membrane potential, enhanced the plateau phase of slow waves, and increased $[Ca^{2+}]_{cyt}$ and tension (see Ozaki *et al.* 1991*b*). ISO (1 μM) repolarized the membrane, inhibited the plateau potential and decreased $[Ca^{2+}]_{cyt}$ and tension. Panel *B* shows these effects on an expanded time scale by displaying events denoted in panel *A* by *a* and *b* (*a*, ACh-stimulated activity; *b*, ISO effects).

first and second phases of contraction. In muscles in which the second phase of contraction was small or undetectable, small doses of ACh (5–20 nM) were used, as noted, to enhance the second phase.

Effect of ISO on electrical activity, $[Ca^{2+}]_{cyt}$ and contraction

Figure 2 shows the effects of ISO on spontaneous electrical activity, Ca^{2+} transients and contractions. ISO (1 μM) produced a small decrease in the amplitude

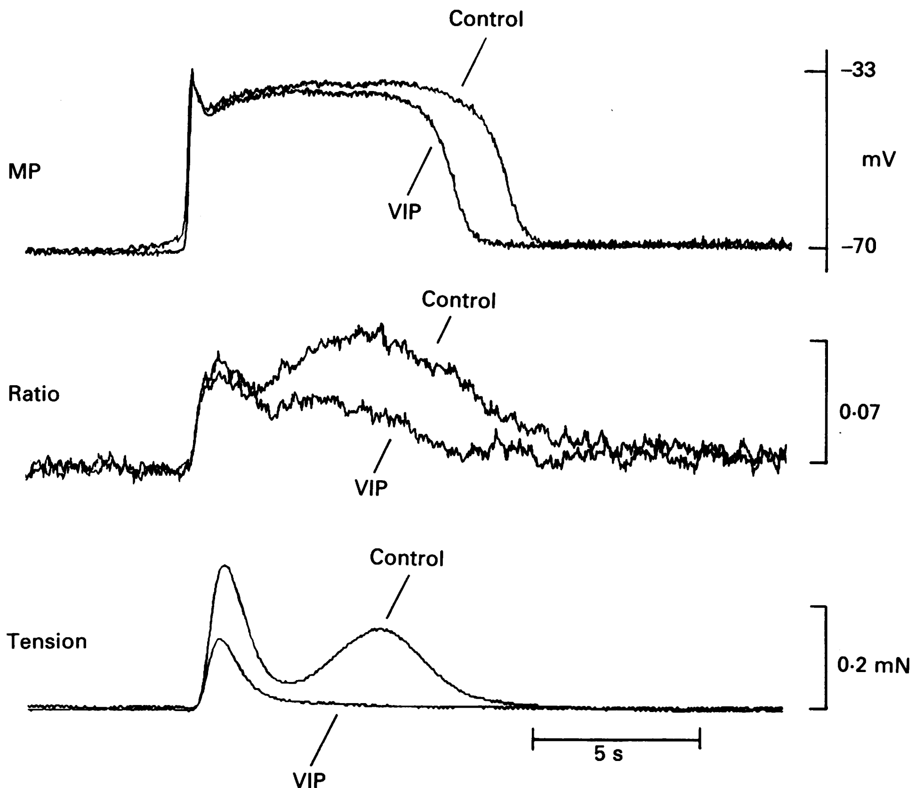


Fig. 5. Effect of VIP on electrical activity, $[Ca^{2+}]_{cyt}$ and tension in unstimulated muscle. VIP (30 nM) decreased the amplitude and duration of the plateau potential of slow waves, decreased the amplitude of the Ca^{2+} transient, and decreased the amplitude of phasic contractions. VIP caused little or no effect on the upstroke phase of slow waves and the first phase of the Ca^{2+} transient, yet significantly depressed the first phase of the contractile response.

of the first phase of the Ca^{2+} transient (average 14% reduction), and a substantial decrease in the amplitude of the first phase of contraction (average 53% reduction). This compound more dramatically decreased the second phase of the Ca^{2+} transients (average 80% reduction) and abolished the second phase of contraction (100% reduction). ISO did not cause significant hyperpolarization, nor did it significantly affect the amplitude of the upstroke depolarization. Removal of ISO restored control electrical, Ca^{2+} and mechanical transients. These results are summarized in Table 1.

The effects of ISO were concentration dependent as demonstrated by a series of six mechanical studies. These studies showed that the effects of ISO on the first and

