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Nonspecific effects of the pharmacological probes commonly used to analyze signal transduction in rabbit parietal cells

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Abstract

In order to examine some possibly misleading conclusions of the pharmacological analysis of the signal transduction pathways of gastric acid secretion, we evaluated various agents including inhibitors of protein kinase C, cyclic AMP-dependent protein kinase, phospholipase C, phospholipase A2, lipoxygenase, casein kinase, calmodulin, myosin light chain kinase, tyrosine kinase, anion exchanger, and protein phosphatase; and activators of protein kinase C. Among them, the cyclic AMP-dependent protein kinase inhibitor *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinylsulfonamide (H-89), the phospholipase A2 inhibitor 2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid (ONO-RS-082), three myosin light chain kinase inhibitors (1-(5-iodonaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine (ML-7), 1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine (ML-9), and wortmannin), the anion exchanger inhibitor 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), the phospholipase C inhibitor neomycin, and most known calmodulin antagonists strongly inhibited [^{14}C]aminopyrine accumulation, an indicator of acid secretion, in isolated rabbit gastric glands stimulated by N^6,2′-*O*-dibutyryl-cyclic AMP. ONO-RS-082, calmidazolium, and DIDS inhibited H^+,K^+-ATPase. Most of the chemicals with antisecretory activity showed protonophore-like activity in gastric microsomes as well as in the mitochondria. It is concluded that H-89, ONO-RS-082, ML-7, ML-9, neomycin, and all calmodulin antagonists tested so far should not be used as tools to analyze gastric acid secretion.

Keywords: Parietal cell; Acid secretion; Signal transduction; Uncoupler; Mitochondria; H^+,K^+-ATPase

1. Introduction

When pharmacologically analyzing cellular signal transduction, investigators usually use compounds which are believed to selectively affect the components of the signaling pathways of interest, e.g., kinases, phosphatases, phospholipases, etc. In the gastric parietal cell, which secretes hydrochloric acid, the signal transduction pathways have also been pharmacologically analyzed by using such compounds. The parietal cell is considered to have at least three types of activating receptors on its basolateral membrane, i.e., histamine H_2, muscarinic M_3, and gastrin. It is already established (for review, Urushidani and Forte, 1997) that the histamine H_2 receptor couples to Gs to activate adenylate cyclase, producing cyclic AMP and subsequent activation of cyclic AMP-dependent protein kinase, whereas both M_3 and gastrin receptors couple to Gq to activate phospholipase C, which produces inositol 1,4,5-trisphosphate and diacylglycerol, with the former releasing Ca^{2+} from the intracellular stores and the latter activating protein kinase C. The main pathway mediating acid secretion is thought to be the cyclic AMP-dependent protein kinase pathway, since N^6,2′-*O*-dibutyryl-cyclic AMP (dbcAMP), a membrane permeable cyclic AMP analog, and forskolin, an activator of adenylate cyclase, stimulate acid secretion to the maximal level (Chew, 1983), and since acid secretion is inhibited by the cyclic AMP-dependent protein kinase inhibitor, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinylsulfonamide (H-89; Nandi et al., 1994, 1996; Urushidani and Nagao, 1996). In contrast, there has been a limited amount of information about the coupling between receptors and phospholipase C (Muto et al., 1997). For Ca^{2+}, definitive targets have not yet been

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elucidated, although Ca^{2+} is believed to be essential for many types of secretory events. One of its targets is calmodulin, because *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), a calmodulin antagonist, inhibits acid secretion stimulated by any type of agonist (Schepp et al., 1987, 1989). For the activation pathway after calmodulin, calmodulin-dependent protein kinase II is suggested to be involved at least in the cholinergic pathway, since 1-(*N*,*O*-bis[5-isoquinolinesulfonyl]-*N*-methyl-L-tyrosyl)-4-phenylpiperazine (KN-62), a calmodulin-dependent protein kinase II inhibitor, inhibits carbachol-stimulated, but not histamine-stimulated, acid secretion (Tsunoda et al., 1992). Myosin light chain kinase, which is another calmodulin-dependent enzyme, was suggested to be involved in the activation process because acid secretion was inhibited by the myosin light chain kinase inhibitors, *n*-butyl-2-(thiazolo[5,4-β]pyrid-2-yl)sulfinylacetate (ME-3407), wortmannin (Urushidani et al., 1997) and 1-(5-iodonaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine (ML-7; Wakasugi et al., 1992). Activation of protein kinase C is the other consequence of phospholipase C activation and it is thought to mediate the inhibitory signal, since 12-*o*-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C activator, inhibits acid secretion (Brown and Chew, 1986), and this inhibitory effect is reversed by 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), a protein kinase C inhibitor (Ostrowski and Bomsztyk, 1989). Protein kinase C was also suggested to be involved in the inhibitory effect of epidermal growth factor (Wang et al., 1996). In addition to these main factors, several modulator proteins have been suggested to be involved in signal transduction. For example, the involvement of tyrosine kinase was postulated on the basis of the observation that genistein, a tyrosine kinase inhibitor, affected the short-term inhibition and long-term stimulation by epidermal growth factor and transforming growth factor α (Tsunoda et al., 1993; Chew et al., 1994). The importance of protein dephosphorylation was also indicated by the observation that okadaic acid and calyculin A, phosphoprotein phosphatase I and IIa inhibitors, affected acid secretion (Goldenring et al., 1992; Urushidani and Nagao, 1996). The involvement of the anion exchanger in the maintenance of acid secretion was suggested because 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), an anion exchanger inhibitor, inhibited the secretion stimulated by histamine and carbachol (Horie et al., 1993). It is also possible that other factors in the parietal cells which have not been suggested are involved, e.g., phospholipase A_2, lipoxygenase, etc. This can be tested by using selective inhibitors.

However, investigators should be circumspect in using these pharmacological tools. *N*-(2-[*N*-(4-chlorocinnamyl)-*N*-methylaminomethyl]phenyl)-*N*-(2-hydroxyethyl)-4-methoxybenzenesulfonamide (KN-93), a calmodulin-dependent protein kinase II inhibitor, was reported to have a potent antisecretory activity in vitro that was not due to the inhibition of the kinase but due to its protonophore-like activity (Mamiya et al., 1993). We recently reported that 1-(6-[([17β]3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl)-1*H*-pyrrole-2,5-dione (U73122), a phospholipase C inhibitor, and its negative control, 1-(6-[([17β]3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl)-2,5-pyrrolidinedione (U73343), have totally unexpected actions on isolated gastric glands (Muto et al., 1997). It was also reported that tricyclic anti-depressants inhibited acid secretion in isolated rabbit gastric cells, possibly due to their protonophore-like action (Batzri, 1985), and a series of anti-depressants and neuroleptics were shown to inhibit acid secretion at low concentrations and H^+,K^+-ATPase activity at high concentrations (Sewing and Beil, 1989). These authors also showed that verapamil and gallopamil inhibited acid secretion by isolated guinea-pig parietal cells not by their Ca^{2+} channel antagonism but by their protonophoric action (Beil et al., 1990). Therefore, we considered it very important to examine whether these commonly used pharmacological tools have any nonspecific effects other than the expected effects which might affect gastric acid secretion. By screening more than 20 chemicals, we found in the present study that many compounds had protonophore-like effects or direct inhibitory effects on the proton pump, H^+,K^+-ATPase. We also examined the effect of these chemicals on the mitochondria, since a protonophore is very likely to be an uncoupler.

