Gastric Acid Secretion Is Augmented by the Replacement of Extracellular Na^+ With K^+ or Other Ions

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ABSTRACT—We examined the validity of the high K^+ condition, widely used as a model to investigate the mechanism of acid secretion, employing isolated rabbit gastric glands. When Na^+ in the nutrient solution was replaced with K^+ , the acid secretion monitored by the accumulation of ^{14}C -aminopyrine increased K^+ -concentration-dependently. Stimulation of carbachol or dbcAMP was also greatly enhanced by K^+ . The responses to forskolin, phorbol ester, gastrin and submaximal dose of histamine were potentiated in the high K^+ condition, but the responses to isobutylmethylxanthine, calyculin A and the maximal dose of histamine were not. The augmented response to carbachol was not dependent on $[\text{Ca}^{2+}]_o$. Replacement of Na^+ with any other ions, including Rb^+ , Cs^+ , Li^+ , tetramethylammonium and choline, increased the basal aminopyrine ratio. Most of these ions were also effective in enhancing the response to dbcAMP, but failed to augment the effect of carbachol. Replacement of Na^+ with K^+ , Rb^+ , Cs^+ and Li^+ transiently elicited acid secretion in the isolated mouse whole stomach. These results suggest that the high K^+ is not a simple model for the direct activation of the proton pump, but the augmentation consists of two components, i.e., the combined effects of low Na^+ and high K^+ . The former augments basal and cAMP-stimulated acid production, whereas the latter augments acid production by any agonist except calyculin A.

Keywords: H^+ , K^+ -ATPase, Alkaline metal, Gastric gland, Aminopyrine accumulation

It has been postulated that gastric acid is created by H^+ , K^+ -ATPase, which is translocated from tubulovesicles to the apical membranes in the parietal cell, exchanging K^+ secreted into the lumen with intracellular H^+ . Therefore, if the luminal K^+ could be increased by some means, acid secretion would be stimulated, bypassing the intracellular events with the direct activation of the enzyme in the secretory membranes. To investigate this idea, advanced work has already been performed by Berglin (1) in the days when the details of H^+ , K^+ -ATPase were still unknown. Using the isolated rabbit gastric gland model, he showed that the removal of K^+ ions from the outside of the glands abolished the stimulated acid secretion, and this was monitored by the accumulation of a weak base, ^{14}C -aminopyrine. On the other hand, the removal of Na^+ did not affect the secretory response, suggesting that extracellular K^+ was essential for gastric acid secretion. He also found that the increment of extra-

cellular K^+ by itself caused acid secretion or aminopyrine accumulation. The latter phenomenon has been utilized, without any critical examination of its mechanism, for the analysis of the actions of proton pump inhibitors (2–4), calmodulin antagonist (5) and epidermal growth factor (EGF) (6), as a way to activate the final step of acid secretion. If the acid secretion stimulated by high K^+ reflects the amount of functional H^+ , K^+ -ATPase on the apical membrane of the parietal cells, it should be quite a useful tool for investigating the mechanism of the membrane-recycling hypothesis, which is still subject to some debate (7, 8). However, there does not appear to be enough basic information about this phenomenon for establishing it as a model system to quantify the functional proton pump on the apical membrane. In the present study, we re-examined the process of gastric acid secretion under the condition of high K^+ and found some critical points that need to be elucidated.

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MATERIALS AND METHODS

Materials

^{14}C -aminopyrine and ^{14}C -inulin were purchased from New England Nuclear (Boston, MA, USA). Omeprazole was generously provided by Yoshitomi-Astra Co. (Osaka). Other chemicals of reagent grade were purchased from Sigma Chemical (St. Louis, MO, USA) or Wako Pure Chemical (Tokyo).

