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A cyclic GMP-dependent housekeeping Cl^- channel in rabbit gastric parietal cells activated by a vasodilator ecabapide

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1 The membrane potential of rabbit gastric parietal cells is dominated by a Cl^- channel with a sub-picosiemens single channel conductance in the basolateral membrane. The effects of 3-[[[2-(3,4-dimethoxyphenyl)ethyl]carbamoyl]methyl]amino-N-methylbenzamide (DQ-2511: ecabapide), a vasodilator, on the opening of this Cl^- channel, the cyclic GMP content and the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) of parietal cells were investigated by whole-cell patch-clamp technique, enzyme immunoassay and Fura 2-fluorescence measurement.

2 Ecabapide stimulated the opening of the Cl^- channel as determined by the reversal potential. This stimulation was concentration-dependent, and its EC_{50} value was 0.2 μM. Both the basal and ecabapide-induced openings of the channel were inhibited by 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB, 500 μM), a Cl^- channel blocker. Another Cl^- channel blocker, niflumic acid (500 μM) was much less effective.

3 The power spectra of the currents before and after the addition of ecabapide (10 μM) were analysed. Both spectra contained only one Lorentzian ($1/f^2$) component.

4 6-Anilino-5,8-quinolinedione (LY83583; 5 μM), which prevents activation of soluble guanylate cyclase, significantly inhibited both the basal and ecabapide (10 μM)-induced openings of the Cl^- channel.

5 Ecabapide (0.01-100 μM) concentration-dependently elevated the cyclic GMP content in the parietal cell-rich suspension. The EC_{50} value was 0.2 μM.

6 In single Fura 2-loaded parietal cells, ecabapide (10-100 μM) did not increase $[Ca^{2+}]_i$.

7 These results indicate that ecabapide stimulates an intracellular production of cyclic GMP in the parietal cell without increasing $[Ca^{2+}]_i$, and leads to an activation of the housekeeping Cl^- channel.

Keywords: Ecabapide; rabbit stomach; parietal cell; guanylate cyclase; cyclic GMP; intracellular Ca^{2+} concentration; Cl^- channel

Introduction

3-[[[2-(3,4-Dimethoxyphenyl) ethyl] carbamoyl] methyl] amino-N-methylbenzamide (DQ-2511: ecabapide) shows potent inhibitory effects on the development of a variety of experimental gastric and duodenal ulcers in rats (Asano *et al.*, 1990). It was suggested that these effects were mediated by an increase in gastric mucosal blood flow and decreases in secretion of gastric acid and pepsin, and also mediated via so far undefined cytoprotective pathways (Asano *et al.*, 1990; Hirohashi *et al.*, 1993). In isolated arteries of dog and rat, ecabapide elicited vasodilatation via an increase in cyclic GMP content, which may result in an increase in gastric mucosal blood flow (Okamura *et al.*, 1995; Hatanaka *et al.*, 1995). It has not been reported whether ecabapide acts directly on gastric parietal cells.

We have recently reported a membrane potential-dominating (housekeeping) Cl^- channel with sub-picosiemens conductance in the basolateral membrane of rabbit gastric parietal cells (Sakai *et al.*, 1992; 1995; Sakai & Takeguchi, 1993; 1994). Prostaglandin E_2 (PGE_2) activates this Cl^- channel via the EP_3 receptor-mediated Ca^{2+}/ nitric oxide (NO)/cyclic GMP pathway: that is, activation of the EP_3 receptor stimulates Ca^{2+}-dependent NO synthase, inducing the subsequent activation of guanylate cyclase (Sakai *et al.*, 1995).

In the present study, we investigated whether ecabapide affected the opening of the housekeeping Cl^- channel in rabbit parietal cells, and found that ecabapide activated the Cl^- channel via an increase in intracellular guanosine 3':5'-cyclic monophosphate (cyclic GMP) content.

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Methods

Isolation of gastric glands

Gastric glands were prepared from male Japanese white rabbits (weighing 1-3 kg) as described previously (Berglindh *et al.*, 1980). Rabbits had free access to water and food until the day of the experiment. Animals were killed by the intraperitoneal administration of an overdose of urethane (> 2 g kg^{-1}). The stomach was removed and the gastric mucosa was digested with 170 units ml^{-1} of collagenase at 35-37°C for 40 min. Isolated glands were further treated with 500 tyrosine units ml^{-1} of Actinase E at 23-25°C for 5 min. As a result of the additional digestion, parietal cells protruded from the base of glands and had no leaky connection between the intracellular canaliculi and the lumen (Sakai *et al.*, 1992). Isolated gastric glands were suspended in a medium (hereafter, called the respiratory medium; composition, mM NaCl 132.4, KCl 5.4, Na_2HPO_4 5, NaH_2PO_4 1, $MgSO_4$ 1.2, $CaCl_2$ 1, HEPES 10, pH 7.35) supplemented with 2 mg ml^{-1} bovine serum albumin and 2 mg ml^{-1} glucose.

Preparation of the parietal cell-rich suspension

Isolated rabbit gastric glands were treated with 4,000 units ml^{-1} Actinase E at 35-37°C for 50 min. Then, parietal cells in the suspension were separated by use of a continuous Percoll gradient as described elsewhere (Kashiwagura *et al.*, 1990). The gradient was formed by centrifuging a mixture of Percoll (Pharmacia, Uppsala, Sweden), the respiratory medium (pH 7.0) and 1.5 M NaCl (45:50:5 in v/v) at 23,000 *g* for 50 min. The fraction of parietal cells was further purified with a Beckmann J2-21M elutriator centrifuge (Soll, 1978). It consisted of 86±1% parietal cells (mean±s.e., 16 animals).

