Consequences of metabolic inhibition in smooth muscle isolated from guinea-pig stomach

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1. In smooth muscle isolated from the guinea-pig stomach, cyanide (CN) and iodoacetic acid (IAA) were applied to block oxidative phosphorylation and glycolysis, respectively. Effects of IAA on generation of spontaneous mechanical and electrical activities were systematically investigated by comparing those of CN. Spontaneous activity ceased in 10–20 min during applications of 1 mM IAA. On the other hand, application of 1 mM CN also reduced the spontaneous activity, but never terminated it. In the presence of CN the negativity of the resting membrane potential was slightly reduced.

2. When spontaneous activity ceased with IAA, the resting membrane potential was not significantly affected. Also, before ceasing, the amplitude and duration of the spontaneous electrical activity were significantly reduced. The amplitude of the electrotonic potential was, however, not changed by IAA. Further, glibenclamide did not prevent the effects of IAA. These results suggest that, unlike cardiac muscle, activation of metabolism-dependent K^+ channels in stomach smooth muscle does not seem to play a major role in reducing and terminating spontaneous activity during metabolic inhibition.

3. Carbachol-induced contraction transiently increased, and subsequently decreased gradually during application of IAA.

4. After 50 min application of IAA, when there was no spontaneous activity, the concentrations of phosphocreatine (PCr) and ATP measured with ^{31}P nuclear magnetic resonance decreased to 60 and 80% of the control, respectively, while inorganic phosphate (P_i) concentration paradoxically fell to below detectable levels. During subsequent prolonged application of IAA, high-energy phosphates steadily decreased. On the other hand, after 50 min CN application, [PCr] and [ATP] decreased to approximately 30 and 80% of the control, respectively, while [P_i] increased by 2·6-fold.

5. In the presence of either CN or IAA, spontaneous mechanical and electrical activities were reduced or eliminated, although amounts of high-energy phosphates sufficient to contract smooth muscle remained. It can be postulated that some mechanism(s) related to energy metabolism, but not including ATP-sensitive K^+ channels, plays an important role in generating spontaneous activity in guinea-pig stomach smooth muscle. During metabolic inhibition the energy metabolism-dependent mechanism(s) would preserve high-energy phosphates, and consequently cell viability, by stopping spontaneous activity.

The smooth muscle isolated from guinea-pig gastric antrum and corpus shows slow regular phasic contractions. The contractions are accompanied by spontaneous electrical oscillations called slow waves (reviewed by Tomita, 1981). Intestinal motility based on the spontaneous contractions is modulated by sympathetic and parasympathetic nervous activity and other hormonal control, but it is not evoked by oscillatory changes in the activities of these factors.

In guinea-pig stomach smooth muscle, a unique characteristic is low voltage sensitivity of slow wave frequency: when the resting membrane potential is altered using a sucrose gas apparatus (Ohba, Sakamoto & Tomita, 1975) or by applying K^+ channel-opening drugs (Katayama, Huang, Tomita & Brading, 1993), the frequency is only slightly affected. Conversely, it has recently been shown that low concentrations of cAMP-related compounds significantly reduced the slow wave frequency despite little alteration in the resting membrane potential (Tsugeno, Huang, Pang, Chowdhury & Tomita, 1995). The process of producing spontaneous excitation in this tissue seems very different from ordinary excitable cells, e.g. action potentials in nerve and cardiac cells can be terminated by a strong hyper-

polarizing current. In these cells voltage-dependent ionic conductances (e.g. slow delayed rectifier K^+ channels; T-type Ca^{2+} channels; hyperpolarization-activated, non-selective inward current, summarized by Hille, 1992) are considered to play a dominant role in pacemaking activity.

It is well known that metabolic changes modulate mechanical and electrical activities in intestinal smooth muscle. For example, the amplitude of slow waves and resultant contractions are very sensitive to hypoxia and metabolic inhibitors such as cyanide (e.g. Job, 1969; Huang, Chowdhury, Kobayashi & Tomita, 1993), and also the frequency is highly temperature dependent (e.g. Job, 1969; Ohba *et al.* 1975). From these features it can be speculated that instead of voltage-dependent ionic conductances, some mechanism(s) linked to the metabolic processes may generate the unique electrical oscillations seen in guinea-pig stomach. Therefore, it is interesting to investigate how energy metabolism alters its mechanical and electrical activities.

In the present study, we applied cyanide (CN) and iodoacetic acid (IAA) to block oxidative phosphorylation and glycolysis, respectively. Slow waves ceased within 20 min during application of IAA. On the other hand, CN does not completely prevent the generation of the slow waves, although it significantly affects their frequency and amplitude, as previously described (Huang *et al.* 1993). Thus, the effects of IAA on slow waves were systematically investigated by comparing those of CN.

METHODS

Animals

Guinea-pigs of either sex, weighing 300–450 g, were stunned and bled using approved humane techniques. The stomach was dissected, and cut into two parts along the greater and lesser curvatures. After the mucosa was removed, the smooth muscle layer in the corpus and antral regions was used in the experiments.

Mechanical activity

Small strips (1·5–2 mm in width, 15 mm in length) were prepared by cutting the stomach smooth muscle tissue along the circular muscle layer (longitudinal muscle layer remaining attached). The strips were mounted in an organ bath and continuously superfused with physiological saline at a constant flow rate of 2 ml min^{-1} (32–35 °C).

Isometric tension development was recorded using a strain gauge and potentiometric pen recorder. To obtain stable spontaneous activity, preparations were equilibrated for at least 1 h before starting experiments.

Electrical activity

The membrane potential was measured using conventional microelectrode techniques, as previously described (Chihara & Tomita, 1987; Huang *et al.* 1993). The resistance of the electrode filled with 3 M KCl was 30–50 MΩ. A high-impedance amplifier (MEZ-8201, Nihon Koden Ltd, Japan) was used. After the mucosa was removed, muscle preparations (containing both circular and longitudinal muscle layers) were pinned down to a silicone rubber bed, and continuously superfused with physiological saline solutions. The electrodes were normally inserted from the mucous (circular muscle) side.

In some experiments, electrotonic potentials were produced by applying the current pulses extracellularly using a partitioned stimulating chamber (Abe & Tomita, 1968) in order to assess if the ionic conductances in the cell membrane were affected by metabolic inhibition.

^{31}P NMR

The methods employed for ^{31}P NMR measurements were essentially the same as those previously used (Nakayama, Seo, Takai, Tomita & Watari, 1988; Nakayama, Nomura & Tomita, 1994; Nakayama & Nomura, 1995). The smooth muscle preparation of the corpus and antral regions, weighing 0·5–0·7 g, was obtained from three guinea-pigs, and was mounted in a 10 mm diameter sample tube. The sample tube was perfused with warmed solutions (32 °C) at a constant rate of 12 ml min^{-1}. After inserting the sample into the bore of the main magnet, the proton signal from water was used for shimming to improve the homogeneity of the magnetic field. For measurements of phosphorous compounds the NMR spectrometers (Bruker WM-360 and JEOL GSX270W) were operated at 145·8 and 109·4 MHz, respectively. Radio frequency pulses corresponding to a flip angle of 30 deg were applied every 0·6 s. ^{31}P NMR spectra were normally obtained by accumulation of 2500 signals (free induction decays, FIDs) over 25 min. Before Fourier transformation, a line broadening of 15 Hz was applied to enhance the signal-to-noise ratio. Spectral peak resonances (frequencies) were measured relative to that of phosphocreatine (PCr) in parts per million (p.p.m.).