2. Materials and methods

2.1. Materials

The compounds used in this study and their expected effects are as follows. Protein kinase C inhibitors: H-7 (Seikagaku Kogyo, Tokyo, Japan), bisindolylmaleimide I, and chelerythrine (Sigma, St. Louis, MO). Protein kinase C activators: TPA and indolactam V (Sigma). Cyclic AMP-dependent protein kinase inhibitor: H-89 (Seikagaku Kogyo). Phospholipase C inhibitor: neomycin (Wako, Osaka, Japan). Phospholipase A2 inhibitors: 2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid (ONO-RS-082; a generous gift from ONO Pharmaceutical, Osaka, Japan) and aristolochic acid (Sigma). Lipoxygenase inhibitor: 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA861; Wako). Casein kinase inhibitor: *N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide (CKI-7; Seikagaku Kogyo). Calmodulin inhibitors: W-7 (Seikagaku Kogyo), calmidazolium, trifluoperazine (Sigma), and chlorpromazine (Wako). Myosin light chain kinase inhibitors: ML-7, 1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine (ML-9; Seikagaku Kogyo), and wortmannin (Wako). Tyrosine kinase inhibitor: genistein (Sigma). Phosphoprotein phosphatase inhibitors: okadaic acid and calyculin A (Wako).

[^{14}C]Aminopyrine was from New England Nuclear. The acetoxymethylester form of 2′,7′-bis(carboxyethyl)5(6)-carboxyfluorescein (BCECF/AM) was from Wako. Db-cAMP, collagenase type I, bovine serum albumin, acridine orange, valinomycin, nigericin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and other chemicals for biological buffers were from Sigma. Japanese white rabbits were obtained from Shiraishi (Tokyo, Japan). In the present study, all the rabbits received humane care in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health.

2.2. Methods

2.2.1. Isolation of rabbit gastric glands and measurement of [^{14}C]aminopyrine accumulation

Rabbit gastric glands were isolated as described (Berglindh and Obrink, 1976). Isolated glands (5% cytocrit) were incubated with secretagogue and inhibitor at 37°C for 30 min in an incubation medium containing 132.4 mM NaCl, 5 mM Na_2HPO_4, 1 mM NaH_2PO_4, 5.4 mM KCl, 1.2 mM $MgSO_4$, 1.0 mM $CaCl_2$, 25 mM Hepes-Na (pH = 7.4), and 11.1 mM glucose, in addition to 1.0 mg/ml bovine serum albumin. Acid secretion was monitored by measuring [^{14}C]aminopyrine accumulation in duplicate samples (Sack and Spenny, 1982). In general, stimulation of acid secretion was expressed as the increase in the aminopyrine ratio above the resting value and as a percentage of the control values. Therefore, when a value lower than the resting value was obtained with the drug treatment, the result had a negative value.

2.2.2. Purification and enzymatic assay of H^+,K^+-ATPase

Microsomal H^+,K^+-ATPase was purified by sucrose density gradient centrifugation of a homogenate of rabbit gastric mucosa as described earlier (Hirst and Forte, 1985). K^+-dependent *p*-nitrophenylphosphatase activity was measured in a total volume of 1 ml containing 7.5 mM Tris, pH = 7.5, 2.5 mM $MgSO_4$, 0.1 mM ouabain, with or without 20 mM KCl, about 5 μg of protein (specific activity: 30–40 μmol h^{-1} mg^{-1} protein), and 5 mM sodium *p*-nitrophenylphosphate. The net increase elicited by KCl was considered to be K^+-*p*-nitrophenylphosphatase activity.

The H^+,K^+-ATPase assay was performed in a total volume of 1.0 ml containing 10 mM piperazine-1,4-bis(2-ethanesulfonic acid), pH = 6.5, 1 mM $MgSO_4$, 0.1 mM ouabain, with or without 20 mM KCl and 1 mM ATP. The net increase elicited by KCl was considered to be H^+, K^+-ATPase activity. Liberated inorganic phosphate was quantified as described (Maeda et al., 1988). Gastric membranes used for these enzyme assays were rendered leaky by three cycles of freezing–thawing. It was confirmed that

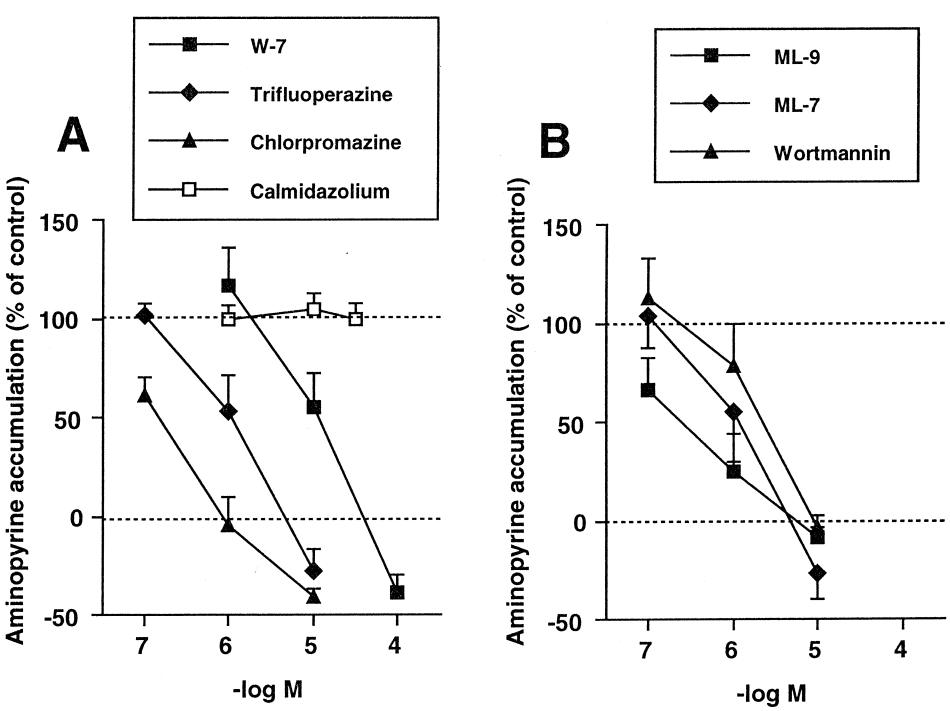

Fig. 1. Effects of W-7, calmidazolium, chlorpromazine, and trifluoperazine (A), and ML-9, ML-7, and wortmannin (B) on [^{14}C] aminopyrine accumulation stimulated by 0.1 mM dbcAMP in isolated rabbit gastric glands. Stimulation of acid secretion is expressed as the increase in the aminopyrine ratio above the resting value, and the effects of drugs on the agonist are expressed as percentages of the control values. Values are means ± S.E. from 4 to 6 separate gland preparations with duplicate measurements.

these samples were not further activated by the potassium ionophore, valinomycin, relative to the activity measured in the presence of KCl.

Proton transport by intact membrane vesicles was monitored by acridine orange quenching as described earlier (Lee and Forte, 1978). An aliquot of membrane suspension (50 μg/ml) was added to the uptake media (125 mM KCl, 50 mM sucrose, 2.5 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid, pH = 6.8, 0.4 mM MgATP, 25 μM EDTA, 10 mM phosphocreatine, 1.5 μM acridine orange). Fluorescence changes (excitation: 493 nm, emission: 540 ± 6 nm band pass filter) were continuously monitored with a spectrophotofluorometer (CAF-110; JASCO, Tokyo) at 37°C.

2.2.3. Isolation of mitochondria and pH measurement

Rabbit liver mitochondria were isolated as described previously (Johnson and Lardy, 1967). Mitochondria were incubated with 10 μM BCECF/AM for 20 min at room temperature. They were diluted, re-centrifuged, and re-suspended as described (Kapus et al., 1989) in a medium containing 100 mM KCl, 80 mM sucrose, 10 mM K^+-3-(*N*-morpholino)-propanesulfonic acid (pH = 7.0), 2.5 μM oligomycin, and 1 μM rotenone. Mitochondrial suspensions were excited at two wavelengths (503/438 nm) and the fluorescence emission ratio was recorded at 25°C using a 540 ± 6 nm band pass filter and CAF-110 to monitor the intra-mitochondrial pH.