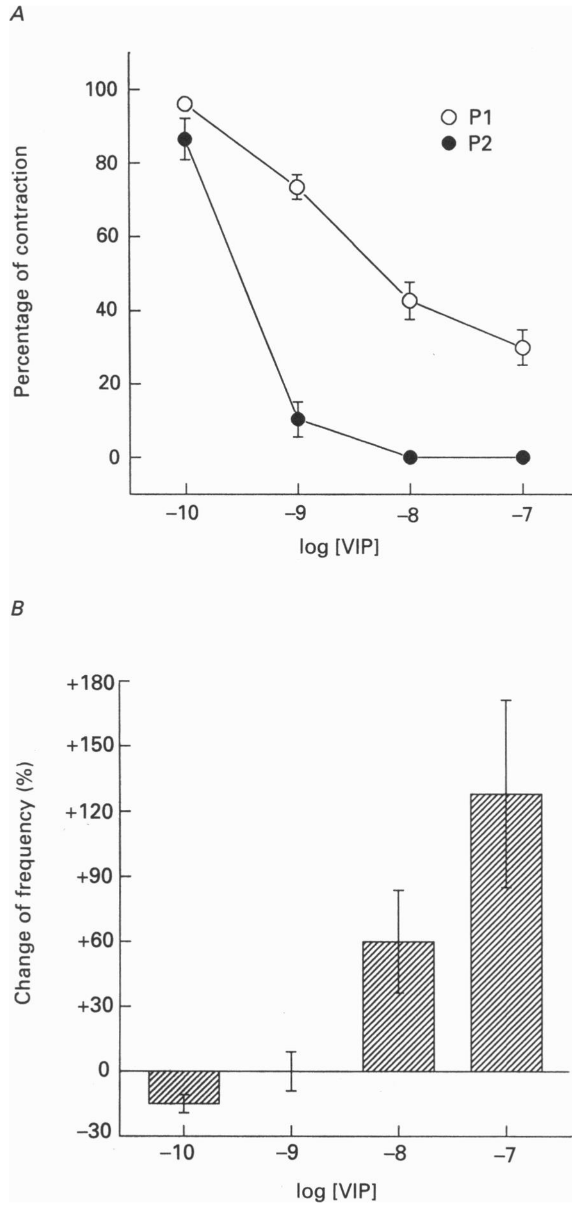


Fig. 6. Concentration–response relationship for VIP for the amplitude (panel *A*) and frequency (panel *B*) of spontaneous phasic contractions. Frequency effects measured as percentage of control (average $3{\cdot}1 \pm 0{\cdot}2$ contractions per minute). VIP had a more potent effect on the second phase of contraction (P2, ●) than the first phase of contraction (P1, ○). At concentrations higher than 10 nM, VIP increased frequency. Data are averages ± S.E.M. from six experiments.

second phases of contraction had different concentration dependencies; the second phase was more sensitive to ISO. Higher concentrations of ISO (at 1 μM) had negative chronotropic effects. These experiments are summarized in Fig. 3.

The effects of ISO on two muscles stimulated by ACh were also characterized. ACh

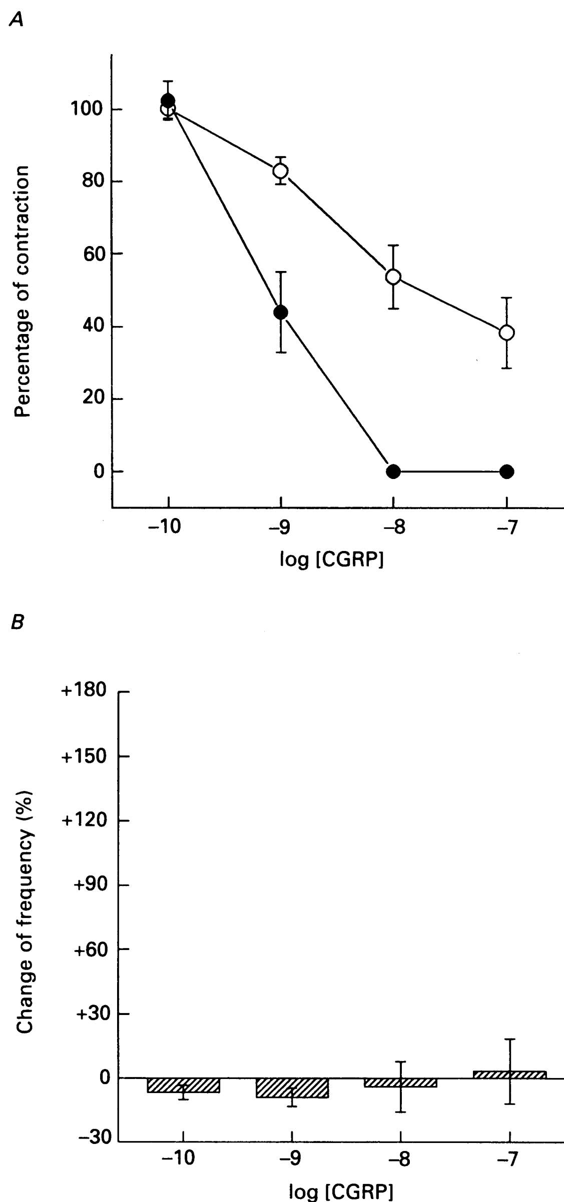


Fig. 7. Concentration–response relationship for CGRP of the amplitude (panel *A*) and frequency (panel *B*) of spontaneous phasic contractions. Frequency effects measured as percentage of control (average 2·8 ± 0·2 contractions/min). CGRP had a more potent effect on the second phase of contraction (P2, ●) than the first phase of contraction (P1, ○). CGRP did not affect frequency in the range of concentrations tested. Data are averages ± S.E.M. from seven experiments.