Patch-clamp experiments

Whole-cell currents recorded from rabbit parietal cells in the isolated gastric glands were previously established to be due to the current through the basolateral membrane (Sakai *et al.*, 1992; Sakai & Takeguchi, 1993). An EPC-7 patch clamp system (List Electronic, Darmstadt, Germany) was used for whole-cell recordings, and necessary corrections were made as described elsewhere (Hamill *et al.*, 1981; Sakai *et al.*, 1992). The following extracellular bathing and intracellular pipette solutions were used (Sakai *et al.*, 1989). The $133K^+ - 142\ Cl^-$ bathing solution contained (in mM): KCl 133, NaCl 7, sodium aspartate 1, $MgCl_2$ 1, $CaSO_4$ 1, ouabain 0.1 and HEPES 10 (pH 7.3). The $133K^+$-$13Cl^-$ pipette solution contained (in mM): potassium aspartate 133, NaCl 7, $MgCl_2$ 3, $CaSO_4$ 0.062, EGTA 0.1, ATP 2 and HEPES 10 (pCa 7, pH 7.3). The $133K^+$-$13Cl^-$ (BAPTA) pipette solution contained (in mM): potassium aspartate 133, NaCl 7, $MgCl_2$ 3, $CaSO_4$ 4.7, BAPTA 5, ATP 2 and HEPES 10 (pCa 7, pH 7.3). After achievement of whole-cell configuration, the seal between the cell and the patch electrode and the cell activity were checked by measuring the cell resistance and the membrane potential, respectively. This procedure took 10–30 s before the start of the current recording. Whole-cell Cl^- currents were recorded continuously at a holding potential of 0 mV, the zero-current potential for K^+ and non-selective cation channels. It was noted that the selectivities of K^+ and Na^+ for the non-selective cation channel of rabbit parietal cells (22 picosiemens) were not significantly different, and the reversal potential of the channel was close to 0 mV (Sakai *et al.*, 1989). To determine the reversal potential for the current opened by ecabapide and closed by NPPB, we recorded whole-cell currents in response to voltage ramps from −100 to +50 mv for 1 s in both the absence and presence of the drug, and measured the potential at which two ramps crossed. The ramp pulses were generated using the pCLAMP 6 programme (Axon Instruments, Inc., Foster City, CA, U.S.A.) installed onto an IBM AT-compatible computer. The computer was equipped with the Digidata 1200A (Axon Instruments, Inc.), that has D/A output and A/D input channels. Experiments were performed at 35–37°C.

Noise analysis of the whole-cell Cl^- current

Whole-cell Cl^- currents were low-pass filtered (1 kHz), amplified via a low-noise preamplifier (Model 113, Princeton Applied Research, Princeton, NJ, U.S.A.) and recorded on a tape-recorder (Sony A-65). For noise analysis of the Cl^- current, the tape was replayed at the same speed and currents were high-pass filtered (0.3 Hz) via the preamplifier. The roll-off frequencies of the preamplifier were switch selectable and the frequency indicated −3 dB point of 6 dB/octave roll-off curve. The power spectrum ($S_{(f)}$) of whole-cell currents was calculated using a signal analyser (SM-2100A, Iwatsu Electric Co., Tokyo, Japan). Data for ten power spectra were collected for 10 s, and the averaged spectrum was obtained. The spectrum was composed of Lorentzian ($1/f^2$) and 1/f components, as previously reported (Sakai *et al.*, 1992). We used the following equation (Conti *et al.*, 1975; Sakai *et al.*, 1992) to simulate the power spectrum, using a self-made BASIC programme based on the non-linear least-squares method:

$$S_{(f)} = \frac{\gamma I(V_m - V_{eq})(1 - P_o)/\pi\, f_c}{1 + (f/f_c)^2} + \frac{A}{f} \qquad (1)$$

where f is the frequency, f_c is the half-power frequency, γ is the single channel conductance, I is the mean membrane current, V_m is the clamped membrane potential, V_{eq} is the equilibrium (zero-current) potential, P_o is the open probability of the single channel, and A is the fitting constant. It is known that statistical open-closed behaviour of ion channels gives $1/f^2$ noise (Anderson & Stevens, 1973) and that fluctuations in the passive ion transport through leakage or open (not open-closed) channels give 1/f noise (Takeguchi *et al.* 1985).

Measurement of [cyclic GMP]$_i$ in parietal cells

Isolated cells rich in the parietal cell were suspended in the $133K^+ - 142Cl^-$ bathing solution (1×10^6 cells ml^{-1}) followed by a 5 min-incubation at 35°C. Trichloroacetic acid (6%, w/v) was added to the cell suspension before (0 min) and after the addition of ecabapide, LY83583 or vehicle (0.1% dimethyl sulphoxide or 0.2% ethanol). The reaction mixture was kept on ice for 10 min, and centrifuged at 8,500 *g* for 10 min at 4°C. Then, the supernatant was collected and washed three times with 2 ml diethyl ether, and freeze-dried for 12–15 h. The cyclic GMP content of the preparation was determined with a cyclic GMP enzyme immunoassay kit (Amersham, Buckinghamshire, U.K.).

Measurement of [Ca^{2+}]$_i$ in single parietal cells

Isolated gastric glands were suspended in the dye-loading buffer (40–50 mg wet wt. ml^{-1}). The buffer contained (in mM) NaCl 100, KCl 5.4, $MgCl_2$ 1.2, $CaCl_2$ 1, HEPES 20, pyruvate 10, glutamate 10, fumarate 10 and ascorbate 10 (pH 7.35). Then, Fura 2-AM (5 μM) with a detergent Pluronic F127 (0.025%, w/v) was added to the suspension, and incubated for 40 min at 22°C. After loading, the glands were washed, and warmed at 35°C in the $133K^+ - 142Cl^-$ bathing solution before the measurement. Fura 2-loaded single parietal cells in gastric glands were observed under an inverted fluorescence microscope (Nikon TMD-EFQ). The total fluorescence intensity from a single parietal cell was monitored at excitation wavelengths of 340 and 380 nm with an emission wavelength of 510 nm (interference filter) using a photon-counting technique (Spex Fluorolog-2 spectrofluorometer, Edison, NJ, U.S.A.). After corrections for background fluorescence, the intensity ratio (340 nm/380 nm) and $[Ca^{2+}]_i$ were calculated as previously described (Grynkiewicz *et al.*, 1985; Koike *et al.*, 1992).