2.2.4. Measurement of passive diffusion of H^+ in the gastric vesicle

The effects of drugs on the passive diffusion of H^+ were measured as previously described (Nandi et al., 1994). Tight gastric vesicles enriched in H^+,K^+-ATPase were preincubated for 1 h at room temperature in a medium containing 150 mM KCl, 10 mM succinate, and 5.6 mM Tris (pH = 4.0). After the addition of 1.5 μM acridine orange, the extravesicular pH was increased from 4.0 to 8.0 by the addition of 25 mM Tris base with continuous monitoring of fluorescence, as described above. Upon extravesicular alkalization, the fluorescence of acridine orange abruptly quenched according to the pH gradient. Thereafter, H^+ ions passively diffused from the inside to the outside. Test drugs were added to the medium 1 min after the addition of Tris base.

2.2.5. Statistical analysis

Parametric data are expressed as the means ± S.E. Multiple comparisons were analyzed by analysis of variance and Dunnet's post-hoc test using a computer program (Super ANOVA®, ABACUS Concepts, Berkeley, CA). The level of significance was uniformly set at $P < 0.05$ and no further calculation of P value was performed. The IC_{50} values were obtained from dose–response curves by using a computer program (GraphPad Prism®, GraphPad).

Table 1
Summary of the effects of calmodulin antagonists and myosin light chain kinase inhibitors on aminopyrine accumulation in isolated gastric glands, H^+,K^+-ATPase activity, proton permeability of gastric vesicles and mitochondria, and data reported in the literature

Drugs	Aminopyrine accumulation in the gastric glands (IC_{50}) (μM)	Protonophoric effect[a]		Enzyme inhibition IC_{50}		IC_{50} in vitro[b]	Max. conc. reported in literature
		Gastric microsomes (μM)	Mitochondria (μM)	K^+-*p*-nitrophenyl-phosphatase (μM)	K^+-ATPase (μM)		
W-7	9.7	100	NT	NT	[140][e]	50 μM	100 μM[g]
Calmidazolium	No (30)	10	100	~ 2	1.8	5 μM	100 μM[h]
Chlorpromazine	0.88	10	NT	[26][f]	[13.3][f]	28 μM	1 mM[i]
Trifluoperazine	1.3	10	NT	[144][f]	[31][f]	85 μM	1 mM[i]
ML-7	0.12	10	100	No (10)	NT	0.3 μM	56 μM[j]
ML-9	0.16	10	100	No (10)	NT	3.8 μM	100 μM[g]
Wortmannin	2.0	No (10)	NT	No (10)	NT	0.3 μM[c]; 3 nM[d]	10 μM[k]

The values in [ ] are taken from the literature as indicated.
No, no effect was observed up to the concentration given in parentheses; NT, not tested.
[a]The concentration required to elicit protonophoric activity is shown since the effect was not dose-dependent but showed a threshold concentration in general.
[b]These values are mostly taken from the manufacturer's data sheets and thus citation is omitted.
[c]For myosin light chain kinase. [d]For phosphatidylinositol 3-kinase.
[e]Schepp et al., 1987; [f]Sewing and Beil, 1989; [g]J. Biol. Chem. 268 (1993) 2277; [h]Am. J. Physiol. 271 (1996) G304; [i]Neurosci. Behav. Physiol. 26 (1996) 435; [j]Am. J. Physiol. 269 (1995) C257; [k]Biochem. J. 320 (1996) 643.

3. Results

3.1. Effects of calmodulin antagonists and myosin light chain kinase inhibitors

3.1.1. Effects on acid secretion by isolated rabbit gastric glands stimulated by dbcAMP

Rabbit gastric glands were stimulated by 0.1 mM db-cAMP and acid secretion was monitored by measuring [^{14}C]aminopyrine accumulation. This concentration of db-cAMP was selected so that both inhibition and augmentation of the response could be sensitively detected. Under routine assay conditions, the aminopyrine ratio in resting glands was 11.1 ± 0.9 (mean $\pm$ S.E.; $N = 12$). This was increased by addition of 0.1 mM dbcAMP to 37.4 ± 6.3 ($N = 12$), which was much less than the maximal value usually obtained in the glands, i.e., 330 ± 35.5 ($N = 5$) by 0.1 mM histamine plus 50 μM isobutylmethylxanthine.

In the first screening, we examined the effects of several putative calmodulin antagonists: W-7, calmidazolium, chlorpromazine, and trifluoperazine. As shown in Fig. 1A, W-7, chlorpromazine, and trifluoperazine markedly inhibited acid secretion, whereas calmidazolium, the most potent calmodulin antagonist in vitro among these drugs, failed to show any inhibitory effect in concentrations up to 30 μM. Fig. 1B shows the effects of inhibitors of myosin light chain kinase, a calmodulin-dependent enzyme. ML-7, ML-9, and wortmannin caused dose-dependent inhibition

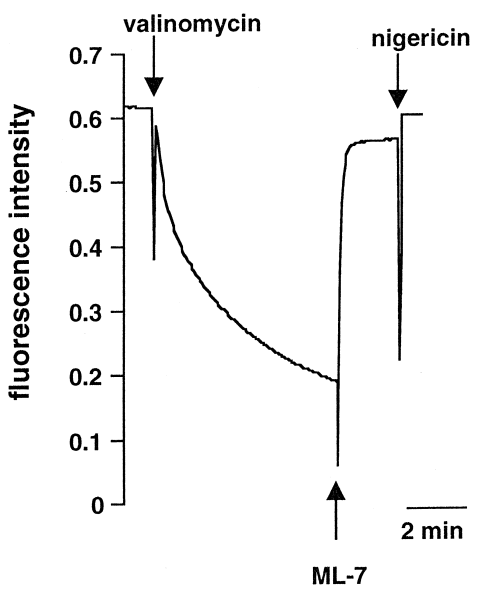

Fig. 2. ML-7 cancels the proton gradient formed by H^+,K^+-ATPase in gastric vesicles. Purified gastric microsomal membranes (50 μg/ml) were added to the cuvette and fluorescence changes were continuously monitored with a spectrophotofluorometer. Subsequent addition of 20 μM valinomycin caused rapid fluorescence quenching, which indicates that the membranes were producing a proton gradient. Addition of 10 μM ML-7 rapidly canceled the formed proton gradient, and the addition of a cation exchanger, nigericin, caused no further recovery of fluorescence. The trace is representative of at least three separate recordings with essentially identical results.