(0·3 μM) slightly depolarized resting membrane potential and increased the maximum level and duration of depolarization achieved during the plateau phase (Fig. 4; see also, Ozaki *et al.* 1991*b*). The increase in slow wave amplitude was closely associated with increases in $[Ca^{2+}]_{cyt}$. After the responses to ACh reached a steady state, ISO

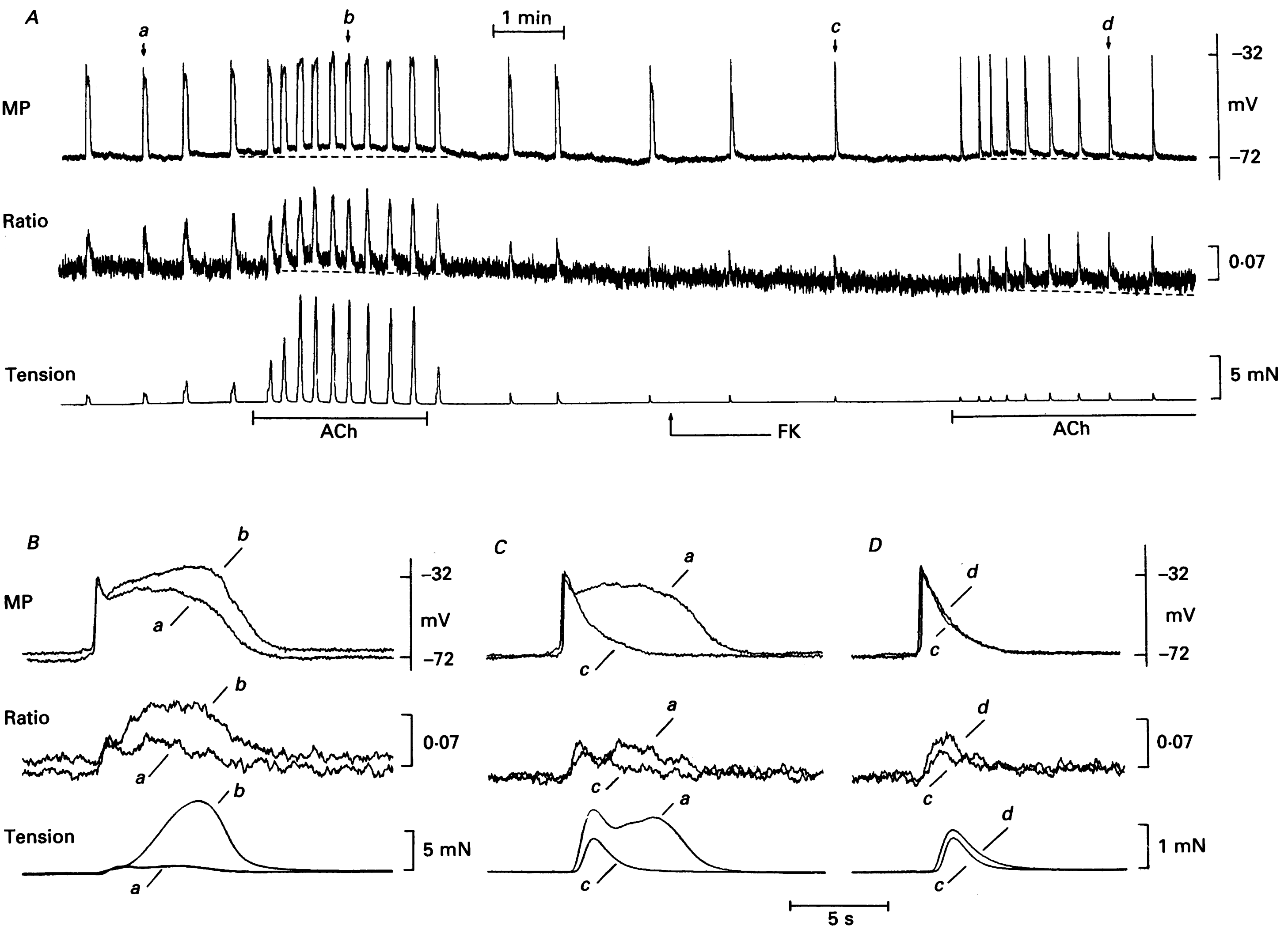


Fig. 8. For legend see facing page.

(1 μM) was added. ISO reduced the plateau depolarizations of slow waves, Ca^{2+} transients and mechanical responses (Fig. 4). In the presence of ISO, these parameters were returned to nearly control (pre-ACh) levels. When muscles were stimulated with higher concentrations of ACh (1 μM; two muscles), the maximum inhibition caused by ISO (1 μM) was reduced.

The inhibitory effects of ISO were blocked by 5 μM-propranolol (non-specific β-antagonist) but not affected by atenolol (β_1-antagonist; 2 μM). Prazosin (α_1-antagonist; 1 μM), which completely abolished the inhibitory effects of noradrenaline (El-Sharkawy & Szurszewski, 1978; H. Ozaki, unpublished observation), had no effect on the effects of ISO. On the other hand, salbutamol (β_2-agonist; 1 μM) had effects similar to ISO. These results indicate that the action of ISO in canine antrum is mediated by β_2-receptors. These results are similar to those in rat gastric muscle (Lefebvre, Verplanke & Bogaert, 1985).

Effect of VIP and CGRP on electrical activity, $[Ca^{2+}]_{cyt}$ and contraction

The magnitude of the Ca^{2+} transients initiated by slow waves appears to be attenuated by stimulation of β_2-receptors. Since these receptors have been shown to be coupled to enhanced adenylate cyclase activity (for reviews, see Stiles, Caron & Lefkowitz, 1984; Bulbring & Tomita, 1987; Daniel, Collins, Fox & Huizinga, 1989), additional experiments were performed to test other agents known to stimulate adenylate cyclase.