Chemicals

Collagenase (Wako Pure Chemical Industries, Osaka, Japan) and Actinase E (Kaken Pharmaceutical Co., Tokyo, Japan) were used for preparing gastric glands. Ecabapide (DQ-2511) was a generous gift from Daiichi Pharmaceutical Co. (Tokyo, Japan). 5-Nitro-2-(3-phenylpropylamino)-benzoate (NPPB) was synthesized in this laboratory following a method described elsewhere (Wangemann *et al.*, 1986). Niflumic acid was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ecabapide, NPPB and niflumic acid were dissolved in dimethyl sulphoxide, and were diluted to final concentrations just before use. 6-Anilino-5,8-quinolinedione (LY83583; Calbiochem-Novabiochem Co., La Jolla, CA, U.S.A.) was dissolved in ethanol, and diluted with the pipette or bathing solution just before use. Dimethyl sulphoxide and ethanol concentrations in the final solutions never exceeded 0.5%, at which concentration the vehicles *per se* did not affect the whole-cell Cl^- current, intracellular cyclic GMP content ([cyclic GMP]$_i$) and intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) of parietal cells. PGE_2 (Toray Industries, Tokyo, Japan) and KT5823 (Kyowa Medex Co., Tokyo, Japan) were generous gifts. 1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, pentaacetoxymethyl ester (Fura 2-AM), O,O′-*bis*(2-aminoethyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and O,O′-*bis*(2-aminophenyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) were obtained from Dojindo Laboratories Co. (Kumamoto, Japan). Pluronic F127 was obtained from Molecular Probes (Eugene, OR, U.S.A.).

Statistics

Results are expressed as mean ± standard error. Statistical significance was evaluated by Student's *t* test. *P* value below 0.05 was considered to be significant. The EC_{50} values of data

shown in Figures 1b and 6b were calculated using the KaleidaGraph programme, version 3.0.4 (Synergy Software, Reading, PA, U.S.A.) installed onto a computer (Power Macintosh 8100/100AV).

Results

Effect of ecabapide on whole-cell Cl^- currents in rabbit parietal cells

We found previously that the whole-cell Cl^- current of a rabbit gastric parietal cell arose from opening of one kind of housekeeping Cl^- channel (Sakai *et al.*,1992; Sakai & Takeguchi, 1993). A unit conductance of the channel was 0.3–0.4 picosiemens (Sakai *et al.*, 1992), which was too small to be detected in patches of single channel recordings (Sakai *et al.*, 1989). Here, the Cl^- channel opening and closure were observed by increase or decrease in the whole-cell outward Cl^- current recorded at the clamped voltage of 0 mV, respectively. Ecabapide increased the outward Cl^- current with a slow time course, and the maximal effect was observed about 6 min after the addition of ecabapide (Figure 1a). The effect of ecabapide ($0.01-100\ \mu M$) was concentration-dependent, and the EC_{50} was 0.2 μM (Figure 1b).

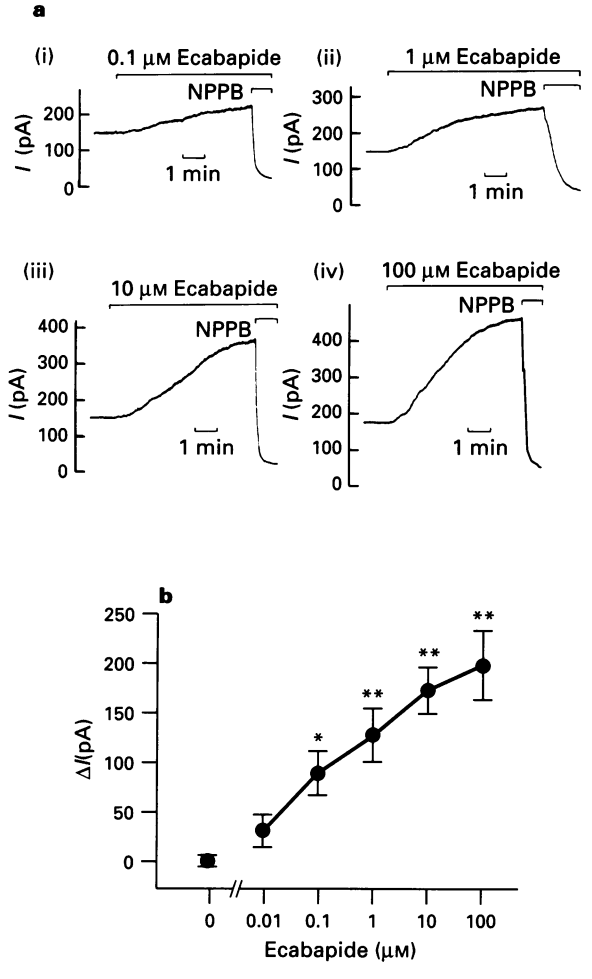

Figure 1 Ecabapide-induced increase in whole-cell outward Cl^- currents recorded from single parietal cells. (a) Representative traces of the whole-cell Cl^- current recorded at 0 mV from single parietal cells in isolated gastric glands. The bathing solutions containing 0.1 μM (i), 1 μM (ii), 10 μM (iii) or 100 μM ecabapide (iv) were perfused as indicated; 500 μM NPPB was used. (b) The concentration-response curve for activation of the whole-cell Cl^- current by ecabapide. The increased current (ΔI) was assessed 6 min after the addition of ecabapide or vehicle ($n = 4-6$). Significantly different from the value at 0 μM: $^*P<0.05$ and $^{**}P<0.01$.

Effects of Cl^- channel blockers on the whole-cell Cl^- current

Both the basal and ecabapide-elicited Cl^- currents were inhibited by 500 μM NPPB (Figure 1a), which inhibits the housekeeping Cl^- channel of rabbit parietal cells with a high concentration ($IC_{50} = 300\ \mu M$) (Sakai *et al.*, 1992). On the other hand, niflumic acid (500 μM) did not significantly decrease either the basal Cl^- current (from 202 ± 9 to 188 ± 7 pA, $n = 4$) or the Cl^- current observed after stimulation with 10 μM ecabapide for 6 min (from 447 ± 53 to 403 ± 48 pA, $n = 4$). Here, the inhibitory effects were determined 30 s after the application of the blocker.

Reversal potentials of the ecabapide-elicited and NPPB-sensitive currents

The reversal potentials of the ecabapide (10 μM)-elicited current (Figure 2a) and the NPPB (500 μM)-sensitive current (Figure 2b) were -58.1 ± 3.9 and -58.0 ± 2.9 mV ($n = 4$), respectively. These values were slightly different from the theoretical Cl^- equilibrium potential at 36°C (−64 mV), but the differences were not significant ($P > 0.05$).

In Figure 3, inward currents were recorded at −64 mV. Although 10 μM ecabapide slightly increased the inward current (Figure 3a and c) and 500 μM NPPB slightly decreased the current (Figure 3b and d), neither of these effects was significant ($P > 0.05$). These very small effects of ecabapide and NPPB at −64 mV may be generated by partial permeation of aspartate ion in the solutions via the Cl^- channel as previously reported in detail (Sakai *et al.*, 1989).