Under control conditions, six major peaks were observed: phosphomonoesters (PME), inorganic phosphate (P_i), PCr and the γ-, α- and β-peaks of ATP. The PME resonance consisted of two peaks (PME 1 and 2) resonating at around 6·8 and 6·3 p.p.m. Concentrations of the phosphorous compounds were estimated by integrating the spectral peaks and by correcting with their saturation factors. The values for saturation correction were as follows: PME 1, 2·14; PME 2, 2·25; P_i, 1·60; PCr, 1·36; β-ATP, 1·07. The saturation factors were obtained by increasing the pulse repetition time to 6 and 12 s. The cytosolic concentration of ATP was estimated from the β-ATP peak, which also includes the contribution of other nucleoside triphosphates (NTPs). Under normal conditions, the ratio of ATP to other NTPs was estimated using high-pressure liquid chromatography. The relative concentrations shown in Figs 6*B* and 7*B* were not corrected by this ratio.

Intracellular pH (pH_i) was normally estimated from the chemical shifts of the P_i peak:

$$pH_i = pK_{a(X)} + \log_{10}((\delta_{o(X)} - \delta_{p(X)})/(\delta_{d(X)} - \delta_{o(X)})), \quad (1)$$

where $pK_{a(X)}$ is the negative logarithm of the dissociation constant in a phosphorous compound (X), and $\delta_{o(X)}$ is its observed chemical shift. $\delta_{p(X)}$ and $\delta_{d(X)}$ are the chemical shifts of the protonated and deprotonated forms of compound X. For P_i, the pK_a, δ_p and δ_d used are 6·70, 3·15 and 5·72, respectively. pH_i was also estimated from the chemical shifts of PME 1 and PME 2 in some cases, e.g. to check whether phosphorylethanolamine (PEt) and phosphorylcholine (PCh) dominate the two PME peaks under normal conditions. The parameters used for the Henderson–Hasselbalch equation were the same as previously described (PME 1: $pK_a = 5·70$, $\delta_p = 3·27$, $\delta_d = 6·95$; PME 2: $pK_a = 5·77$, $\delta_p = 2·65$, $\delta_d = 6·44$; Nakayama, Hachisuka, Itoh, Matsumoto & Tomita, 1995).

High-pressure liquid chromatography

Tissue extracts were made by homogenizing with 6% perchloric acid solution after freezing (Nakayama *et al.* 1995), or by boiling (Ishida & Shibata, 1982). The smooth muscle tissue (100–150 mg) was incubated in 'normal' solution (see Solutions and chemicals) for 1 h before extraction. The contents of nucleoside triphosphates were quantified using high-pressure liquid chromatography (HPLC) with an anion exchange column (TSK gel DEAE-2SW). The eluent was pumped at 1 ml min^{-1} in 100 mM potassium phosphate buffer (pH 6·0) and HPLC grade acetonitrile (15 : 85, v/v). Peaks were detected at 260 nm.

Oxygen consumption

Muscle strips from the guinea-pig stomach were prepared as described above. The solubility of oxygen was taken to be 0·21 μmol ml^{-1}. Oxygen consumption (J_{O_2}) was measured using a Yellow Springs apparatus following the procedures described previously (Clark *et al.* 1994). The muscle strips were stretched and placed in 8 ml of Hepes buffer, equilibrated with 22% ambient oxygen at 35 °C. Basal oxygen consumption under unstimulated conditions was then established for a minimum of 30 min. Mean J_{O_2} was calculated for each of the three phases of the experiment and rates of respiration are reported as moles per minute per gram wet weight.

Solutions and chemicals

The 'normal' solution had the following composition (mM): NaCl, 117; $KHCO_3$, 5·9; $CaCl_2$, 2·4; $MgCl_2$, 1·2; glucose, 11·8; Tris-Cl, 20, pH being adjusted to 7·4–7·5 at 35 °C. When the ionic composition was modified, Na^+ was isosmotically substituted. In NMR experiments 5 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid) was used instead of Tris-Cl, and the solutions were bubbled with oxygen gas. DL-β-Hydroxybutyric acid (sodium salt), iodoacetic acid (sodium salt) and carbamylcholine chloride were purchased from Sigma. ATP (adenosine triphosphate, disodium salt), GTP (guanosine triphosphate, trisodium salt), CTP (cytidine triphosphate, trisodium salt) and UTP (uridine triphosphate, trisodium salt) were from Seikagaku Kogyo (Tokyo, Japan).

Statistics

Numerical data are expressed as means ± S.D. Differences between means were evaluated by Student's paired *t* test, and a test value of less than 0·05 was taken as a statistically significant difference.

RESULTS

Effects of metabolic inhibitors on mechanical activities

In the regions of corpus and antrum of the guinea-pig stomach, as previously described by many authors (e.g. Tomita, 1981; Huang *et al.* 1993), the smooth muscle strips showed spontaneous mechanical activities repeated at a constant interval in each preparation. The frequency range was 3–6 cycles min^{-1} in the muscle strips used in the experiments. Figure 1 shows the effects of cyanide (CN) and iodoacetic acid (IAA) on spontaneous mechanical activities. IAA is known to irreversibly inhibit the glycolytic enzyme, glyceraldehyde-3-dehydrogenase, while CN reversibly inhibits oxidative phosphorylation at the end of the electron transport chain. After observing control mechanical activities, 1 mM CN was applied to normal solution (Fig. 1*A*). The amplitude of the spontaneous tension development was decreased to 11 ± 3% of the control ($n = 8$) within the first minute of CN application. During exposure to the CN-containing solution the amplitude of the spontaneous tension development gradually increased (to 142 ± 27% of the control 30 min after application of CN). However, spontaneous activities occurred at irregular intervals, and the intervals were longer than those seen in the control.

In Fig. 1*B*, the effects of IAA on mechanical activity are shown. β-Hydroxybutyrate (β-HB), directly metabolized through the tricarboxlic acid cycle, was added to the solution

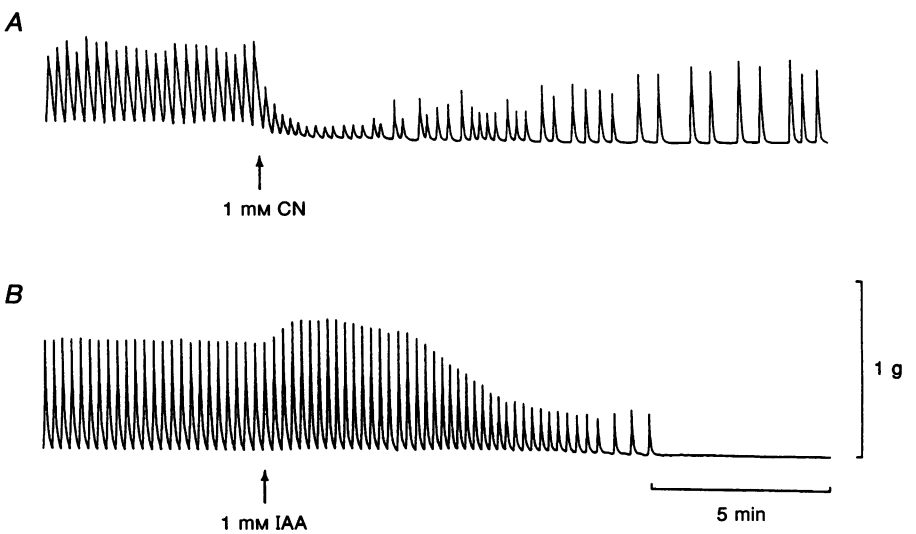

Figure 1. Effects of metabolic inhibition on spontaneous mechanical activity

Applications of 1 mM NaCN (*A*) and 1 mM IAA (*B*) are indicated by arrows. In *B*, 25 min prior to the application of IAA, β-hydroxybutyrate (11·8 mM) was added to the solution.