VIP (30 nM) decreased the amplitude of the plateau potential without significantly affecting the upstroke depolarization. VIP also decreased $[Ca^{2+}]_{cyt}$ and reduced the amplitude of contractions (Fig. 5). The effects of VIP were concentration dependent as demonstrated by a series of mechanical studies. These studies also showed that the effects of VIP on the first and second phases of contraction had different concentration dependencies, similar to the effects of ISO. In contrast to the effects of ISO, VIP had positive chronotropic effects at concentrations of 10 and 100 nM. These experiments are summarized in Fig. 6 and Table 1. The effects of VIP on electrical and mechanical activities differ from a previous report (Morgan *et al.* 1978) in which it was shown that VIP relaxed canine antral muscles without affecting electrical parameters. At present, we have no explanation for this difference.

We also examined the effects of CGRP (30 nM) on membrane potential, $[Ca^{2+}]_{cyt}$ and tension. Like VIP, CGRP (30 nM) decreased plateau potential, $[Ca^{2+}]_{cyt}$ and tension with little effect on the first upstroke phase (Table 1). The inhibitory effect of CGRP on tension was also concentration dependent and it more strongly inhibited the second phase of contraction than the first phase (Fig. 7*A*). CGRP had no effect on frequency (Fig. 7*B*).

Fig. 8. Effect of FK on membrane potential (MP), $[Ca^{2+}]_{cyt}$ and tension in muscle stimulated by ACh. Panel *A* shows continuous recording on a compressed time scale. ACh (0·3 μM) depolarized the membrane potential and increased the plateau phase of slow waves, $[Ca^{2+}]_{cyt}$ and tension. Panel *B* shows these effects on an expanded time scale by displaying slow waves denoted by *a* and *b* in panel *A* (i.e. *a*, control traces; *b*, ACh effects). FK (0·5 μM) markedly inhibited the plateau potential and decreased $[Ca^{2+}]_{cyt}$ and tension (panel *C*). Panel *D* shows the effect of ACh in the presence of FK (*c*, FK effects; *d*, ACh in presence of FK).

Effect of forskolin on electrical activity, $[Ca^{2+}]_{cyt}$ and contraction

The action of β-agonists, VIP and CGRP, is thought to involve an increase in cyclic AMP formation by stimulating adenylate cyclase in smooth muscle (see Introduction). Therefore we compared the effects of forskolin (FK), a direct activator of adenylate cyclase, on membrane potential, $[Ca^{2+}]_{cyt}$ and muscle tension.

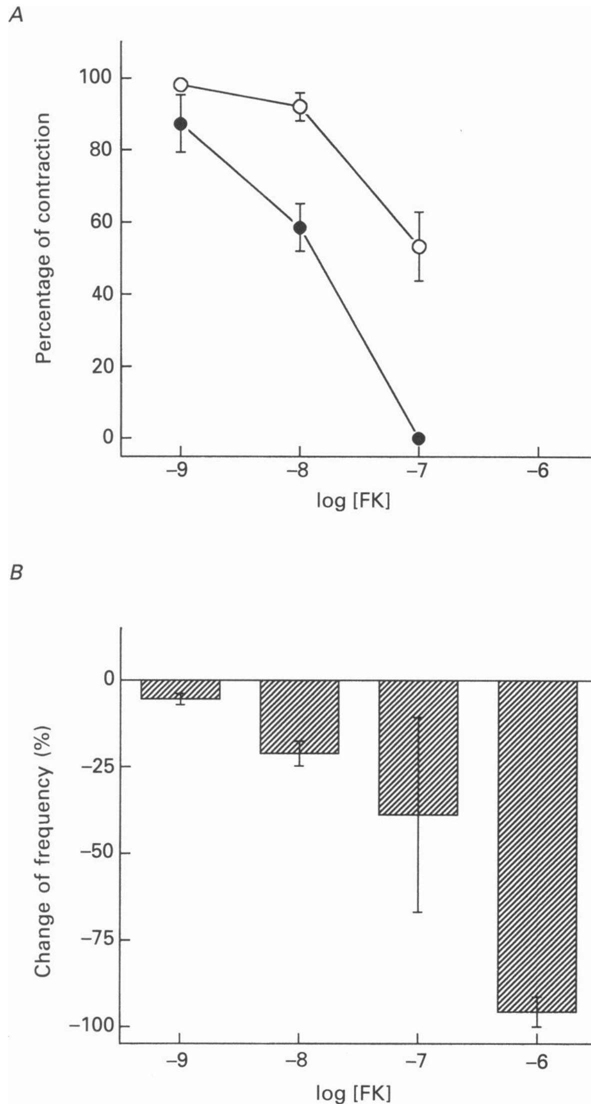
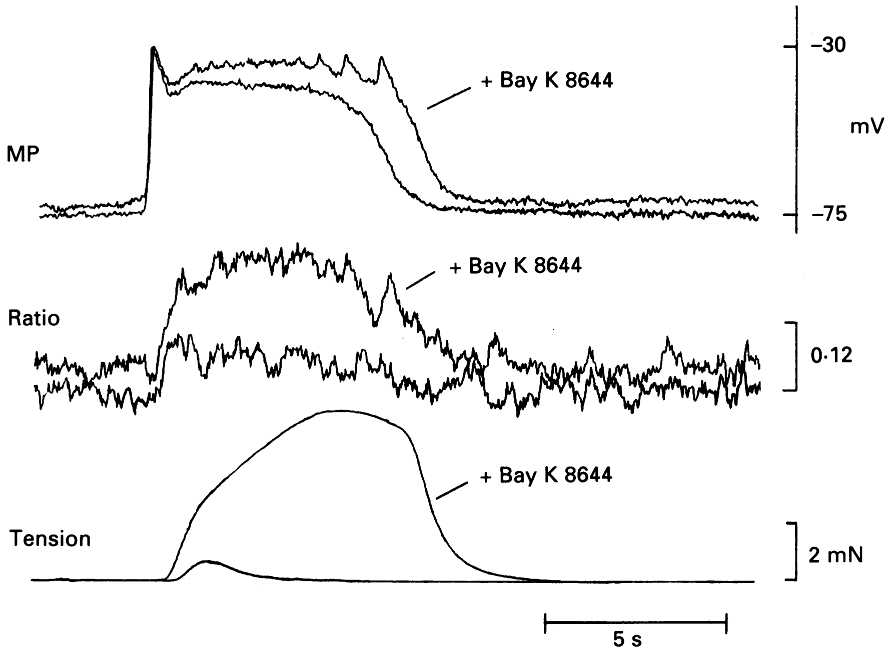