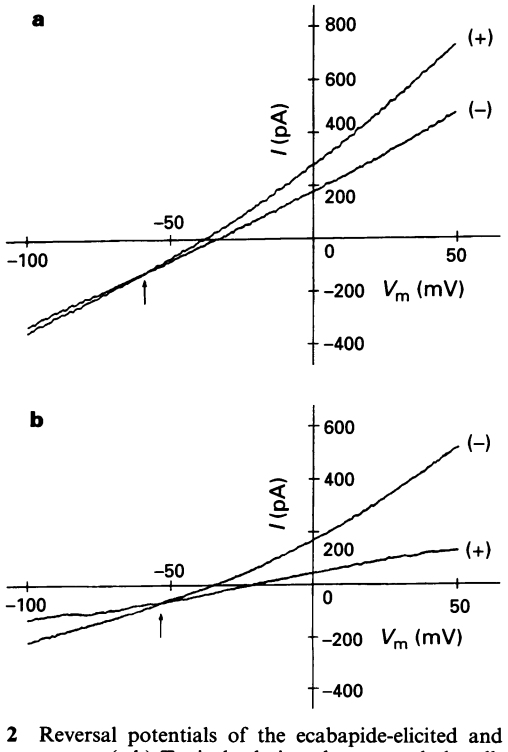

Figure 2 Reversal potentials of the ecabapide-elicited and NPPB-sensitive currents. (a,b) Typical relations between whole-cell currents (I) and membrane potential (V_m). The currents in response to voltage ramps from −100 to +50 mV for 1 s were recorded before (−) and 6 min (a,+) or 30 s (b,+) after the addition of 10 μM ecabapide (a) or 500 μM NPPB (b). The reversal potential at which two ramps cross is indicated by the arrow.

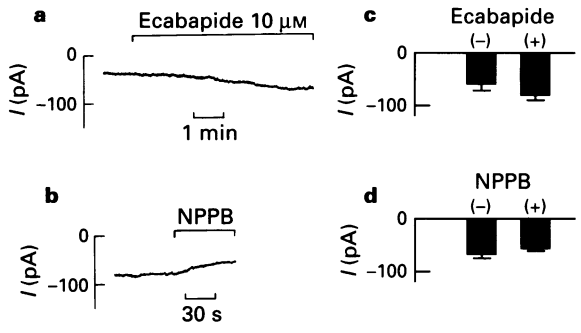

Figure 3 Effects of ecabapide and NPPB on whole-cell inward currents recorded at the Cl^- equilibrium potential. (a,b) Representative traces of the whole-cell current recorded at the Cl^- equilibrium potential (−64 mV). The bathing solutions containing 10 μM ecabapide (a) or 500 μM NPPB (b) were perfused as indicated. (c,d) The Cl^- currents were measured before (−) and 6 min (c, +) or 30 s (d, +) after the addition of ecabapide (c) or NPPB (d) ($n = 4-5$).

Noise analysis of the whole-cell Cl^- current

To examine whether the Cl^- channel activated by ecabapide is the same as the channel that contributed to the basal Cl^- current, we analysed power spectra of the whole-cell Cl^- current recorded at 0 mV. The current fluctuation increased after the addition of 10 μM ecabapide (Figure 4a(i) and b(i)). The power spectra before (Figure 4a(ii), line O) and 6 min after (Figure 4b(ii), line ○) the addition of ecabapide could be simulated with the sum of $1/f^2$ (line △) and $1/f$ (line ▲) noises (see eqn (1)). The slope values of spectra (line ○) in the range from 20 to 50 Hz were −1.5 (Figure 4a(ii)) and −1.6 (Figure 4b(ii)) on logarithmic scales. The non-linear least-squares curve-fitting showed both $1/f^2$ spectra (Figure 4a(ii) and b(ii)) have only one corner frequency. The corner frequencies before and after the stimulation were 9.1 ± 0.7 and 3.3 ± 0.6 Hz, respectively ($n = 3$). These results indicate that one kind of open-closed Cl^- channels contributes to both basal and the ecabapide-elicited Cl^- currents. The significant shift of the corner frequency (f_c) after the stimulation reflects an increase of the open probability of the channel (P_o) (see Eqn (1)).

Inhibition of the basal and ecabapide-elicited Cl^- currents by LY83583

To see reproducible effects of ecabapide, it had to be added within 10 min after the start of current recording. LY83583, which prevents activation of soluble guanylate cyclase (Schmidt *et al.*, 1985; Mülsch *et al.*, 1988), was reported to be effective when it was added to the extracellular solution (Mülsch *et al.*, 1989; Cherry *et al.*, 1990; Kontos & Wei, 1993), because LY83583 rapidly permeated into the cells and transformed into an active inhibitor by intracellular reduction (Mülsch *et al.*, 1988; 1989; Kontos & Wei, 1993). In the present patch-clamp experiments, however, extracellular LY83583 (10 μM) had no effects on the Cl^- current during 10 min (4 observations, data not shown). Under our experimental conditions, permeation of extracellular LY83583 into rabbit parietal cells for 10 min was considered not to be sufficient to have an effect. We, therefore, added LY83583 to the intracellular pipette solution.

As shown in Figure 5b, the intracellular addition of LY83583 (5 μM) significantly decreased the basal Cl^- current from 215 ± 19 to 164 ± 18 pA ($n = 5$, $P < 0.05$). The control basal current was measured 21 ± 7 s after the whole-cell configuration was achieved (see Methods). The maximal effect (plateau phase) was observed 170 ± 33 s ($n = 5$) after the start of current recording. The effect of 10 μM ecabapide in the presence of LY83583 (Figure 5b and c) was significantly smaller than that in the absence of LY83583 (Figure 5a and c).