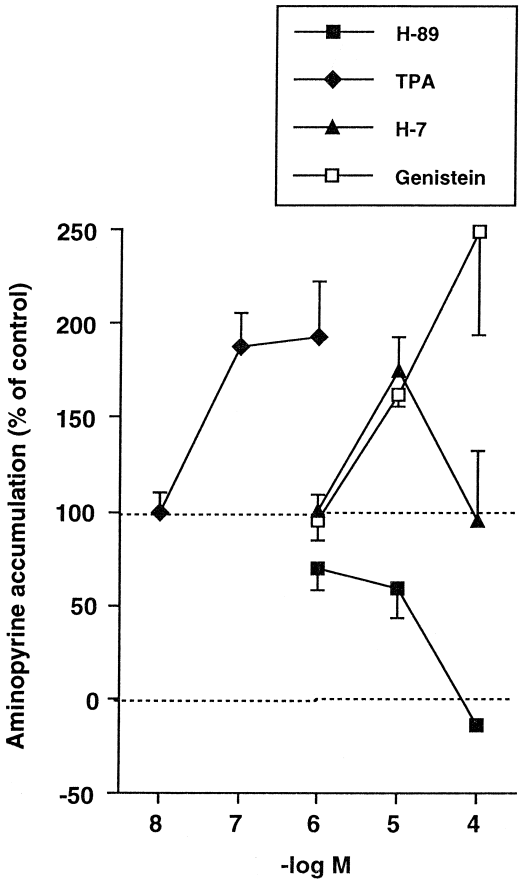

Fig. 3. Effects of H-89, 12-*o*-tetradecanoylphorbol-13-acetate (TPA), H-7, and genistein on [^{14}C] aminopyrine accumulation stimulated by 0.1 mM dbcAMP in isolated rabbit gastric glands. Stimulation of acid secretion is expressed as the increase in the aminopyrine ratio above the resting value, and the effects of drugs on the agonist are expressed as percentages of the control values. Values are means $\pm$ S.E. from 4 to 6 separate gland preparations with duplicate measurements.

of acid secretion in micromolar concentrations (Fig. 1B). All but calmidazolium caused a strong inhibition, with acid secretion at maximal inhibition being lower than the resting value.

These results revealed an apparent discrepancy between the potency against calmodulin in vitro and the anti-secretory effects in vivo (see Table 1). This suggested that the inhibitory effect of these agents might not be due to their postulated mode of action. We examined this point in the following experiments.

3.1.2. Effects on the ATP-driven H^+ gradient in the gastric vesicles

Changes in the proton gradient were monitored by using the acridine orange quenching technique. In the presence of KCl and ATP, valinomycin (20 μM), a K^+ ionophore, formed a proton gradient by activating H^+,K^+-ATPase on gastric vesicles. When the proton pump was inhibited in this way, the fluorescence gradually recovered because of the spontaneous leakage of protons from the inside of the vesicle. When the gradient was rapidly canceled by the

addition of agents with protonophoric activity, the fluorescence promptly recovered. As the latter effect was not dose-dependent but showed a threshold concentration in general, it was impossible to calculate an ED_{50} value. Therefore, we recorded the minimum concentration needed to cancel the whole gradient for each compound. The following agents, at the concentrations indicated, canceled the proton gradient in the gastric vesicles: 100 μM W-7, 10 μM calmidazolium, 10 μM chlorpromazine, 10 μM trifluoperazine, 10 μM ML-7, and 10 μM ML-9. Fig. 2 shows the results of a typical experiment with ML-7. Although the rate of fluorescence recovery was much faster than the passive leak of protons from the vesicles when proton pumping by H^+,K^+-ATPase was inhibited, it was very difficult to distinguish between them clearly. Therefore, we designated the activity that canceled out the proton gradient in the gastric vesicles as 'protonophore-like activity'. This point is elucidated later in detail (Section 3.5).

3.2. Effects of various protein kinase inhibitors, activators, and phosphoprotein-phosphatase inhibitors on acid secretion by isolated rabbit gastric glands and the ATP-driven H^+ gradient in the gastric vesicle

In the previous section, it was revealed that the antisecretory effect of each of the calmodulin antagonists and two of the three myosin light chain kinase inhibitors could not be explained by their anticipated action, but could be by their protonophore-like activity. We thus extended our screening to other pharmacological probes frequently used in the study of cellular signal transduction. For protein kinase C, we selected H-7, bisindolylmaleimide I, and chelerythrine as inhibitors, and TPA and indolactam V as activators. We also examined H-89 as a cyclic AMP-dependent protein kinase inhibitor, KN-62 as a calmodulin kinase II inhibitor, genistein as a tyrosine kinase inhibitor, and CKI-7 as a casein kinase inhibitor.

Fig. 3 depicts the effects of drugs that showed a significant effect on dbcAMP-stimulated acid secretion in the gastric glands. In agreement with previous reports (Ostrowski and Bomsztyk, 1989; Urushidani and Nagao, 1996), H-7 significantly augmented the acid secretion at 10 μM, but was seen to be inhibitory at 100 μM. At 100 μM H-7 seemed to be at a threshold between causing augmentation and inhibition of acid secretion since two preparations showed strong inhibition while three preparations showed marked augmentation, out of a total of five preparations. Ostrowski and Bomsztyk (1989) used 50 μM of H-7 to obtain the maximal augmentation in their experiments.

Among activators of protein kinase C, indolactam V showed no effect up to 10 μM. In contrast, TPA aug-

Table 2
Summary of the effects of protein kinase inhibitors, activators, and phosphatase inhibitors on aminopyrine accumulation in isolated gastric glands, H^+,K^+-ATPase activity, proton permeability of gastric vesicles and mitochondria, and data from the literature

Drugs	Aminopyrine accumulation in the gastric glands (μM)	Protonophoric effect[a]		Enzyme inhibition[b]		IC_{50}, EC_{50} in vitro[c] (μM)	Max. conc. reported in literature (μM)
		Gastric microsomes (μM)	Mitochondria	K^+-*p*-nitrophenyl-phosphatase (μM)	K^+-ATPase (μM)		
H-7	Potentiation: ~10 Inhibition: ~100	100	NT	No (10)	NT	6.0[d] 3.0[e]	100[g]
Bisindolyl-maleimide I	No (10)	No (10)	NT	No (10)	NT	0.48	5[h]
Chelerythrine	No (10)	No (10)	NT	No (10)	NT	0.7	200[i]
TPA	Potentiation: 1–10	No (10)	NT	43% (10)	No (10)	0.01	1[j]
Indolactam V	No (10)	No (10)	NT	No (10)	NT	0.02	1[k]
H-89	IC_{50} = 16	10	100 μM	44% (100)	No (100)	0.048	40[l]
KN-62	No (60)	No (10)	NT	No (60)	NT	0.9	60[m]
KN-93	[IC_{50} = 0.6][f]	[10][f]	NT	NT	[35% (20)][f]	0.37	60[f]
Genistein	Potentiation: 10–100	100	NT	No (10)	NT	2.6	100[n]
CKI-7	No (100)	No (300)	NT	No (100)	NT	9.5	100[o]
Okadaic acid	No (1)	No (1)	NT	No (1)	NT	0.06	10[p]
Calyculin A	Potentiation: ~0.1	No (1)	NT	No (1)	NT	0.002	3[g]

The values in [ ] are taken from the literature as indicated.
No, no effect was observed up to the concentration given in parentheses; NT, not tested.
[a]The concentration required to elicit protonophoric activity is shown since the effect was not dose-dependent but showed a threshold concentration in general.
[b]The value indicates % inhibition of the drug on the enzyme actvity at the concentration shown in parentheses.
[c]These values are mostly taken from the manufacturer's data sheets and thus citation is omitted.
[d]For protein kinase C; [e]For cyclic AMP-dependent protein kinase.
[f]Mamiya et al., 1993; [g]J. Gen. Physiol. 105 (1995) 249; [h]Neurosci. 74 (1996) 927; [i]J. Physiol. 491 (1996) 423; [j]Hematopathol. Mol. Hematol. 10 (1996) 223; [k]Biochem. 31 (1992) 3824; [l]J. Neurochem. 68 (1997) 2241; [m]Tsunoda et al., 1992; [n]Am. J. Physiol. 269 (1995) G874; [o]J. Cell. Biochem. 60 (1996) 387; [p] Cell. Mol. Biol. 42 (1996) 547.