Fig. 9. Concentration–response relationship for FK of the amplitude (panel *A*) and frequency (panel *B*) of spontaneous phasic contractions. Frequency effects measured as percentage of control (average $2{\cdot}6 \pm 0{\cdot}3$ contractions/min). FK was more potent in inhibiting the second phase of contractions (P2, ●) than the first phase of contractions (P1, ○). At 1 μM, spontaneous activity was abolished in four of five muscles. Data are averages ± S.E.M. from five experiments.



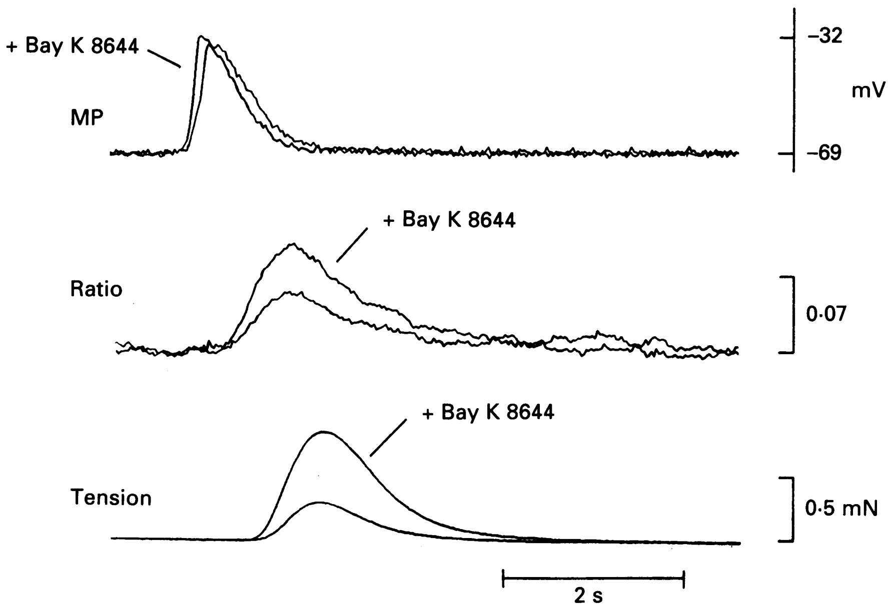


Fig. 10. Effects of FK on membrane potential (MP), $[Ca^{2+}]_{cyt}$ and tension in muscle stimulated by Bay K 8644. Panel *A* shows the responses to Bay K 8644 (0·1 μM). Panel *B* shows the effects of Bay K 8644 on muscle pre-treated with FK (0·5 μM) for 10 min. In the presence of FK, the stimulatory effects of Bay K 8644 on the plateau potential were completely suppressed. In the presence of FK, Bay K 8644 increased the rate-of-rise of the upstroke depolarization and increased Ca^{2+} and contractile transients.