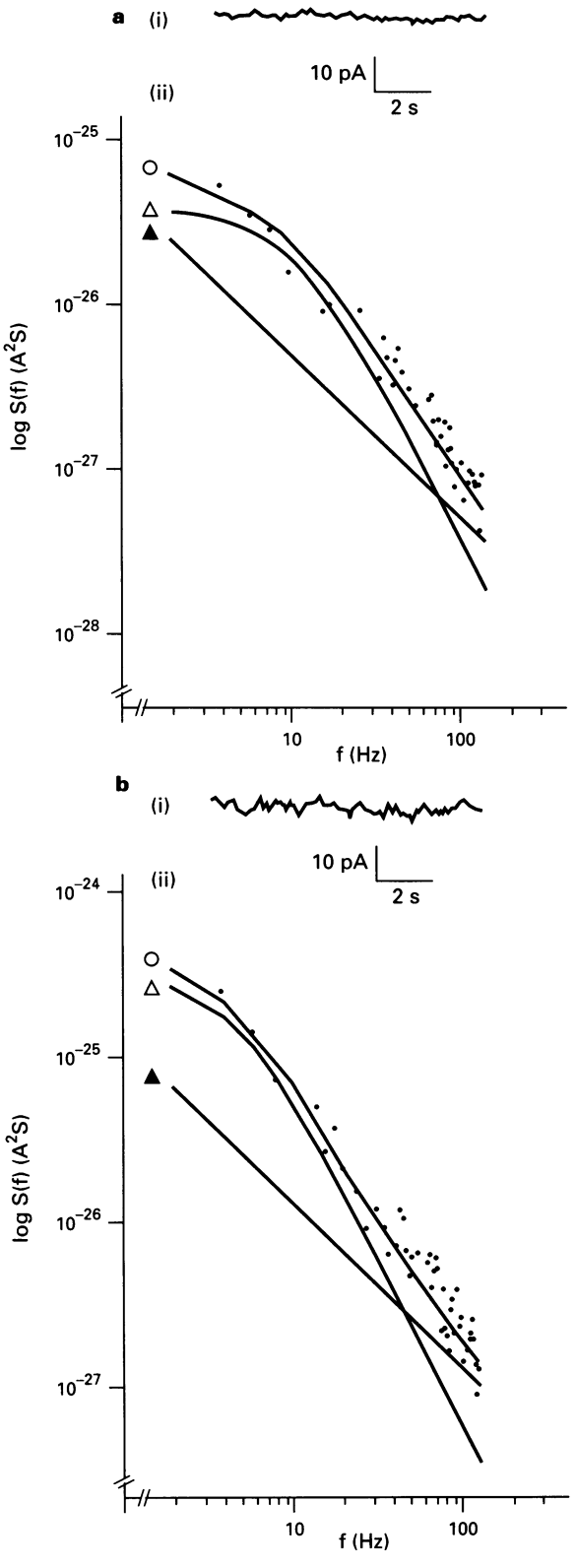

Figure 4 Whole-cell Cl^- current noises and the power spectra. (a,b) Representative traces of whole-cell Cl^- currents recorded at 0 mV (i) and the corresponding power spectra of the Cl^- currents (ii). The data were obtained before (a) and 6 min after (b) the addition of 10 μM ecabapide. In (ii), the fitted lines (○) were drawn as described in Methods. Lines (△) and (▲) represent $1/f^2$ (Lorentzian) and $1/f$ components, respectively.

These results indicate that production of cyclic GMP is involved in the ecabapide-induced activation of the Cl^- channel. Furthermore, the basal Cl^- current was also affected by the

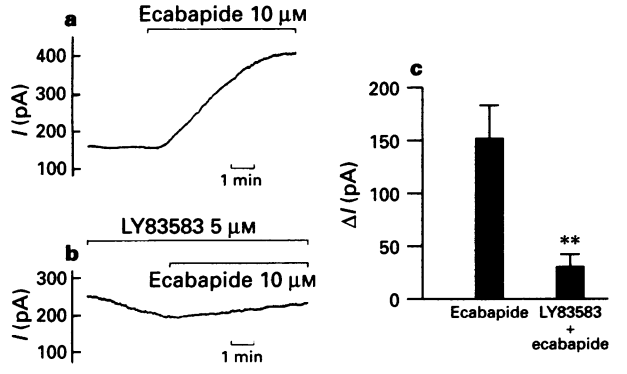

Figure 5 Inhibition of the ecabapide-induced Cl^- current by LY83583. (a,b) Typical traces of the whole-cell Cl^- current recorded at 0 mV. In (b), the intracellular pipette solution contained 5 μM LY83583. The bathing solution containing 10 μM ecabapide was perfused as indicated. (c) The ecabapide-elicited currents (ΔI) were assessed 6 min after the addition of ecabapide ($n=4-5$). **Significantly different **($P<0.01$) from the effect of 10 μM ecabapide alone.

cyclic GMP level. However, the LY83583-induced inhibition of the basal current (−24%) was smaller than that of the ecabapide-elicited current (−79%).

Increase of [cyclic GMP]$_i$ by ecabapide in the parietal cell

From results obtained with whole-cell recording (Figure 5), ecabapide was speculated to increase [cyclic GMP]$_i$. Here, we found that ecabapide (10 μM) increased [cyclic GMP]$_i$ in the parietal cell-rich suspension in a time-dependent manner, and the significant increases were observed 1, 3 and 5 min after the addition of ecabapide (Figure 6a). In Figure 6b, ecabapide-induced net increases of [cyclic GMP]$_i$ assessed 5 min after the stimulation are plotted as a function of ecabapide (0.01–100 μM). The EC_{50} value was 0.2 μM.

Inhibition of the basal and ecabapide-increased [cyclic GMP]$_i$ by LY83583

We tested the effect of LY83583 on both the basal and ecabapide-increased [cyclic GMP]$_i$ in the parietal cell-rich suspension. The addition of LY83583 (10 μM) did not significantly change the basal [cyclic GMP]$_i$ during 10 min (5 observations, data not shown). On the other hand, 30 min incubation of the cells with LY83583 (10 μM) partially but significantly decreased the basal [cyclic GMP]$_i$ (22% of the total; Figure 7b). The subsequent addition of ecabapide (10 μM) did not cause an elevation of [cyclic GMP]$_i$ (Figure 7b), while the ecabapide-induced effect was significant in the control experiment (Figure 7a). These changes correspond with the results from the patch-clamp experiments (Figure 5).

Effect of ecabapide on [Ca^{2+}]$_i$ in single parietal cells

In Fura 2-loaded single parietal cells, ecabapide (10 μM) did not increase [Ca^{2+}]$_i$ (Figure 8), whereas prostaglandin E_2 (PGE_2, 10 μM) induced a significant increase in [Ca^{2+}]$_i$ (Figure 8) as reported recently (Sakai *et al.*, 1995). Higher concentrations of ecabapide (100 μM) also did not affect the [Ca^{2+}]$_i$ level (Δ[Ca^{2+}]$_i$ was 0±1 nM, $n=6$).