25 min prior to the application of IAA. Applications of 1 mM IAA transiently increased the amplitude of the spontaneous tension development (to 134 ± 28% of the control), and during the exposure to IAA its amplitude gradually decreased and spontaneous activity ceased in 11·0 ± 4·1 min ($n = 20$) (with a range of 2·9–22·0 min). Before cessation, in some preparations the cycle of the spontaneous activity became irregular, as shown in Fig. 1*B*. Also, when 2-deoxyglucose (11·8 mM) was applied in the absence of glucose to block glycolysis, spontaneous mechanical activities were similarly eliminated, but it required a longer time (13·1 ± 6·3 min, $n = 10$) than by IAA application.

Applications of carbachol (CCh, 10 μM), a muscarinic agonist, in normal solution caused a phasic mechanical contraction followed by a tonic component. In order to check the potency of contraction, applications of CCh for 5 min were repeated four times at 20 min intervals (total 25 min cycle) from 12 min after application of IAA (1 mM) ($n = 8$). Figure 2*A* shows an example of such experiments. In six out

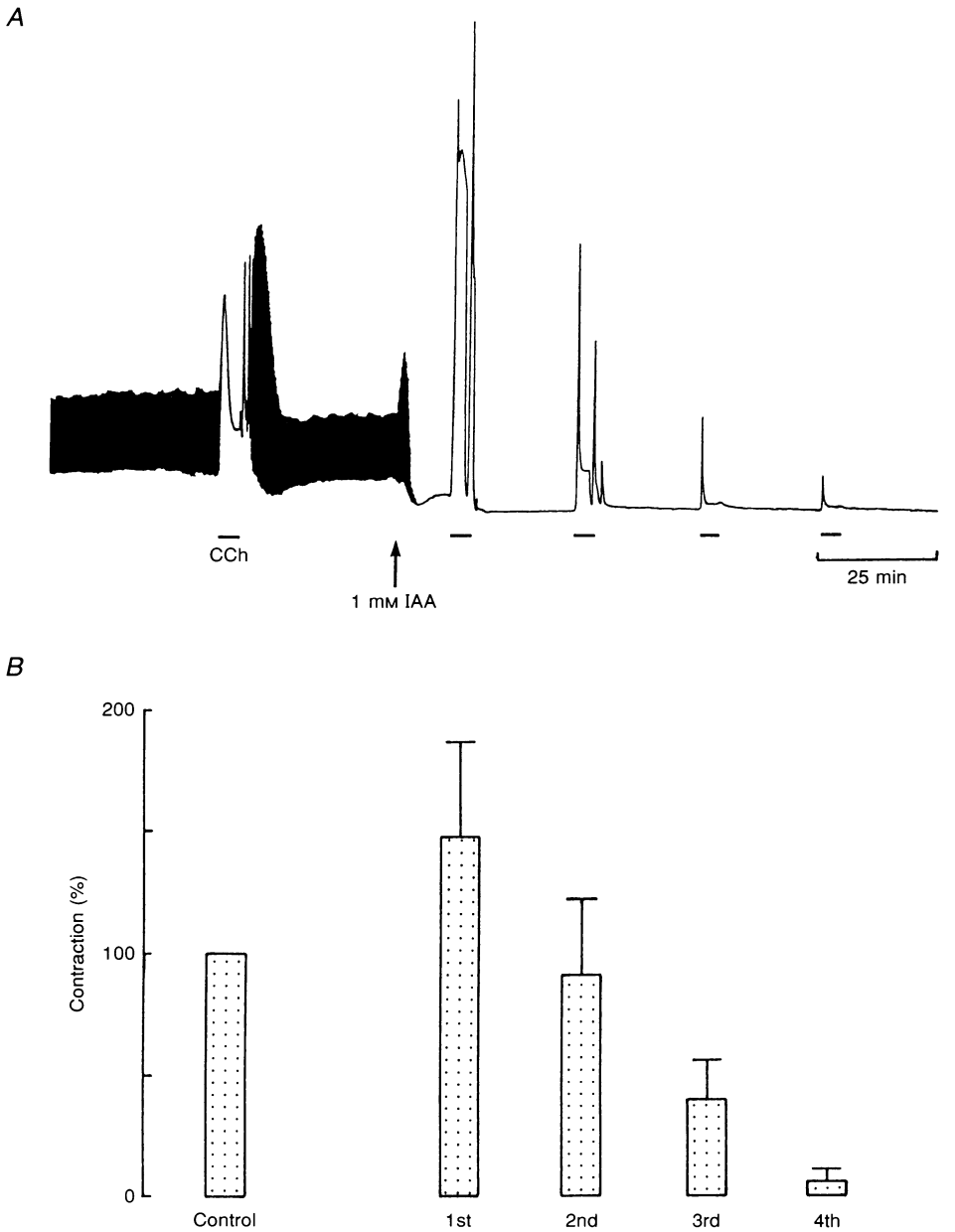

Figure 2. Effects of IAA on CCh-induced contraction

A, example of pen recording. In normal solution, CCh (10 μM, 5 min) was applied to obtain a control response. 12 min after application of IAA (1 mM), CCh-induced contractions (5 min) were reproduced at 20 min intervals. *B*, a graph showing averaged responses ($n = 5$) of the changes in maximum amplitude of the contractions induced by CCh ($n = 8$). The amplitude was normalized by taking the control response as 100%.

of eight preparations, spontaneous mechanical activity was eliminated before application of CCh. The peak tension development induced by CCh was increased to about 150% of the control in the first application of CCh in the presence of IAA (Fig. 2*B*). However, the CCh-induced contraction readily decreased during exposure to IAA, and the amplitude of the fourth contraction fell to below 10% of the control. The first and second CCh-induced contractions had a sizeable tonic component.

Effects on electrical activities

Using the conventional microelectrode technique, changes in the membrane potential upon applications of IAA and CN were measured (Fig. 3). As described by Huang *et al.* (1993), applications of CN (1 mM) depolarized the cell membrane up to several millivolts (with a range of 0–8 mV), and the amplitude of the slow waves was quickly reduced (Fig. 3*A*). During prolonged exposure to CN (25 min) the intervals between slow waves were irregular; however, the generation of slow waves did not cease.

During applications of IAA (1 mM), it has been observed in all preparations that the amplitude and duration of slow waves were gradually reduced, and subsequently ceased ($n = 23$). However, as also described below, the shapes of slow waves varied. It was, therefore, difficult to calculate the mean values of these two parameters. (The area of the slow wave, considered to be most suitable, decreased to 61 ± 21% after 5 min and to 32 ± 14% 10 min after IAA application.)