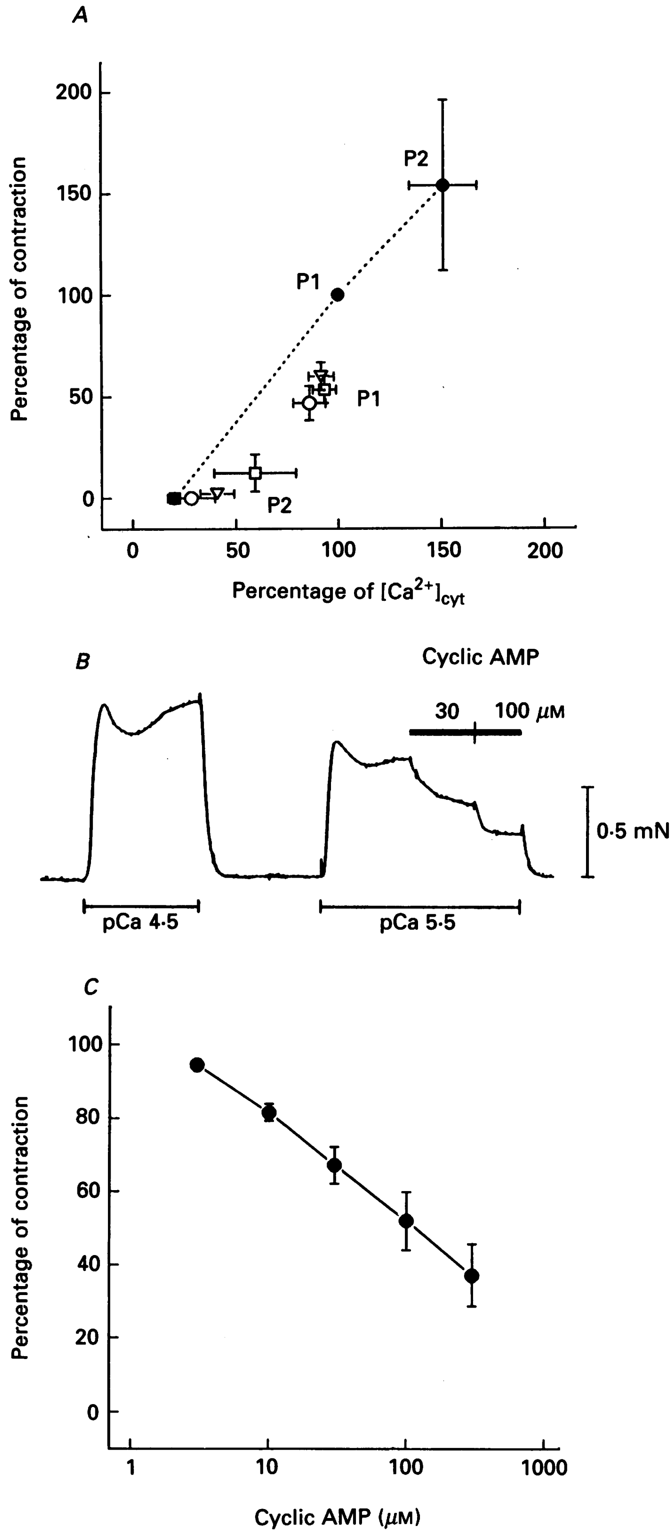


Fig. 11. For legend see facing page.

Before adding FK, a control response to ACh (0·3 μM) was recorded. When control spontaneous activity was restored after the exposure to ACh, FK (0·5 μM) was added for 4 min. In the presence of FK, the plateau potential was completely abolished and corresponding changes in $[Ca^{2+}]_{cyt}$ and contraction were inhibited (Fig. 8*A*. Typical responses are shown in Fig. 8*B–D* on an expanded time scale. While still in the presence of FK, ACh was added again. FK greatly reduced the effects of ACh. The effects of FK were concentration dependent (Fig. 9). The second phase of contraction was more sensitive to FK than the first phase. At 1 μM, FK blocked spontaneous activity in four of five muscles tested.

We also examined the effect of FK on electrical, Ca^{2+} and mechanical activities of antrum stimulated by the Ca^{2+} channel agonist Bay K 8644 in two muscles. This compound (0·1 μM) markedly increased the amplitude and duration of the plateau potential and caused parallel augmentation in $[Ca^{2+}]_{cyt}$ and muscle tension in myenteric muscles (see Fig. 10*A* and Ozaki *et al.* 1991*b*). Bay K 8644 also increased the amplitude of the upstroke depolarization. In muscle pre-treated with FK (0·5 μM), the effects of Bay K 8644 on plateau potential, $[Ca^{2+}]_{cyt}$ and tension were completely suppressed (Fig. 10*B*). In the presence of FK, Bay K 8644 augmented the rate of rise of the upstroke potential and increased the Ca^{2+} and mechanical transients. These findings are consistent with the concept that dihyropyridine-sensitive Ca^{2+} channels participate in both the upstroke and plateau components of slow waves (Ward, Blondfield & Sanders, 1990).

Relationship between $[Ca^{2+}]_{cyt}$ and tension

At least some of the effects of ISO, VIP and CGRP appear to be mediated by changes in the ionic conductances which contribute to electrical slow waves (Sanders, Burke, Carl, Cole, Langton & Ward, 1990). This is evidenced by the observation that decreases in slow wave plateau amplitude and duration result in decreases in Ca^{2+} and mechanical transients. But we also noted that the effects of these agonists on contractile force were not necessarily well correlated with a decrease in $[Ca^{2+}]_{cyt}$. For example, the amplitude of the first phase of contraction was reduced by 40–50 % by these agents (Table 1), whereas the decrease in the Ca^{2+} transient was only 7–15 %. Figure 11*A* is a plot of the $[Ca^{2+}]_{cyt}$–tension relationship in the presence and absence of these agents. Control responses and the average $[Ca^{2+}]_{cyt}$ at which force was initiated have been connected by line segments (although this is not meant to imply a linear $[Ca^{2+}]_{cyt}$–tension relationship). All of the responses to ISO, VIP and CGRP are below the line, suggesting that the Ca^{2+} sensitivity of the contractile mechanisms is decreased by these agents. Similar findings have been reported from studies of rat