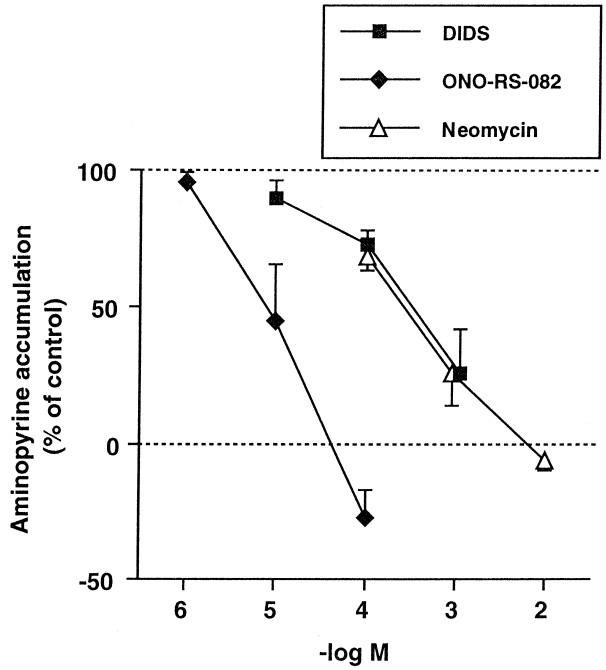

Fig. 4. Effects of 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), ONO-RS-082, and neomycin on [^{14}C] aminopyrine accumulation stimulated by 0.1 mM dbcAMP in isolated rabbit gastric glands. Stimulation of acid secretion is expressed as the increase in the aminopyrine ratio above the resting value, and the effects of drugs on the agonist are expressed as percentages of the control values. Values are means ± S.E. from 4 to 6 separate gland preparations with duplicate measurements.

mented dbcAMP-stimulated secretion at 100 nM or more, in accordance with previously published observations (Brown and Chew, 1986; Akagi et al., 1998).

As expected, the cyclic AMP-dependent protein kinase inhibitor H-89 strongly and dose dependently inhibited dbcAMP-stimulated acid secretion with an IC_{50} value of 16 μM. As already reported (Tsunoda et al., 1992), the calmodulin kinase II inhibitor, KN-62, had little effect up to 60 μM. The potentiating effect of genistein reported by others (Tsunoda et al., 1993; Chew et al., 1994, Nakamura et al., 1996) was also confirmed in the present study. The casein kinase inhibitor CKI-7 was without effect up to 100 μM, suggesting no involvement of these kinases in acid secretion.

Okadaic acid, a phosphoprotein phosphatase IIa inhibitor, showed little effect up to 1 μM. Calyculin A (an inhibitor of phosphatase I and IIa), however, stimulated acid secretion by itself and augmented dbcAMP-stimulated secretion, as has been previously reported (Urushidani and Nagao, 1996). The effect of calyculin A was omitted from Fig. 3 since the stimulatory effect of calyculin A by itself was larger than that of dbcAMP.

In order to examine the possible protonophore-like effects of these agents, we tested them in the acridine orange quenching assay, using gastric microsomes. Among these agents, H-7 at 100 μM, H-89 at 10 μM, and genistein at 100 μM showed protonophore-like activity. These data are summarized in Table 2.

3.3. Effects of other drugs on acid secretion by isolated rabbit gastric glands and on the ATP-driven H^+ gradient in the gastric vesicles

For this section, we examined the effects of the phospholipase C inhibitor, neomycin; two phospholipase A2

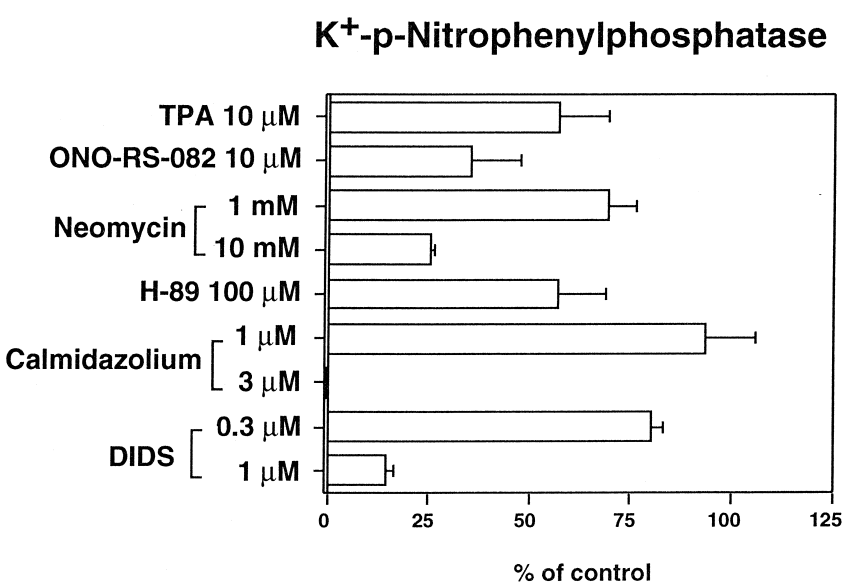

Fig. 5. Effects of 12-*o*-tetradecanoylphorbol-13-acetate (TPA), ONO-RS-082, neomycin, H89, calmidazolium, and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) on gastric K^+-*p*-nitrophosphatase activity. K^+-*p*-nitrophenylphosphatase activity is defined as the activity in the presence of 20 mM KCl after subtraction of the rate in the absence of KCl. Values are means ± S.E. of three separate experiments in a duplicate manner. All of the values except 1 μM calmidazolium were all statistically significant from the control value ($P < 0.05$).

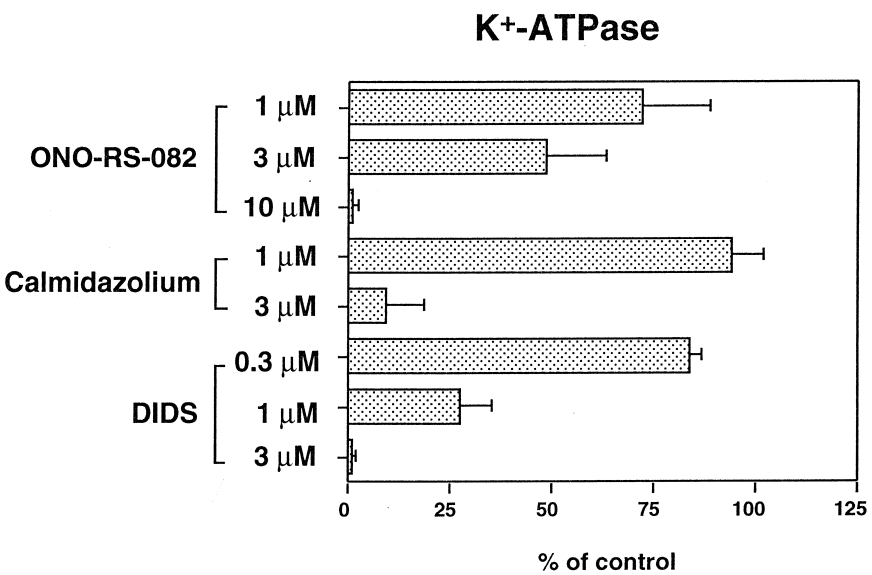

Fig. 6. Effects of ONO-RS-O82, calmidazolium, and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) on K^+-ATPase activity. K^+-ATPase activity is defined as the activity in the presence of 20 mM KCl after subtraction of the rate in the absence of KCl. The effects of drugs are expressed as % of vehicle control. Values are means ± S.E. of three separate experiments in duplicate manner. The values except 1 μM ONO-RS-082 and 1 μM calmidazolium are all statistically significant from the control value ($P < 0.05$).

inhibitors, ONO-RS-082 and aristolochic acid; the lipoxygenase inhibitor, AA861; and the anion exchanger inhibitor, DIDS. Whereas aristolochic acid up to 100 μM and AA861 up to 50 μM failed to show any significant effect, neomycin, ONO-RS-082, and DIDS dose dependently inhibited dbcAMP-stimulated acid secretion by the glands (Fig. 4). Considering the commonly used ranges, the concentrations of neomycin and DIDS were raised to the millimolar range.