Effect of ecabapide on the Cl^- current does not require a rise in [Ca^{2+}]$_i$

Since the conditions used to study the effect of ecabapide on [Ca^{2+}]$_i$ were different from those used for the whole-cell recording, it was possible that ecabapide could induce a rise in

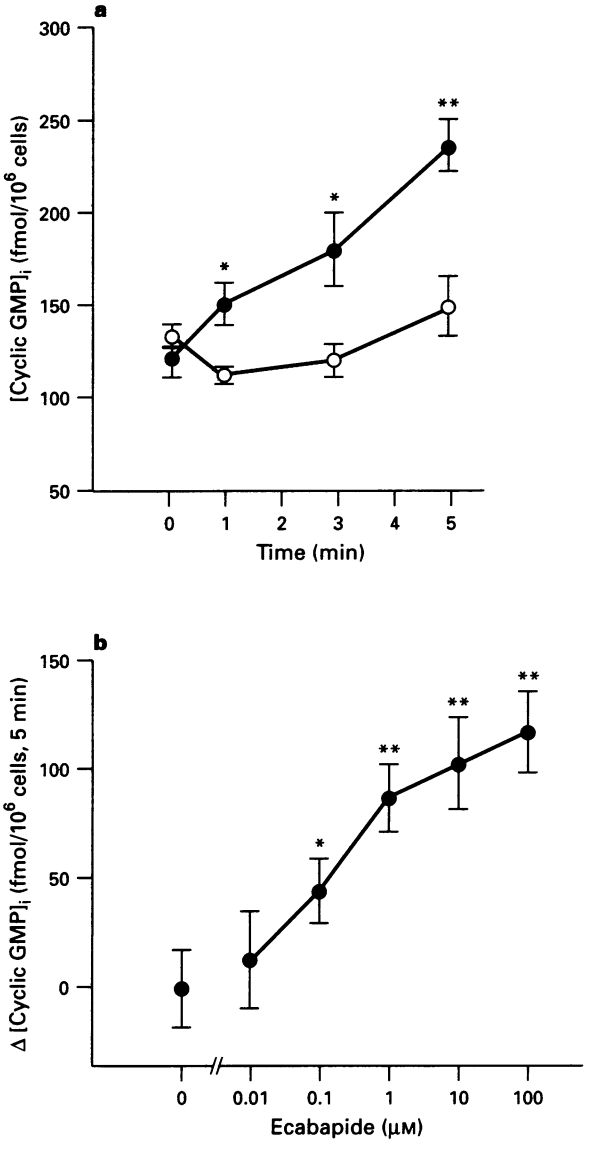

Figure 6 Ecabapide-induced increase in [cyclic GMP]$_i$ in parietal cell-rich suspensions. (a) [Cyclic GMP]$_i$ was measured 1, 3 and 5 min after the addition of 10 μM ecabapide (●) or vehicle (○) (n = 4–5). Significantly different from the value at 0 min *$P<0.05$ and **$P<0.01$. (b) The concentration-response curve for increase in [cyclic GMP]$_i$ by ecabapide. The ecabapide-induced net increase of [cyclic GMP]$_i$ (△ [cyclic GMP]$_i$) was assessed 5 min after the addition of ecabapide or vehicle (n = 5–6). Significantly different from the value at 0 μM *$P<0.05$ and **$P<0.01$.

[Ca^{2+}]$_i$ under whole-cell conditions. We therefore checked this possibility using the 133 K^+ – 13 Cl^- (BAPTA) pipette solution in which Ca^{2+} was chelated strongly with 5 mM BAPTA (pCa 7). However, the effect of ecabapide in the presence of BAPTA was not significantly different from that in the absence of BAPTA: that is, when assessed 6 min after the addition of 10 μM ecabapide, the ecabapide-elicited Cl^- current (ΔI) in the presence of BAPTA was 167±25 pA ($n=3$) and that in the absence of BAPTA was 174±23 pA ($n=6$, Figure 1b).

Discussion

Recently, we reported that the sub-picosiemens (pS) Cl^- channel in rabbit gastric parietal cells was activated via a prostaglandin EP_3 receptor-mediated Ca^{2+}/NO/cyclic GMP pathway (Sakai *et al.*, 1995), and inhibited by GTPγS-induced

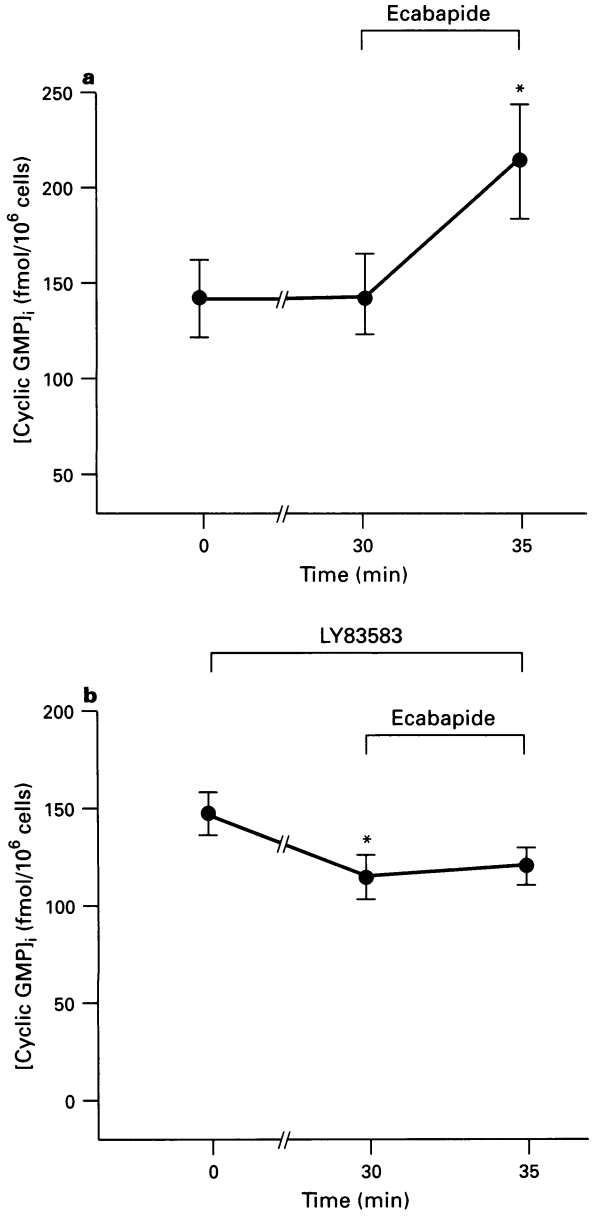

Figure 7 Inhibition of the basal and ecabapide-increased [cyclic GMP]$_i$ by LY83583. (a,b) [Cyclic GMP]$_i$ in parietal cell-rich suspensions was measured before (0 min) and 30 min after the addition of 10 μM LY83583 (b) or vehicle (a) (n = 5). At 30 min, 10 μM ecabapide was subsequently added and then [cyclic GMP]$_i$ was measured at 35 min (5 min after the addition of ecabapide). * Significantly different from the value at 30 min (a) or 0 min (b): ($P<0.05$).