In the preparation shown in Fig. 3*B*, at around 5 min of IAA application, the peak amplitude (including initial spike) and duration of half-maximal amplitude of the slow waves were 83 and 22% of the control, respectively. When slow

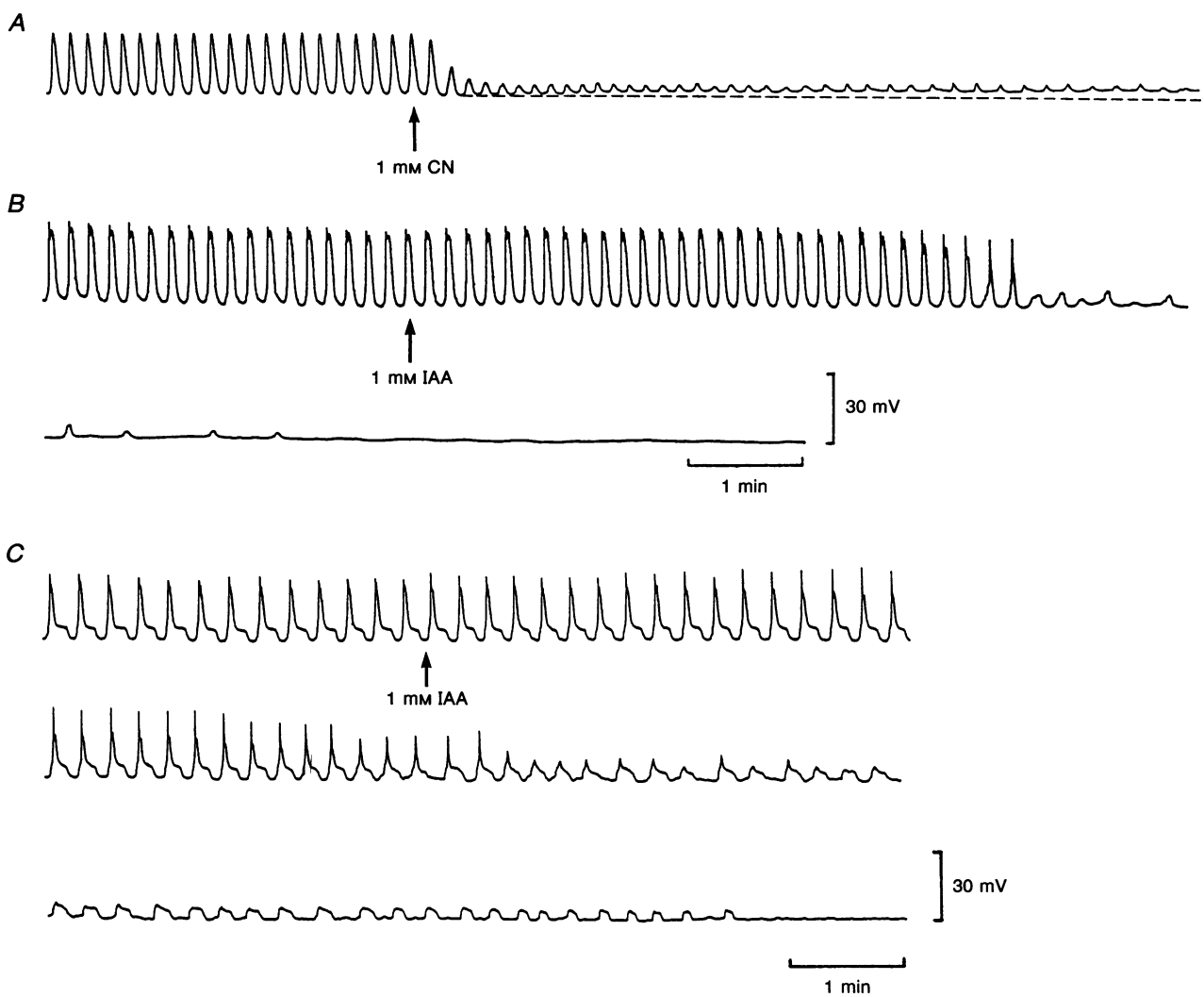

Figure 3. Pen recordings of the membrane potential during metabolic inhibition

Applications of 1 mM NaCN (*A*) and 1 mM IAA (*B*) are indicated by arrows. The dashed line in *A* corresponds to the resting potential under control conditions. The solution used in *B* contained β-HB. In *C*, IAA (1 mM) was applied to slow waves in which two components are distinguishable from the shape.

waves ceased, the resting membrane potential was normally unchanged. However, small depolarizations (~5 mV) were observed in some of the preparations. Also, relative increases in the spike component were usually observed during an early period of IAA application (Figs 3*B* and *C*, and 5), and irregular intervals between slow waves were seen particularly before stopping. The former phenomenon may contribute to the temporary increases in tension development during IAA application (Fig. 1*B*).

A small number of preparations showed slow waves in which two components were clearly distinguished: the first (voltage insensitive) component forms the bottom of the slow wave and the second (voltage sensitive) component is riding on the first component (Ohba *et al.* 1975). Figure 3*C* shows an example pen recording of such slow waves. When IAA was applied to this type of slow wave, reduction of the second component (upper part) was seen prior to changes in the first (bottom) component. Subsequently, slow waves ceased during 10–20 min of IAA application, not accompanied by a significant change in the resting membrane potential.

To confirm whether the cessation of the spontaneous electrical activity was accompanied by changes in the membrane property, effects of IAA were further examined using a partition chamber. In two preparations prolonged depolarizations and hyperpolarizations (for approximately 1 min) were elicited by applying electric current in normal solution. The hyperpolarizing and depolarizing pulses had little effect on the frequency of slow wave generation (only 12% reduction at 30 mV hyperpolarization). The phenomena (including changes in the amplitude) were consistent with those observed using a double sucrose gap technique (Ohba *et al.* 1975).

In Fig. 4, using the partition technique, short depolarizing or hyperpolarizing electric currents of the same magnitude were alternately applied at resting potential (at approximately 50–60 s intervals). During the application of IAA, the amplitude and duration of slow waves were gradually reduced (to 87 and 36% of the control, respectively, after 5 min), while the amplitude of the tonic potential induced by current application was not changed, even up to the time when slow waves ceased. Also, in the presence of glibenclamide (1·5 μM), which completely blocks cromakalim (5 μM)-induced hyperpolarization, the effects of IAA on slow waves remained unchanged (Fig. 5). These results suggest that the changes in amplitude and duration of slow waves were not due to an increase in K^+ conductance (ATP-sensitive K^+ channels) as seen in cardiac muscle during metabolic inhibition.