The possible protonophore-like activity of these agents was examined in the acridine orange quenching assay, using gastric microsomes. Aristolochic acid and DIDS could not be tested because of their own fluorescence. AA861 did not affect the proton permeability of the vesicles. ONO-RS-082 at 10 μM and neomycin at 10 mM promptly canceled the proton gradient formed in the gastric vesicles, suggesting that they had protonophore-like activity.

3.4. Effects on H^+,K^+-ATPase activity

The potential effects of the compounds on H^+,K^+-ATPase activity were first screened by measuring K^+-*p*-nitrophenylphosphatase activity. Then the effects on K^+-ATPase activity were examined for the compounds that showed significant effects. Among the drugs tested, 10 μM TPA, 10 mM neomycin, and 100 μM H-89 significantly inhibited K^+-*p*-nitrophenylphosphatase activity (Fig. 5). However, these agents did not affect K^+-ATPase activity at the same concentration (data not shown). This showed a differentiation between the effects on K^+-*p*-nitrophenylphosphatase and K^+-ATPase, as we previously reported in the case of U-73122 (Muto et al., 1997). ONO-RS-082, calmidazolium, and DIDS inhibited K^+-*p*-nitrophenylphosphatase activity (Fig. 5) as well as K^+-ATPase activity (Fig. 6). In order to examine the inhibitory kinetics of K^+-ATPase by these compounds, enzyme assays were performed with various concentrations of K^+.

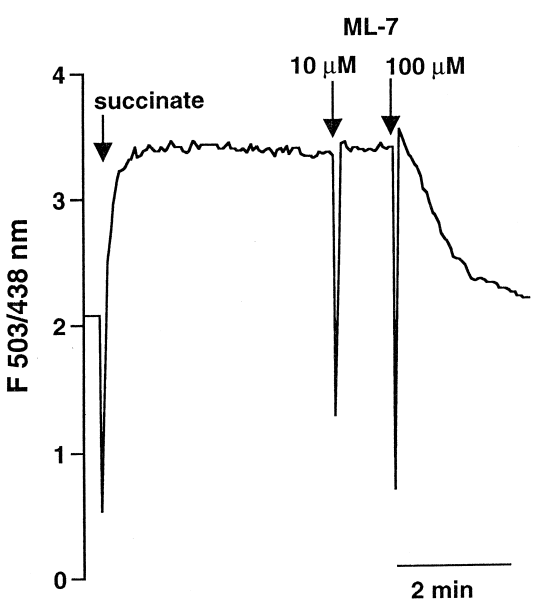

Fig. 7. ML-7 cancels the proton gradient formed in the mitochondria. Mitochondria were loaded with a pH indicator, BCECF, and the pH of the matrix was elevated by the addition of 5 mM succinate as indicated by an increase in the fluorescence ratio. The addition of 10 μM ML-7, which showed a protonophore-like action on the gastric vesicles, did not affect the fluorescence ratio. When its concentration was increased to 100 μM, the fluorescence ratio decreased to the basal level, indicating that the proton gradient was canceled. The trace is a representative of at least three separate recordings with essentially identical results.

However, it was quite difficult to get consistent results with these compounds because the inhibition curves were so steep that the inhibition around the IC_{50} fluctuated highly, especially with calmidazolium.

3.5. Effects on mitochondrial pH gradient

It is well known that protonophores act as uncouplers in mitochondria. We examined the effects on rabbit liver mitochondria of the compounds which showed protonophore-like activity in the gastric vesicles. Using BCECF-loaded mitochondria, the pH of the matrix was elevated by the addition of 5 mM succinate, and then test drugs were added. As shown in Fig. 7, 10 μM ML-7, which showed protonophore-like activity in the gastric vesicles, did not affect the fluorescence ratio. However, when its concentration was increased to 100 μM, the fluorescence ratio was decreased to the basal level; that is, the proton gradient was canceled. ML-9, H-89, and calmidazolium (100 μM each) also showed protonophore-like activity in the mitochondria at a concentration ten times higher than that at which they showed protonophore-like activity in the gastric vesicles. Both neomycin (10 mM) and ONO-RS-082 (10 μM) showed protonophore-like activity at the same concentration as that in gastric vesicles.

3.6. Effects on the passive leakage of protons through gastric membranes

For ML-7, ML-9, H-89, and calmidazolium, but not for ONO-RS-082 or neomycin, the concentrations showing protonophore-like activity were different between gastric vesicles and mitochondria. We thought that this effect might be due to the presence of valinomycin, because the effect of protonophore-like uncouplers is known to be generally potentiated by valinomycin (Levy et al., 1995). Therefore, the effects of these compounds were examined in gastric vesicles in the absence of valinomycin.

Gastric vesicles were preincubated and equilibrated in pH 4.0 buffer, and the pH gradient was formed by the addition of Tris base so that the external pH increased to 8.0. The gradient, which was monitored by acridine orange quenching, gradually disappeared due to the proton leak. It was examined whether ML-7, ML-9, H-89 and calmidazolium accelerated the rate of the disappearance of the proton gradient, and if this was the case, whether the reaction was further augmented by valinomycin. Fig. 8A shows typical fluorescence changes in the case of ML-7. This compound accelerated the disappearance of the formed proton gradient. Valinomycin failed to potentiate the effect of ML-7 (data not shown). Practically the same results