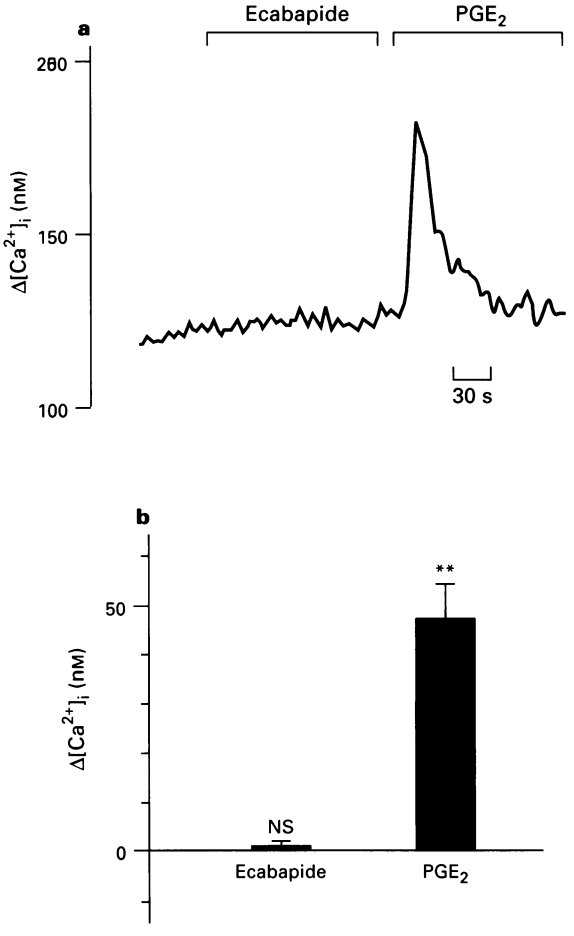

Figure 8 Effects of ecabapide and PGE_2 on $[Ca]_i$ in single parietal cells. (a) A typical trace of change in $[Ca^{2+}]_i$ of single parietal cells in gastric glands. The bathing solutions containing 10 μM ecabapide or 10 μM PGE_2 were perfused as indicated. (b) The averaged values of $\Delta[Ca^{2+}]_i$ (=peak value−basal value) from 5 experiments. NS, not significantly different ($P>0.05$) from the level before the addition of ecabapide. **Significantly different ($P<0.01$) from the level before the addition of PGE_2.

intracellular production of superoxide anion (Sakai & Takeguchi, 1993; 1994; 1995). This Cl^- channel is physiologically important because it has a housekeeping role via dominating the cell membrane potential (Sakai *et al.*, 1989; 1992).

In the present study, we suggest that ecabapide, a vasodilator, directly acted on parietal cells and activated the housekeeping Cl^- channel via an increase in [cyclic GMP]$_i$ because (1) the reversal potential of the ecabapide-elicited current was not significantly different from the theoretical Cl^- equilibrium potential (−64 mV) (Figure 2a), and ecabapide had little effect on the current recorded at −64 mV (Figure 3a and c), (2) the EC_{50} value for the ecabapide-induced activation of the Cl^- channel (Figure 1b) was the same as that for the ecabapide-induced increase in [cyclic GMP]$_i$ (Figure 6b), and (3) both the ecabapide-elicited Cl^- current (Figure 5) and ecabapide-increased [cyclic GMP]$_i$ (Figure 7) were abolished by LY83583, which prevents activation of soluble guanylate cyclase. This is the first report that ecabapide has a physiological function in parietal cells. Cyclic GMP-induced activation of small-conductance Cl^- channels have been reported in human colonic carcinoma cells such as T84 (10 pS) (Lin *et al.*, 1992) and HT29 (< 4 pS) (Kunzelmann *et al.*, 1992).

The characteristics of the Cl^- channels observed before and after the stimulation by ecabapide were the same, because the Cl^- currents in both cases were inhibited by NPPB (Figure 1a), and both power spectra of the basal and ecabapide-stimulated Cl^- current fluctuations consisted of not several but one Lorentzian ($1/f^2$) component (Figure 4). It is noted that the Cl^- currents in both cases were not significantly inhibited by niflumic acid, which is established as an inhibitor of Ca^{2+}-activated Cl^- currents (White & Aylwin, 1990; Korn *et al.*, 1991; Hogg *et al.*, 1994; Currie *et al.*, 1995) and cyclic AMP-activated Cl^- currents (Hughes & Segawa, 1993).

LY83583 has been reported to block activation of a guanylate cyclase via generation of oxygen radicals which react with NO and endothelium-derived relaxing factor (Mülsch *et al.*, 1989; Cherry *et al.*, 1990; Kontos & Wei, 1993). In the present study, LY83583 significantly decreased the basal [cyclic GMP]$_i$, but the inhibition was incomplete (−22%; Figure 7b). On the other hand, the ecabapide-induced increase in [cyclic GMP]$_i$ was almost completely in-