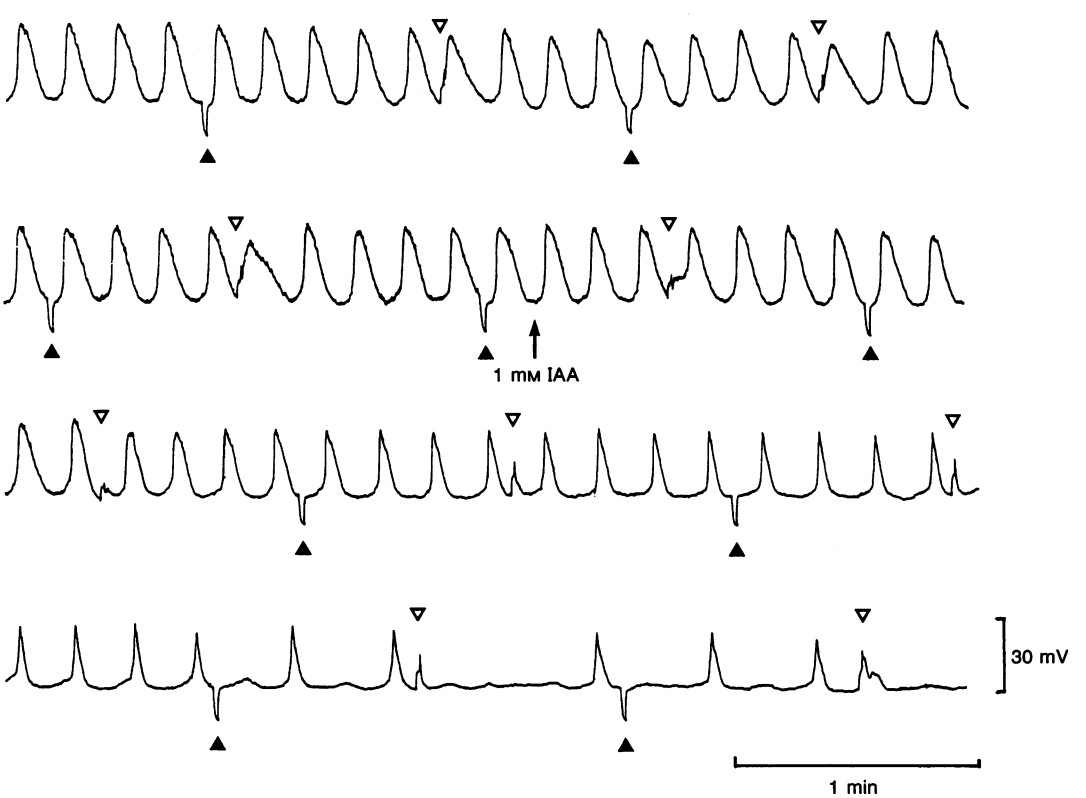

Figure 4. Slow waves recorded in a partition chamber

Short (1200 ms) depolarizing (▲) and hyperpolarizing (▽) currents (of the same magnitude) were alternately applied between slow waves at about 50–60 s intervals. Application of 1 mM IAA is indicated by an arrow.

^{31}P NMR spectrum in stomach smooth muscle

To assess the effects of metabolic inhibition shown above, intracellular phosphorous compounds were measured using ^{31}P NMR. Figures 6*Aa* and 7*Aa* show control spectra obtained from guinea-pig stomach smooth muscle under superfusion with normal solution. As shown in the guinea-pig taenia caeci (Nakayama *et al.* 1988, 1994), six major peaks were observed in stomach smooth muscle: α-, β- and γ-ATP (nucleoside triphosphate); PCr (phosphocreatine); P_i (inorganic phosphate); and PME (phosphomonoesters). PME was normally divided into two peaks (PME 1, PME 2). Compared with the taenia caeci, the PCr peak was significantly smaller and the PME peak larger in the stomach.

From the chemical shifts of the PME peaks (PME 1: 6·78 ± 0·02 p.p.m.; PME 2: 6·27 ± 0·02 p.p.m., $n = 8$), major compounds of the two peaks are presumed to be phosphorylethanolamine and phosphorylcholine, respectively (Nakayama *et al.* 1995). The chemical shift of P_i was 4·96 ± 0·04 p.p.m. ($n = 8$), and the subsequently calculated pH_i from the chemical shift value was 7·08 ± 0·03. [ATP] was estimated from the β-ATP peak area in the ^{31}P NMR spectrum. Taking [ATP] = 1, the relative concentrations of PCr, P_i and PME were 0·92 ± 0·09, 0·54 ± 0·05 and 3·09 ± 0·32, respectively ($n = 10$). Approximately 45% of [PME] was attributed to [PME 1] and 55% to [PME 2].

The ATP peaks in the ^{31}P NMR spectrum are known to contain contributions from other nucleoside triphosphates (NTPs), i.e. GTP, CTP and UTP. Using HPLC, the tissue content of NTP was estimated to be 0·94 ± 0·12 μmol (g wet weight)$^{-1}$. The ATP content was 75·8% of the NTP content. The ratio of ATP to the total NTP was similar to that obtained in other smooth muscles (73–78% in rabbit uterus and urinary bladder: Kushmerick, Dillon, Meyer, Brown, Krisanda & Sweeney, 1986; 80% in guinea-pig taenia caeci: Nakayama & Tomita, 1990). The contents of GTP, CTP and UTP were 10·5, 7·5 and 6·2%, respectively.

Effects of metabolic inhibitors on phosphorous compounds

Figure 6*A* shows the effects of CN on the NMR spectrum. Each spectrum was obtained by the accumulation of 2500 signals over 25 min. Changes in pH_i and phosphorous compounds are plotted in Fig. 6*B*. After observing a control spectrum (Fig. 6*Aa*), 1 mM CN was applied for 50 min. In the spectrum obtained during the 25–50 min period of CN application (Fig. 6*Ac*), [PCr] and [ATP] decreased to 27 and 83% of the control (on average, $n = 4$), respectively, while [P_i] correspondingly increased by 2·6-fold (Fig. 6*B*, lower graph). The pH_i estimated from the chemical shift of P_i, decreased by 0·21 pH units (from 7·07 ± 0·03 to 6·86 ± 0·11) (Fig. 6*B*, upper graph). Washout of CN nearly completely (85–95%) restored high-energy phosphates in 25–50 min. In separate experiments, the effect of CN on oxygen consumption (J_{O_2}) was examined. The J_{O_2} was 0·40 ± 0·16 μmol min^{-1} (g wet weight)$^{-1}$ under normal conditions ($n = 8$) and was decreased to below detectable levels by applications of 1 mM CN.

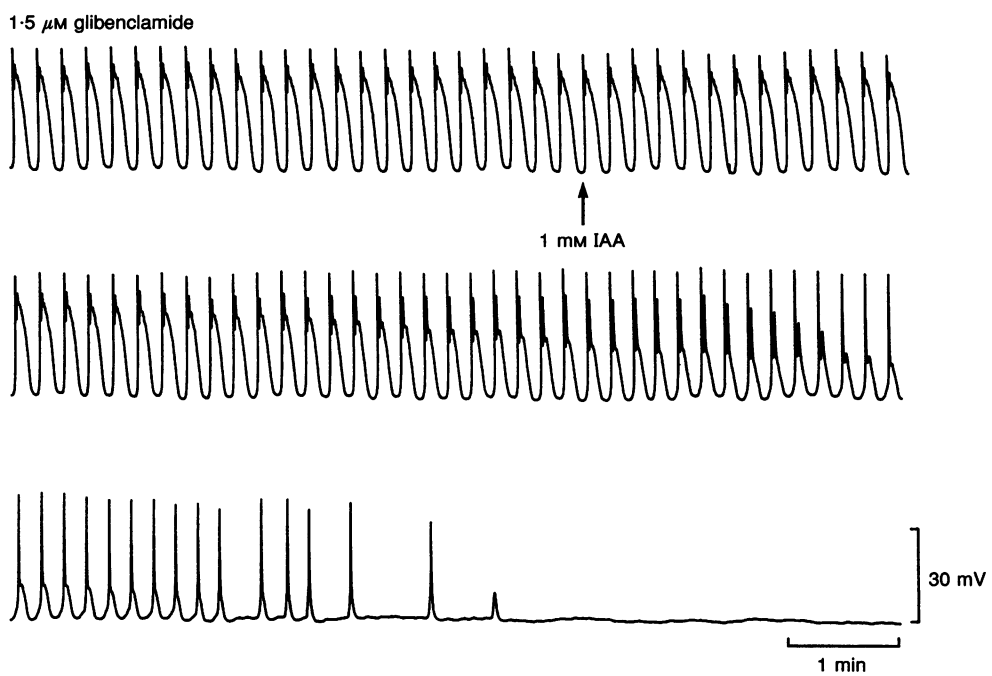

Figure 5. Effects of IAA in the presence of glibenclamide

Slow waves were recorded with the conventional microelectrode technique used in Fig. 3. Glibenclamide (1·5 μM) was added to the solution approximately 5 min prior to application of IAA (1 mM).