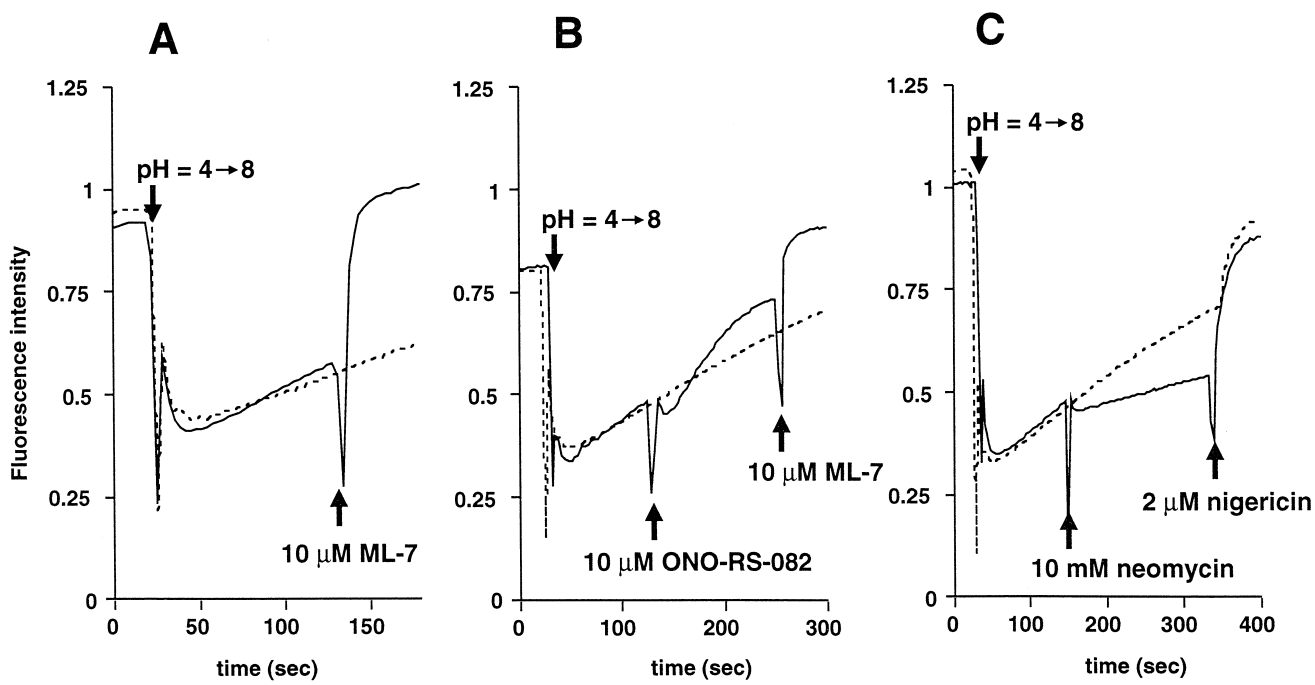

Fig. 8. ML-7, ONO-RS-082, but not neomycin, accelerates passive diffusion of protons through gastric microsomal membranes. Gastric vesicles were preincubated and equilibrated in pH 4.0 buffer, and the pH gradient was formed by the addition of Tris base, which raised the external pH to 8.0, as indicated by the first arrow. The gradient, monitored by the acridine orange quenching, gradually disappeared due to proton leakage (control; dotted line in each panel). (A) When 10 μM ML-7 was added, the rate of the disappearance of proton gradient was accelerated (solid line). (B) When 10 μM ONO-RS-082 was added, a relatively small acceleration was observed compared with that seen with ML-7. (C) Neomycin did not accelerate, but rather inhibited, proton leakage. This seemed to affect proton permeability, since the addition of nigericin restored fluorescence to the same level as that of the control (thin line). Each set of traces is a representative of at least three separate recordings with essentially identical results.

were obtained for ML-9 and H-89. In contrast, a typical protonophore, CCCP (1 μM), accelerated the recovery of quenching, which was further accelerated by the addition of valinomycin (data not shown).

ONO-RS-082 also accelerated the proton leak, but its effect was much weaker than that of ML-7 (Fig. 8B). Neomycin did not accelerate, but rather prevented, the proton leak, as shown in Fig. 8C. This clearly indicated that neomycin itself is not a real protonophore, and that the underlying mechanism for canceling the proton gradients in the gastric vesicles and mitochondria is due to something other than proton conductance. Calmidazolium could not be assayed in this system because it affects the fluorescence of acridine orange at this pH.

4. Discussion

Drugs that inhibit acid secretion stimulated by dbcAMP are thought to affect steps downstream to cyclic AMP generation, energy metabolism, H^+,K^+-ATPase activity, and the maintenance of the pH gradient. As acid secretion in vitro can be monitored by the accumulation of a weak base ([^{14}C]aminopyrine) in the acidic space, it is expected that any agent with protonophore-like activity or proton pump inhibiting activity should be highly effective in inhibiting the acid secretory response. In the present study, we found that many of the agents widely used to investigate intracellular signal transduction could exert non-specific effects in the concentration range in which they are expected to exert a specific effect. Our present data are summarized together with previously published data in Tables 1–3. Table 1 contains the calmodulin antagonists and myosin light chain kinase inhibitors. Table 2 contains the inhibitors of various kinases, protein kinase C activators, and phosphoprotein phosphatase inhibitors. The agents in Table 3 belong to other categories, i.e., the inhibitors of phospholipase C, phospholipase A2, lipoxygenase, and anion exchangers. The EC_{50} value of each drug in vitro for the anticipated target and the maximal concentration used in the literature are also listed. When these compounds are used in cells or tissues, their concentrations are usually higher than the EC_{50} values in vitro because their membrane permeability is limited. When the compound acts as a competitor of the substrate, the compound should be used at a concentration equivalent to that of the intracellular substrate, which is sometimes much higher than that used in the in vitro assay system. It should be noted that the concentration which showed protonophore-like activity in this study did not differ much from the concentration at the cell level.

4.1. Calmodulin antagonists and related compounds

As shown in Table 1, all the calmodulin antagonists except calmidazolium strongly inhibited dbcAMP-stimulated acid secretion. The concentrations required to elicit complete inhibition of aminopyrine accumulation coincided with those showing marked protonophore-like activity. For a naphthalenesulfonate-type antagonist, this was somewhat apparent in the early reports (Schepp et al., 1987, 1989) proposing the involvement of calmodulin in the final common step of acid secretion, based on the effects of W-7. Using isolated rat parietal cells, Schepp et

Table 3
Summary of the effects of the inhibitors of phospholipases, lipoxygenase, and anion-exchanger, on aminopyrine accumulation in isolated gastric glands, H^+,K^+-ATPase activity, proton permeability of gastric vesicles or mitochondria, and data from literature

Drugs	Aminopyrine accumulation in the gastric glands (IC_{50}) (μM)	Protonophoric effect[a]		Enzyme inhibition		IC_{50} in vitro[c]	Max. conc. reported in literature
		Gastric microsomes (μM)	Mitochondria	K^+-p-nitrophenyl-phosphatase[b]	K^+-ATPase (IC_{50})		
Neomycin	320	No?[d]	10 mM	75% (10 mM)	No (10 mM)	0.01–1 mM	10 mM[f]
U-73122	[Potentiation: ~ 3][e]	[No (10)][e]	NT	[1 μM][e]	[No (10 μM)][e]	1–40 μM	100 μM[g]
U-73343	[3][e]	[10][e]	NT	[26 μM][e]	[13.3 μM][e]	(negative control)	10 μM[h]
ONO-RS-082	7.1	10	10 μM	65% (10 μM)	2.3 μM	3.5 μM	10 μM[i]
Aristolochic acid	No (100)	?	NT	NT	NT	40 μM	200 μM[j]
AA861	No (10)	No (10)	NT	NT	NT	0.8 μM	100 μM[k]
DIDS	290	?	NT	94% (10 μM)	0.4 μM	0.1 ~ 200 μM	500 μM[l]

The values in [ ] are taken from the literature as indicated.
No, no effect was observed up to the concentration given in parentheses; NT, not tested.
?: Acridine orange quenching assay could not be performed because of the fluorescent nature of the drug.
[a]The concentration required to elicit protonophoric activity is shown since the effect was not dose-dependent but showed a threshold concentration in general.
[b]As the first screening, dose-response was not examined. The value indicates % inhibition of the drug on the K^+-p-nitrophenylphosphatase actvity at the concentration shown in parentheses.
[c]These values are mostly taken from the manufacturer's data sheets and thus citation is omitted.
[d]Protonophore-like activity was evident in the acridine orange quenching whereas it was absent in the experiment of proton passive diffusion.
[e]Muto et al., 1997; [f]Biochem. J. 298 (1994) 87; [g]J. Pharm. Exp. Ther. 253 (1990) 688; [h]Br. J. Pharmacol. 120 (1997) 841; [i]Am. J. Physiol. 269 (1995) G435; [j]Pancreas 14 (1997) 301; [k]Prostagland. Leuko. Essent. Fatty Acid. 53 (1995) 355; [l]Biochim. Biophys. Acta 1330 (1997) 172.

al. showed that W-7 uniformly inhibited aminopyrine accumulation stimulated by any agonist, including histamine, forskolin, dbcAMP, and K^+, with the IC_{50} values being around 1 μM. Moreover, they observed that W-7 inhibited aminopyrine accumulation in digitonin-permeabilized parietal cells with an IC_{50} of 10 μM. It is doubtful that these effects are all critically dependent upon calmodulin. In fact, the reported IC_{50} of W-7 for calmodulin in vitro is around 50 μM, and that in vivo is usually even higher (Asano et al., 1982; see Table 1). Although there is a 10-fold difference in the IC_{50} values between those reported by Schepp et al. and our own (which might be due to the species difference), their data clearly indicate that W-7 was not working as a calmodulin antagonist, but rather as a non-specific inhibitor, like a protonophore. This would support our present conclusion.