Fig. 11. Panel *A* shows the $[Ca^{2+}]_{cyt}$–tension relationship under control conditions (●) and in the presence of ISO (1 μM, ○), VIP (30 nM, ▽) or CGRP (30 nM, □). Values were obtained from Table 1. Filled square indicates the level of $[Ca^{2+}]_{cyt}$ at which contraction is initiated ($20·3 \pm 2·9$ %, $n = 15$). In the presence of ISO, VIP and CGRP, the $[Ca^{2+}]_{cyt}$–tension relationship shifted to the lower right, indicating a decreased Ca^{2+} sensitivity. Panel *B* shows the effect of cyclic AMP on Ca^{2+} induced contraction of α-toxin-treated antral muscle. Addition of cyclic AMP (30 and 100 μM) inhibited the contraction induced by pCa 5·5, demonstrating a cyclic AMP-dependent decrease in Ca^{2+} sensitivity. Panel *C* shows the average inhibition of contraction produced by cyclic AMP over the range from 3 to 300 μM ($n = 4$).

aorta (Abe & Karaki, 1989) and canine trachea (Ozaki *et al.* 1990*b*) in which FK and ISO inhibited agonist-induced contractions with little change in $[Ca^{2+}]_{cyt}$.

From studies on α-toxin-treated rat mesenteric arteries, it has been suggested that a cyclic AMP-dependent decrease in Ca^{2+} sensitivity of the contractile proteins also contributes to the effects of some inhibitory agonists (Nishimura & Van Breemen, 1989). We tested whether cyclic AMP-dependent Ca^{2+} desensitization occurs in antral muscles treated with α-toxin (30 μg/ml for 10–15 min). Elevation of Ca^{2+} (to pCa 4·5 and 5·5) induced a biphasic contractile response (Fig. 11*B*). Such biphasic responses were observed even after muscles were treated with α-toxin for 60 min. Figure 11*B* shows the inhibition of contraction upon the addition of 30 and 100 μM-cyclic AMP at a fixed pCa (5·5). Average responses to cyclic AMP in α-toxin-treated muscles ($n = 4$) over the range from 3 to 300 μM are shown in Fig. 11*C*. The effects of cyclic AMP were reversible (data not shown).

DISCUSSION

Several studies in the past have developed the hypothesis that phasic contractions of gastric muscles are regulated by the amplitude and duration of electrical slow waves (Morgan & Szurszewski, 1980; Morgan, Muir & Szurszewski, 1981; Bauer, Reed & Sanders, 1985; Ozaki *et al.* 1991*b*; for reviews see Szurszewski, 1987; Sanders & Publicover, 1989). The data from the present study are consistent with this hypothesis: the various agonists studied decreased the amplitude and duration of the plateau phase and reduced the amplitude of Ca^{2+} and mechanical transients. Since these agonists are thought to enhance cyclic AMP production, it is possible that the inhibitory effects may be mediated by cyclic AMP-dependent activation of protein kinase A (PKA). This idea is strengthened by the finding that FK, a direct activator of adenylate cyclase (see Seamon & Daly, 1983), had similar effects to ISO, VIP and CGRP on slow waves, $[Ca^{2+}]_{cyt}$ and contractions.

There is no extensive information about the mechanism of inhibition caused by the β-adrenergic agonists, VIP and CGRP, in gastric muscles (see Bulbring & Tomita, 1987; Sanders & Publicover, 1989). VIP caused hyperpolarization and inhibited spike potentials in rat fundic and antral smooth muscles and these effects were associated with an increase in cyclic AMP levels (Ito, Kurokawa, Ohga, Ohta & Sawabe, 1990). In contrast, it has been reported that VIP produced a reduction in force without affecting electrical activity of canine antral muscles (Morgan *et al.* 1978). This is contradictory to the results of the present study (i.e. VIP clearly reduced the amplitude and duration of slow waves; see Table 1). CGRP inhibited carbachol-stimulated contraction of single cells isolated from guinea-pig gastric muscle and this inhibition was closely associated with cyclic AMP level (Maton *et al.* 1988). It has also been reported that CGRP is a potent relaxant of other gastrointestinal smooth muscles (Lorland, Lembeck & Holzer, 1987).

In cardiac muscles, stimulation of β-adrenergic receptors is excitatory, leading to more forceful contractions. At least part of this response is mediated by an increase in cyclic AMP levels, activation of PKA, and phosphorylation of certain membrane proteins. This leads to an increase in the magnitude of L-type Ca^{2+} current (Reuter, 1983; for review see Trautwein & Hescheler, 1990). Similar to the heart, in most muscles of the gastrointestinal tract β-adrenergic stimulation increases cyclic AMP

production. But in contrast, β-adrenergic stimulation is coupled to inhibition of electrical and contractile activity in gastrointestinal muscles (Stiles *et al.* 1984; Bulbring & Tomita, 1987; Daniel *et al.* 1989). This suggests that channels in gastrointestinal muscles may be regulated in a different manner than cardiac channels by PKA-dependent phosphorylation.