The effects of IAA on phosphorous compounds were also examined. Figure 7*A* shows ^{31}P NMR spectra obtained during application of IAA. β-HB (11·8 mM) was added to the solution 25 min prior to application of IAA (1 mM). During the first 25 min application of IAA (Fig. 7*Ab*) [PCr], [ATP] and [P_i] peaks decreased to 81, 93 and 72% of the control (on average, $n = 4$), respectively, while pH_i estimated from the P_i peak was only slightly increased (from 7·08 ± 0·03 to 7·11 ± 0·03; Fig. 7*B*), but the increase was statistically significant. In the second spectrum obtained during application of IAA (after 25–50 min, Fig. 7*Ac*; when it can be assumed that there was no spontaneous activity), [PCr] and [ATP] decreased further to 59 and 79%, respectively, and the P_i peak became undetectable.

During the subsequent 50 min application of IAA (Fig. 7*Ae*), [PCr] and [ATP] decreased to 19 and 30% of the control. On the other hand, [PME] gradually increased to approximately 280% of the control. Aerobic glycolysis is a well-known feature in smooth muscle metabolism (e.g. Paul, 1983). This increase in [PME] is presumably due to an increase in intermediate metabolites in the glycolytic pathway caused by inhibition of glyceraldehyde-3-dehydrogenase. Since the PME peaks contained a considerable amount of phosphorous compounds other than phosphorylethanolamine and phosphorylcholine under exposure to IAA, these peaks were not used to estimate pH_i. Also, since the P_i peak was in the noise level, changes in pH_i were unclear after 25 min application of IAA. In one of the experiments using IAA, 75–100 min after its application the P_i peak became visible again, but was smaller than the control. The pH_i estimated from the chemical shift of P_i (obtained during 75–100 min) was lower (about 0·15 pH units) than the control value.

In one experiment, to prevent glycolysis, 2-deoxyglucose was applied for 100 min instead of IAA. After 50 min the deoxyglucose-6-phosphate (DOG-6-P) peak became large enough to estimate pH_i (Bailey, Williams, Radda & Gadian, 1981). During the subsequent 75 min, a small shift of the DOG-6-P peak to lower frequency (from 7·27 to 7·22 p.p.m.) was observed, while all ATP peaks gradually decreased as seen in the presence of IAA. This result suggested intracellular acidification during prolonged inhibition of glycolysis.

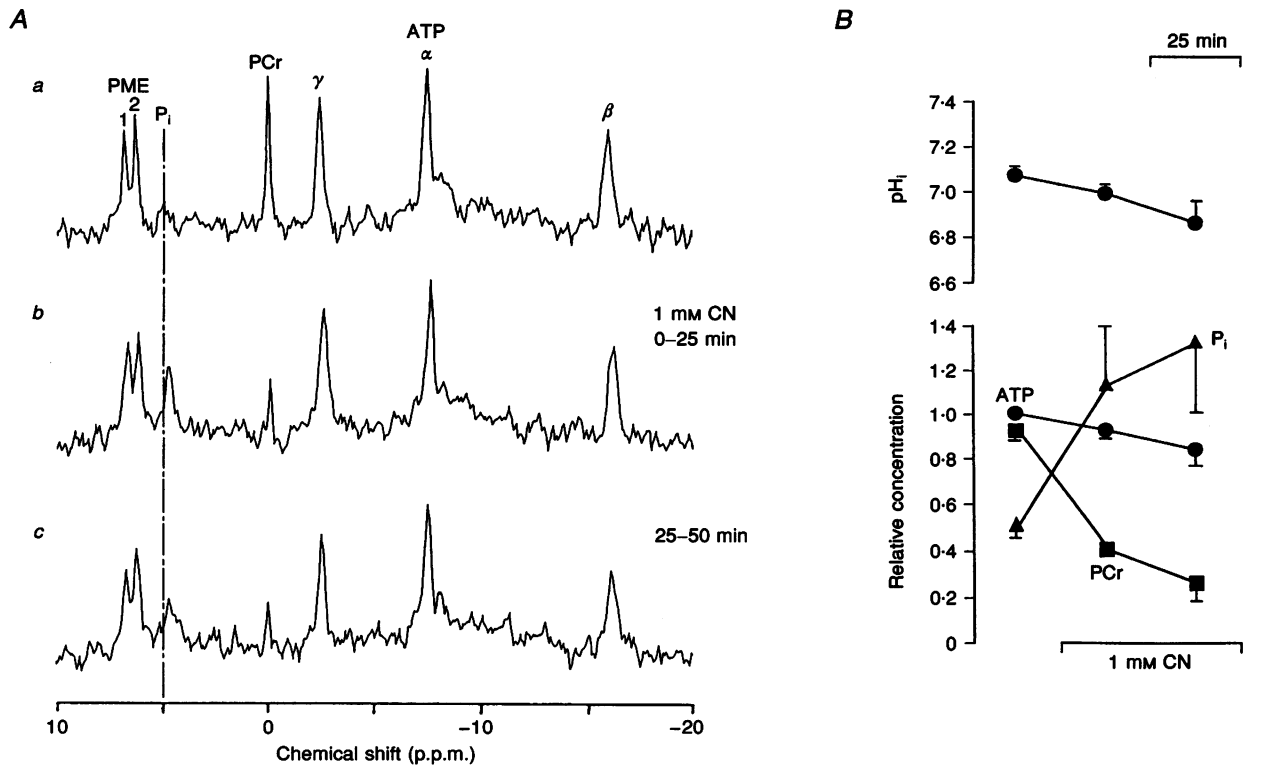

Figure 6. Effects of cyanide (CN) on ^{31}P NMR spectrum and estimated changes in phosphorous compounds

A, effects of CN on ^{31}P NMR spectrum. Each spectrum was obtained by accumulating 2500 FIDs (over 25 min). After observing the control spectrum (*a*), 1 mM CN was applied for 50 min. The spectra *b* and *c* were obtained during 0–25 and 25–50 min applications of CN. The vertical interrupted line indicates the initial chemical shift of the P_i peak. *B*, the time courses of the changes in pH_i (upper graph) and phosphorous compounds (lower graph) are shown. Changes in [PCr] (■), [P_i] (▲) and [ATP] (●) are expressed relative to the initial [ATP] taken to equal 1. The mean values of the data ($n = 4$) are plotted ± S.D. if S.D. values are larger than the size of the symbols.