For phenothiazine derivatives, we confirmed in the present study that chlorpromazine and trifluoperazine were potent antisecretory agents. This is consistent with the report by Sewing and Beil (1989) showing that a number of tricyclic antidepressants (including chlorpromazine, triflupromazine, trifluoperazine, etc.) uniformly inhibit histamine- or dbcAMP-stimulated [^{14}C]aminopyrine uptake in isolated guinea-pig parietal cells at sub-micromolar concentrations. Although some of these compounds have inhibitory activity against H^+,K^+-ATPase, their IC_{50} values are higher than 10 μM (Sewing and Beil, 1989; see Table 1). Sewing and Beil supposed that the inhibitory activity of these compounds could be attributed to the inhibition of H^+,K^+-ATPase after these compounds had accumulated in the acidic space as lipophilic bases. We also suggest, based upon our present study and the discussion of Batzri (1985) regarding the effect of amitriptyline in isolated rabbit gastric glands, that these tricyclic antidepressants, which are frequently used as calmodulin antagonists, also work as protonophores. It is evident from Table 1 that the antisecretory activity of the so-called calmodulin antagonists does not correlate with their anti-calmodulin activity. Based on these arguments, it should be concluded that there is no real pharmacological evidence of calmodulin involvement in gastric acid secretion. The development of a new, specific pharmacological tool is eagerly expected.

Among the calmodulin antagonists, calmidazolium was unusual. This compound, with the lowest IC_{50} in vitro, showed not only protonophore-like activity in the gastric vesicles but also inhibitory activity against the H^+,K^+-ATPase at micromolar concentrations. However, it did not inhibit acid secretion at all in isolated gastric glands in concentrations up to 30 μM. This suggests that calmidazolium does not penetrate into the parietal cell and is only useful in cell-free experiments.

In the search for a pharmacological tool for myosin light chain kinase, a calmodulin dependent enzyme, only wortmannin survived as a candidate from the present analysis—though ML-7 and ML-9 acted as protonophores. This of course does not prove that the antisecretory effect of wortmannin was due to its inhibition of myosin light chain kinase. Although it is well known that wortmannin inhibits phosphatidylinositol 3-kinase with an IC_{50} of 3 nM (Yano et al., 1993), the IC_{50} for acid secretion (2 μM) was out of the range where one would expect suppression of acid secretion to be due to inhibition of this enzyme.

4.2. Protein kinase inhibitors

It was clearly shown in the present study that all of the pharmacological tools used to analyze the involvement of cyclic AMP-dependent protein kinase, protein kinase C, or phospholipase C pathways in acid secretion need to be reconsidered. Although there is no doubt about the involvement of cyclic AMP-dependent protein kinase in the signal transduction of acid secretion (Urushidani and Forte, 1997), it would be very risky to assume the involvement of cyclic AMP-dependent protein kinase based on the modulation of acid secretion by H-89, whose IC_{50} for dbcAMP-stimulated secretion was close to the concentration showing protonophore-like activity. For this purpose, Rp-adenosine-3′,5′-cyclic monophosphorothioate would be better, but its efficacy is quite weak (Li et al., 1995) and it is costly.

For the involvement of protein kinase C, the situation is more complex. The putative ‘protein kinase C-inhibitor’ H-7 potentiated acid secretion in the present study as previously reported (Ostrowski and Bomsztyk, 1989; Urushidani and Nagao, 1996), but this might be interpreted as a protein kinase C-mediated inhibitory signal (for review, see Urushidani and Forte, 1997). It was reported earlier (Chew et al., 1994) that pharmacological analysis of the involvement of protein kinase C in acid secretion was difficult because of the presence of various types of protein kinase C and also because of the lack of specificity of the inhibitors. Chew et al. reported that the bisindolylmaleimide-type inhibitor, Ro31-8220, potentiated both carbachol- and histamine-stimulated aminopyrine accumulation, in contrast to the finding of an earlier report (McKenna and Hanson, 1993). It seems to be difficult to conclude that the potentiation by H-7 was due to its protein kinase C inhibition, since the IC_{50} of this compound in vitro for protein kinase C (6 μM) was almost the same as that for cyclic AMP-dependent protein kinase (3 μM). The concentration needed to inhibit dbcAMP-stimulated secretion was over 100 μM and at this concentration it showed protonophore-like activity in gastric microsomes. Moreover, other protein kinase C inhibitors, bisindolylmaleimide I and chelerythrine, which have no inhibitory effect on cyclic AMP-dependent protein kinase, did not show the obvious potentiating effects that H-7 did. TPA, an activator of protein kinase C, clearly exhibited a potentiating interaction with dbcAMP. In addition, genistein potentiated acid secretion, possibly not through inhibition of tyrosine kinase. So, it might be postulated that some types of kinase inhibitors work as potentiators of

acid secretion via the modulation of some other kinases or unknown factors in the parietal cell.

4.3. Miscellaneous agents

The physiological role of phospholipase C is also known, but there are no appropriate pharmacological tools to assess its involvement in gastric acid secretion. As we reported previously (Muto et al., 1997), the widely used phospholipase C inhibitor, U73122, cannot be used, at least in the parietal cell. In the present study, neomycin was also found to be useless for this purpose. At present, the mechanism by which neomycin inhibits acid secretion is unknown. This compound canceled the proton gradient formed in the gastric microsomes and in the mitochondria, but it did not affect H^+,K^+-ATPase activity by itself and did not accelerate, but rather inhibited, the passive proton diffusion in the gastric vesicles. It can only be concluded that the inhibitory effect of neomycin is not due to inhibition of receptor-coupled phospholipase C activity.

With similar arguments, it would be quite risky if one were to conclude from the results for ONO-RS-082 and DIDS that phospholipase A2 and the anion exchanger are both essential for acid secretion. ONO-RS-082 itself is a protonophore as well as a proton pump inhibitor, and this is enough to explain its antisecretory effect. For DIDS, it is established that the anion-exchanger plays an important role in the acid secretory process (Horie et al., 1993). As DIDS is thought to act on the outside of the cell membrane, and its IC_{50} values varied from 0.02 to 280 μM depending upon the type of the exchanger or channel (Cabantchik and Greger, 1992), many researchers usually use this compound at 100 μM or more. In the present study, the IC_{50} values of DIDS for acid secretion and H^+,K^+-ATPase were found to be 290 μM and 0.4 μM, respectively. This means that the intracellular concentration of DIDS should be lower than 1/1000 of the extracellular concentration if one does not want to alter the activity of H^+,K^+-ATPase. This is critical because some types of stilbene derivatives are possibly transported by the exchangers or channels (Cabantchik and Greger, 1992).

The results shown in Fig. 5 are somewhat remarkable since inhibition of H^+,K^+-ATPase by ONO-RS-082, calmidazolium, and DIDS occurred at such low concentrations that they might be lead compounds for new types of proton pump inhibitors, or at least tools for analyzing the enzymatic reaction of H^+,K^+-ATPase. Before they are used for these purposes, their specificity should be examined.

5. Conclusion

Although most of the compounds tested needed to be used at 10 times higher concentrations in the mitochondria than in the gastric vesicles or glands, some of them were still active in the commonly used concentration range. It is thus proposed that in some cases the uncoupling effect of a 'specific' agent on the mitochondria was accidentally interpreted as the result of its anticipated effects. Furthermore, it is also possible that researchers using these kinds of agents are misled into supposing the involvement of the intracellular signal when the effects are actually due to the loss of proton gradient in the lysosomes or endosomes, where the maintenance of the pH gradient is physiologically important (Al-Awqati, 1995).

We present Tables 1–3 as important guidelines for pharmacological investigations of acid secretion. Furthermore, they should be referred to when analyzing other physiological effects which might be affected by a protonophore. In some cases, we propose that previously established theory should be reconsidered.

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