In antral smooth muscles two types of channel regulation could produce the type of electrical responses caused by ISO, VIP, CGRP and FK: (i) inhibition of voltage-dependent Ca^{2+} current; or (ii) an increase in voltage- or Ca^{2+}-dependent K^+ currents (see Sanders *et al.* 1990). A decrease in voltage-dependent Ca^{2+} current would reduce the inward current that balances with outward currents during the plateau phase of slow waves (Sanders *et al.* 1990). This would reduce the duration of the plateau, reduce the amount of Ca^{2+} entry, and reduce the magnitude of Ca^{2+} and mechanical transients. Regulation of outward currents could produce similar results. An increase in the non-Ca^{2+}-dependent, voltage-dependent K^+ current that balances the inward current during the plateau phase would tend to decrease the level of depolarization during the plateau and could shorten this phase. An increase in Ca^{2+}-dependent K^+ current could also shorten the plateau phase since this current is thought to be involved in repolarization (Carl, McHale, Publicover & Sanders, 1990). There is evidence to suggest that inward and outward currents are regulated by phosphorylation in gastrointestinal smooth muscles. Okadaic acid and calyculin-A (specific phosphatase inhibitors; Bialojan & Takai, 1988; Ishihara, Martin, Brautigan, Karaki, Ozaki, Kato, Fusetani, Watabe, Hashimoto, Uemura & Hartshorne, 1989) inhibit L-type Ca^{2+} currents in antral and colonic smooth muscle cells (Ward, Vogalis, Blondfield, Ozaki, Fusetani, Uemura, Publicover & Sanders, 1991). These phosphatase inhibitors would be expected to increase protein phosphorylation; therefore, it is possible that the increase in cyclic AMP production in response to ISO, VIP, CGRP and FK might inhibit Ca^{2+} channels via PKA-mediated phosphorylation. Other studies have shown that the open probability of Ca^{2+}-activated K^+ channels was increased by the catalytic subunit of PKA in tracheal and colonic smooth muscle cells (Kume, Takai, Tokuno & Tomita, 1989; Carl, Kenyon, Uemura, Fusetani & Sanders, 1991). This effect was enhanced by okadaic acid and calyculin-A. These data suggest that enhanced cyclic AMP production could increase Ca^{2+}-dependent outward current, and this could contribute to the inhibitory effect of the agonists investigated in the present study.

The inhibitory effects of ISO, VIP and CGRP do not appear to be limited to an electrophysiological mechanism. These compounds had little effect on the upstroke phase of slow waves and produced only a 7–14% decrease in the first phase of the Ca^{2+} transient. Despite these minor changes, the three agonists greatly reduced the amplitude of the first phase of contraction. These data suggest some dissociation between $[Ca^{2+}]_{cyt}$ and tension. Others have suggested that activation of PKA can reduce the Ca^{2+} sensitivity of the contractile proteins in vascular and tracheal smooth muscles (Abe & Karaki, 1989; Nishimura & Van Breemen, 1989; Ozaki *et al.* 1990*b*). It is possible that a similar mechanism exists in gastric muscles and cyclic AMP-dependent activation of PKA might reduce Ca^{2+} sensitivity. We tested this by investigating the effects of cyclic AMP on tension in muscles permeabilized with α-toxin in which $[Ca^{2+}]_{cyt}$ was clamped. These experiments demonstrated a large reduction in the Ca^{2+} sensitivity in response to cyclic AMP and suggested that PKA

may be an important regulator of contractions in these muscles. This is likely to be a second level of the inhibitory effects of agonists that increase cyclic AMP levels.

Another site of cyclic AMP-dependent actions may be pacemaker activity. The muscles used in these experiments contained the myenteric half of the circular layer, the myenteric plexus region and the entire longitudinal layer. The myenteric region has previously been reported to house the pacemaker in antral muscles (Bauer *et al.* 1985). We found that ISO reduced the frequency of gastric slow waves (see Fig. 3*B*). The chronotropic effects were caused by higher concentrations of ISO than the effects on contractile amplitude. In a previous report in which adrenergic effects on electrical activity of the canine antrum were investigated, noradrenaline reduced the slow wave plateau amplitude, but *increased* slow wave frequency (El-Sharkawy & Szurszewski, 1978). We have confirmed these findings and found that the positive chronotropic effect was blocked by prazosin (1 μM; H. Ozaki, N. G. Publicover & K. M. Sanders, unpublished observations). From these results, it appears that α- and β-adrenergic stimulation mediate contrasting chronotropic effects. The mechanism by which α-stimulation might enhance frequency is unknown, but the β-effect may be mediated by an elevation of cyclic AMP since FK also induced negative chronotropic effects. In contrast to the effects of ISO and FK, VIP increased frequency, thus confirming a previous finding (Morgan *et al.* 1978) and CGRP had no effect on frequency (see Figs 6*B* and 7*B*). If the effects of VIP are exclusively mediated by enhanced cyclic AMP production, then it is not clear why this compound increased slow wave frequency. It is possible that receptors for VIP may be coupled to additional second messengers or that VIP may affect additional cell types (e.g. nerves) causing release of agents with positive chronotropic effects.

In conclusion, the results suggest that ISO, VIP and CGRP, all agonists that are thought to increase the level of cyclic AMP, inhibit contractile activity by decreasing the influx of Ca^{2+}. This could occur directly via inhibition of voltage-dependent Ca^{2+} channels or indirectly by increasing a K^+ conductance. The effects of these agonists also appear to be mediated by a cyclic AMP-dependent decrease in the Ca^{2+} sensitivity of the contractile element and effects on the pacemaker process.

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