DISCUSSION

In the present study, we examined the effects of metabolic inhibition on spontaneous mechanical and electrical activities in guinea-pig stomach smooth muscle. ^{31}P NMR was used to monitor the metabolic changes during glycolytic and oxidative inhibition. The spectrum obtained from stomach smooth muscle has five major peaks, as seen in other smooth muscles (e.g. the rabbit and guinea-pig taeniae caeci: Hellstrand & Vogel, 1985; Nakayama *et al.* 1988; rat uterus: Wray, 1990; porcine carotid artery: Clark & Dillon, 1995). Compared with the ^{31}P NMR spectrum obtained from the smooth muscle of guinea-pig taenia caeci which we measured

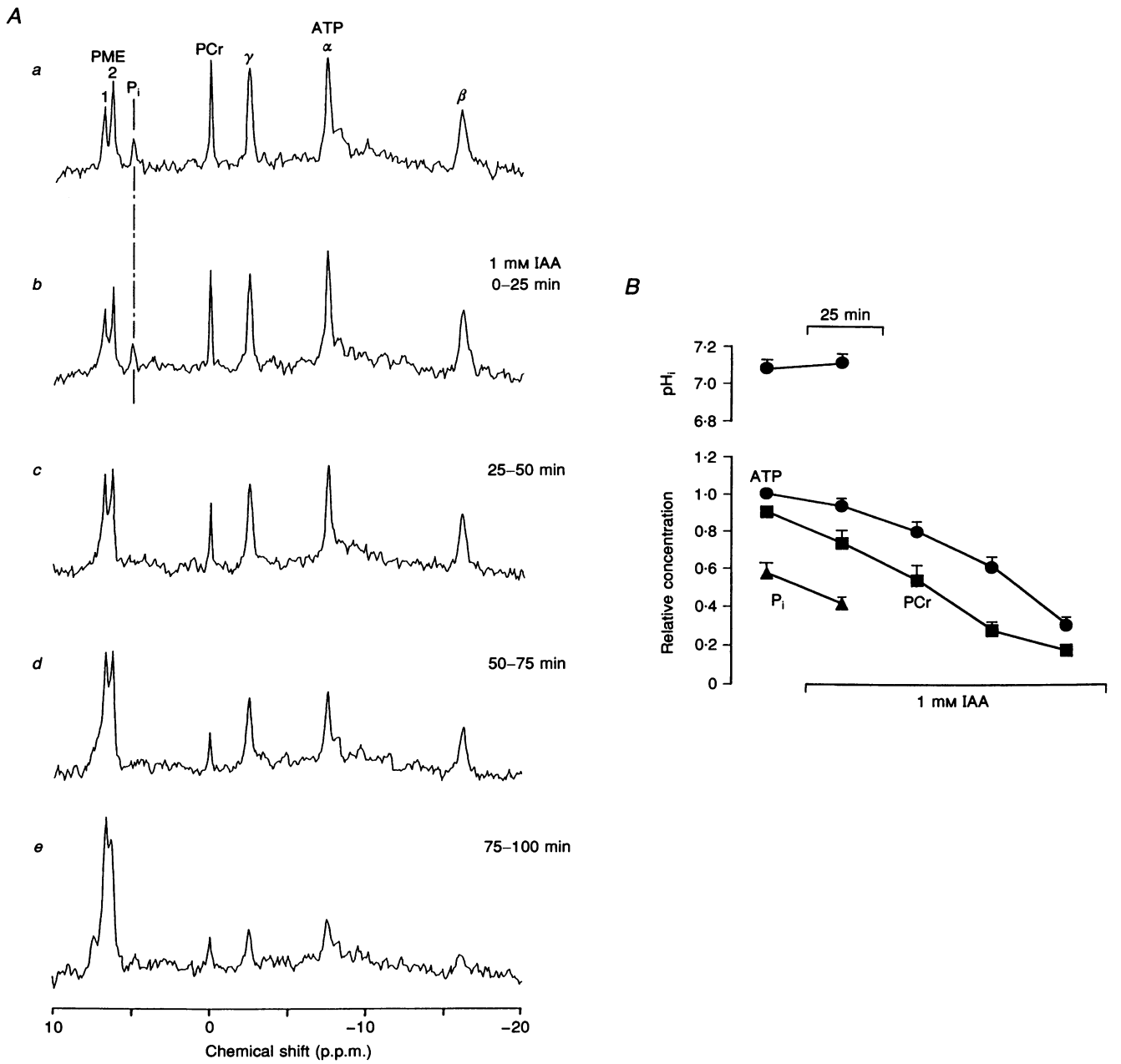

Figure 7. Effects of IAA on ^{31}P NMR spectrum and estimated changes in phosphorous compounds

A, after observing control spectrum (*a*) 1 mM IAA was applied for 100 min (*b–d*). The vertical interrupted line indicates the initial chemical shift of the P_i peak. β-HB (11·8 mM) was contained in the solutions throughout the experiment. *B*, the time courses of the changes in pH_i (upper graph) and phosphorous compounds (lower graph) during application of IAA (1 mM). The P_i peak was not large enough to estimate $[P_i]$ and pH_i 25–100 min after application of IAA. Changes in [PCr] (■), $[P_i]$ (▲) and [ATP] (●) are expressed relative to the initial [ATP] taken to equal 1. The mean values of the data ($n = 4$) are plotted ±S.D. if S.D. values are larger than the size of the symbols.

previously ([PCr]/[ATP] = 1·71, Nakayama *et al.* 1988; [PME]/[ATP] = 1·45, unpublished data of S. Nakayama & T. Tomita), the features of the spectra in stomach smooth muscle are a relatively greater concentration of PME ([PME]/[ATP] = 3·08) and smaller concentration of PCr ([PCr]/[ATP] = 0·92). The chemical shifts of the two PME peaks in conjunction with their pH dependency (Nakayama *et al.* 1995) indicates that under normal conditions these peaks mainly arise from phosphorylethanolamine (PEt) and phosphorylcholine (PCh).

Since the P_i levels were similar in smooth muscles of guinea-pig stomach ($[P_i]/[ATP] = 0{\cdot}54 \pm 0{\cdot}05$) and taenia caeci (0·5–0·6: Nakayama *et al.* 1988; Nakayama & Tomita, 1990, 1991), the smaller [PCr] to [ATP] ratio in stomach is not due to the breakdown of PCr. Therefore, our NMR measurements may indicate the normal [PCr] to [ATP] ratio. Also, it has been reported that smooth muscles which show phasic contractions have a higher [PCr] to [ATP] ratio (e.g. 1·4 in rabbit taenia caeci: Hellstrand & Vogel, 1985; 1·69 in rabbit urinary bladder: Kushmerick *et al.* 1986; approximately 1·7 in guinea-pig urinary bladder: unpublished observations of S. Nakayama and L. M. Smith), while the [PCr] to [ATP] ratio is smaller in tonic smooth muscle (e.g. 0·47 in porcine carotid artery: Clark & Dillon, 1995; 0·7 in sheep aorta: Hardin, Wiseman & Kushmerick, 1992*b*). Stomach smooth muscle also shows phasic contraction, but the time course and frequency of the action potentials are much slower and lower in frequency compared with those seen in taenia caeci and urinary bladder. It is interesting to note that stomach smooth muscle has a [PCr] to [ATP] ratio between those of phasic and tonic smooth muscles.