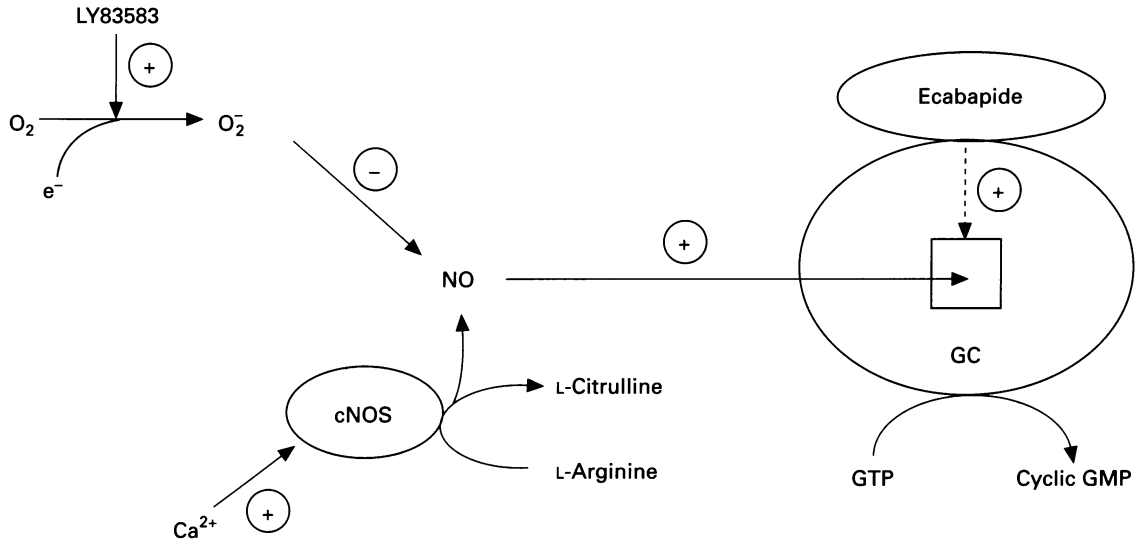

Figure 9 A tentative scheme of action of ecabapide in rabbit parietal cells. cNOS, constitutive NO synthase; GC, guanylate cyclase; + and −, activation and inhibition, respectively.

hibited by LY83583 (−93%; Figure 7). Corresponding with these results, LY83583-induced inhibition of the basal Cl^- current (−24% Figure 5b) was much smaller than that of the ecabapide-elicited Cl^- currents (−79%; Figure 5b and c). Similarly, LY83583 partially decreased basal [cyclic GMP]$_i$ in mouse isolated parotid glands (−14%; Watson *et al.*, 1990), rabbit isolated left atrial strips (−18%; MacLeod & Diamond; 1986), rabbit isolated right ventricular muscles (−46%; MacLeod & Diamond; 1986), guinea-pig isolated lung strips (−3%; Wong *et al.*, 1995) and rat RINm5F insulinoma cells (−66%; Laychock *et al.*, 1991), while it almost completely inhibited the increase in [cyclic GMP]$_i$ caused by carbachol (−76−100%; Watson *et al.*, 1990; MacLeod & Diamond; 1986), sodium nitroprusside (−92%; Wong *et al.*, 1995) or L-arginine (−100%; Laychock *et al.*, 1991) in the corresponding preparations.

Activation of a guanylate cyclase via Ca^{2+}-dependent production of NO has been reported in a variety of cells (Reiser, 1990; Ishii *et al.*, 1991; Leurs *et al.*, 1991; Xu *et al.*, 1994; Sakai *et al.*, 1995). These reports showed that rise of $[Ca^{2+}]_i$ by endothelium-1 (Reiser, 1990; Ishii *et al.*, 1991), histamine (Leurs *et al.*, 1991), CCK8 (Xu *et al.*, 1994) or PGE_2 (Sakai *et al.*, 1995) stimulated NO synthases. In the present study, however, ecabapide did not elevate $[Ca^{2+}]_i$ (Figure 8) and the activation of Cl^- channel by ecabapide did not depend on increase in $[Ca^{2+}]_i$. Our results indicate that the Ca^{2+} is not involved as an intracellular messenger in the ecabapide-induced activation of the Cl^- channel.

Figure 9 shows one plausible scheme of action of ecabapide. In this scheme, ecabapide enhances the sensitivity of guanylate cyclase to NO or the binding activity of NO to guanylate cyclase, resulting in an increased production of cyclic GMP. At present, it is unknown whether the effect of ecabapide on guanylate cyclase is direct or indirect. Ecabapide does not increase either the intracellular free Ca^{2+} concentration, or the production of NO. LY83583 inhibits the effect of ecabapide via quenching NO. It is unlikely that ecabapide acts as a phosphodiesterase inhibitor in the present rabbit parietal cell, because the addition of ecabapide did not stimulate the LY83583-insensitive (NO-independent) basal production of cyclic GMP (Figure 7b). Apparently, more complicated schemes must also be considered. Further study is required to clarify the action of ecabapide in detail. Similarly, ecabapide was reported to potentiate markedly the nitroprusside-induced increase in cyclic GMP content in dog arteries (Okamura *et al.*, 1995).

At present, the mechanism of activation of the Cl^- channel after the elevation of [cyclic GMP]$_i$ is unknown. Two major types of vertebrate cyclic GMP-dependent protein kinase exist, the soluble type I (cGK I) and the membrane-bound type II (cGK II) forms (Butt *et al.*, 1993). In the present mechanism, at least, cGK I was not involved because an intracellular addition of 1 μM KT5823, a cGK I inhibitor (Kase *et al.*, 1987; Tien *et al.*, 1994), did not affect the ecabapide (10 μM)-elicited Cl^- current (6 observations, data not shown). Recently, cGK II (not cGK I) was suggested as a mediator of cyclic GMP-dependent Cl^- secretion in rat intestinal mucosa (Markert *et al.*, 1995). The activation of the present housekeeping Cl^- channel may require cGK II. However, there is currently no specific inhibitor for cGK II (Tien *et al.*, 1994).

Ecabapide showed an antiulcer action against ethanol in rats at a non-antisecretory dose (30 mg kg^{-1}, oral administration) (Asano *et al.*, 1990). Pharmacokinetic study in rats revealed that the maximal concentration of ecabapide in blood after oral administration (30 mg kg^{-1}) was about 5 μM (Y. Ryokawa, Daiichi Pharmaceutical Co.; personal communication). Ecabapide-induced vasolidatation in isolated arteries of dog and rat was reported to be mediated via increase in [cyclic GMP]$_i$ (Okamura *et al.*, 1995; Hatanaka *et al.*, 1995). In these systems, however, higher concentrations of ecabapide ($\geqslant$ 100 μM) were required to induce significant increases in the cyclic GMP content. In contrast, lower concentrations of ecabapide (0.1 μM) significantly increased [cyclic GMP]$_i$ in rabbit parietal cells (Figure 6b).

Our present study suggests that the ecabapide strengthens the housekeeping function of gastric parietal cells via increase in the open probability of the membrane potential-dominating (housekeeping) Cl^- channel that is positively regulated by cyclic GMP.

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