Application of CN to the guinea-pig stomach stopped regular spontaneous mechanical and electrical activity. These effects of CN were not accompanied by hyperpolarization, but rather the cell membrane was depolarized, despite energy levels being reduced. In this smooth muscle, cromakalim-induced hyperpolarization has previously been shown (Katayama *et al.* 1993). Patch clamp experiments revealed that cromakalim-activated K^+ channels are identical to the K^+ channels which are inhibited by ATP and activated by nucleoside diphosphates (NDP): ATP-sensitive K^+ channels (K_{ATP} channels: Beech, Zhang, Nakao & Bolton, 1993*a*, *b*).

Assuming that stomach smooth muscle contains 1–3 mM ATP under normal conditions and that PCr is 50–75% of the total creatine (Butler & Siegman, 1985), the maximum [ADP] during exposure to CN in the present study is estimated to be 30–40 μM using the creatine kinase equilibrium (e.g. Clark, Kemp & Radda, 1995). This estimated [ADP] value seems high enough to cause some metabolic effects, compared with that reported for half-maximal activation of ATP-sensitive K^+ channels (0·1 mM: Beech *et al.* 1993*a*). However, during 50 min exposure to CN, 80% ATP (which can inhibit opening of K_{ATP} channels; Kajioka, Kitamura & Kuriyama, 1991) still remained.

Application of IAA eliminated spontaneous activities in the guinea-pig stomach smooth muscle in approximately 10–20 min (Fig. 1*B*). However, immediately after its application a small increase in the amplitude of spontaneous mechanical activity was always observed. This was presumably caused by a decrease in $[P_i]$, which attenuates Ca^{2+}-induced tension by shifting the Ca^{2+}-tension relationship (e.g. Itoh, Kanmura & Kuriyama, 1986), and by an increase in the free-energy change for ATP hydrolysis. Similar potentiation of the tension has been reported in sheep aorta under exposure to 2-deoxyglucose-containing solutions (Hardin *et al.* 1992*b*). Alternatively, the increase in tension development could be attributed to modulation of Ca^{2+} movements: a relative increase in the spike component (Fig. 3*B*) and inhibition of the Ca^{2+} pump by blocking glycolysis (Hardin, Raeymaekers & Paul, 1992*a*).

After a transient increase, the amplitude of spontaneous mechanical activity gradually decreased and eventually ceased. During application of IAA, the concentration of high-energy phosphates decreased gradually. However, even after 50 min, about 80% of [ATP] still remained. This amount of ATP was substantially higher than that required to produce contraction in skinned fibre experiments (Iino, 1981; Arner & Hellstrand, 1985). Furthermore, in the presence of IAA, $[P_i]$ decreased to below its control level. Thus, it seems plausible that the decrease in tension development was not due to inhibition of contractile machinery, but probably due to the gradual decrease in the size of the action potential (slow waves in Figs 3*B* and *C*, 4 and 5). The fact that CCh-induced contraction was larger immediately after spontaneous activity was eliminated by IAA (1st contraction in Fig. 2) also supports this hypothesis.

In cardiac muscle, activation of ATP-sensitive K^+ channels is generally thought to cause shortening of the action potential duration during metabolic inhibition (e.g. Nichols, Ripoll & Lederer, 1991), although some dissociation between the fall in ATP and shortening of action potential duration has also been demonstrated (Elliott, Smith, Eisner & Allen, 1992). In guinea-pig stomach smooth muscle, however, cromakalim, an ATP-sensitive K^+ channel opener, induces membrane hyperpolarization, which is often accompanied by increases in the size of the action potential (slow wave), but does not affect its frequency (Katayama *et al.* 1993). In the present experiments, although slow waves decreased in size and eventually ceased during application of IAA, both the membrane potential and the amplitude of the electrotonic potential were relatively unchanged (Figs 3*B* and *C*, and 4). The changes brought about by IAA were distinct from the electrical properties upon activation of ATP-sensitive K^+ channels. This observation is supported by the fact that the effects of IAA on slow waves is reproducible even in the presence of glibenclamide (Fig. 5). Furthermore, compared with CN application, the rate of [PCr] decrease was smaller in the presence of IAA, suggesting a relatively low increase in [ADP], which activates ATP-sensitive K^+ channels. Metabolism-dependent mechanisms other than ATP-sensitive

K^+ channels, therefore, seem to be responsible for the elimination of the spontaneous electrical activities.

Also, with respect to CN poisoning, neither spontaneous mechanical nor electrical activity is restored by glibenclamide (Huang *et al.* 1993). This fact implies, together with the present measurements of phosphorous compounds, that the ATP concentration is high enough to keep ATP-sensitive K^+ channels inactive during application of CN. This suggests that impairment of spontaneous activity is not mainly due to the activation of ATP-sensitive K^+ channels. This interpretation can be compared with the report in rat uterus that glibenclamide does not restore spontaneous contraction during application of CN (Heaton, Wray & Eisner, 1993), although in that particular smooth muscle the CN-induced increase in $^{86}Rb^+$ efflux is partially suppressed by glibenclamide (suggesting some activation of ATP-sensitive K^+ channels under exposure to CN).

During application of IAA the γ- and α-ATP peaks as well as the β-ATP peak, from which the concentration of ATP is estimated, decreased by similar degrees (Fig. 7*A*). ADP contributes to the γ- and α-ATP peaks. Thus, the decrease in all ATP peaks seen in the presence of IAA implies the depletion of the adenylate pool itself and release of some adenosine to the extracellular space (Nakayama *et al.* 1988). Purinoceptor stimulation is known to modulate contractile behaviour in intestinal smooth muscles (Burnstock, 1981). However, in guinea-pig stomach smooth muscle, applications of adenosine (1–300 μM) never eliminate spontaneous electrical activities (unpublished observations of S. Nakayama & S.-M. Huang).

Intracellular pH is also an important factor involved in the modification of smooth muscle contractility (Wray, 1988). Although precise estimation of pH_i was difficult from the present NMR measurements, pH_i seemed to increase slightly (by 0·03 pH units) in the early period of exposure to IAA-containing solution, and then gradually decrease (by 0·15 units). During application of CN pH_i decreased by 0·21 units, while spontaneous activity was disordered but not terminated. Thus, changes in pH_i alone do not explain the termination of spontaneous activity seen in the presence of IAA. The discrepancy that changes in pH_i and spontaneous tension development do not correlate during application of CN has also been described in rat uterine smooth muscle (Taggart & Wray, 1995).

Intracellular acidification during prolonged inhibition of glycolysis was also seen in the case of 2-deoxyglucose application. Both applications of IAA and 2-deoxyglucose significantly decreased [ATP]. Similar phenomena have been reported in uterine smooth muscle (Wray, 1990). The intracellular acidification could be explained by (1) proton release associated with breakdown of ATP (Eisner, Nichols, O'Neill, Smith & Valdeolmillos, 1989) and/or (2) ATP dependence of pH-regulatory mechanisms, e.g. Na^+–H^+ exchange (Demaurex, Romanek, Orlowski & Grinstein, 1997).

Taken together, the results shown in the present study suggest that some energy metabolism-related mechanism(s), although we cannot specify which, plays an important role in generating spontaneous activity in guinea-pig stomach smooth muscle. In particular, when glycolysis was inhibited by IAA, spontaneous activity ceased with no significant change in the membrane potential. Also, after application of either CN or IAA, spontaneous activity was reduced or eliminated while the amounts of high-energy phosphates were still kept above a minimum required concentration for contraction, suggesting that some mechanism(s) was operating to maintain high-energy phosphates and cell viability.

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