Isolation of rabbit gastric glands and measurement of their functions

Isolated gastric glands were prepared from Japanese White rabbits weighing about 2.5 kg (Shiraishi Co., Tokyo) by a combination of high pressure perfusion and collagenase digestion (9). Acid secretion of the glands was monitored by accumulation of a weak base, ^{14}C -aminopyrine, setting the water content of the glands at a constant, 2.0 $\mu\text{l}/\text{mg}$ dry weight (9). We reconfirmed this value in the resting glands using ^{14}C -inulin as an extracellular marker. When Na^+ ions were isotonicity replaced with other ions, or the glands were highly stimulated, the glandular water content was often increased up to about 3 $\mu\text{l}/\text{mg}$ as reported (9). We did not determine whether the increment was due to the swelling of the cells because of the intracellular ionic unbalance or to the expansion of the glandular lumen because of osmotic flow. We decided not to employ the increased value, but to use the constant value of 2.0 for the following reasons. If the former case is true (the glandular water does not change with the ionic replacement), there is no need to revise the aminopyrine ratio. If the latter case is true, it means that the acidic space was increased. Suppose the aminopyrine uptake/mg dry weight of the glands is doubled and the water content is also doubled. If the aminopyrine ratio is corrected by the increased water content, the aminopyrine accumulation would be concluded to be unchanged. However, we consider the case to be that the acid space is doubled, namely, the acid secretion is stimulated to be twice as much. Therefore, we employed the aminopyrine ratio based on the uniform value of 2.0 $\mu\text{l}/\text{mg}$ dry weight, which is not the real ratio but rather the uptake corrected by the dry weight of the glands and in this way is considered to be a closer measure of the actually secreted acid.

Drugs were all dissolved in dimethylsulfoxide in appropriate concentrations so that the final concentration of the vehicle assay was less than 0.5% v/v. The normal medium contained 132.6 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), 11.1 mM glucose and 1 mg/ml bovine serum albumin. The replacement of the ions were performed within the sum of the concentrations of NaCl and KCl, 138 mM: e.g., high K^+

medium with 120 mM KCl contained 17.8 mM NaCl, 5 mM Na_2HPO_4 , 1 mM NaH_2PO_4 , 120 mM KCl, 1.2 mM MgSO_4 , 1.0 mM CaCl_2 , 25 mM HEPES-Na, 11.1 mM glucose and 1 mg/ml bovine serum albumin.

Measurement of cyclic AMP contents (10)

Isolated glands were suspended and stimulated as above and isolated by centrifugation at the appropriate time. The pelleted glands were quickly suspended in 400 μl of 0.1 N HCl and boiled for 5 min. After centrifugation, the pellet was assayed for protein, and the supernatant was quantified for cyclic AMP (cAMP) by a radioimmunoassay using a kit obtained from Yamasa Co., Ltd. (Tokyo). The content of cAMP was expressed as pmol/mg protein.

Acid secretion in the isolated whole mouse stomach (11)

Male ddY strain mice (Japan SLC Inc., Shizuoka), weighing 22–32 g were anesthetized by urethane (15 mg/10 g, intraperitoneally) and the stomach was cannulated and removed. The isolated stomach was placed in a 20-ml organ bath containing a serosal side nutrient solution of the same composition as that for isolated glands and gassed with 100% O_2 at 37°C. The stomach lumen was perfused at the rate of 1 ml/min with a mucosal side nutrient solution (158.6 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO_4 , 1.0 mM CaCl_2 1.0, 11.1 mM glucose, adjusted to pH 5.0 with 0.1 N HCl). Acid output was measured by titrating hydrogen ion with 1/500 N NaOH to the end point of pH 5.0 with an automatic titrator (HM-5ES and HSM-10A; Toa Electronics Co., Tokyo). The digital pulse (2 $\mu\text{l}/\text{pulse}$) from the titrator was sent to a personal computer (FM-77; Fujitsu, Tokyo) equipped with a pulse counter. Vagal stimulation (5 Hz, 0.3 msec, 10 V for 5 min) was applied via a pair of platinum ring electrodes (0.25-mm-thick, 1.8 mm diameter; distance of the electrodes, 1.5 mm) at the position of the lower esophagus. Square-wave pulses were delivered from an electronic stimulator (SEN-7203; Nihon Kohden, Tokyo).

Statistical analyses

Parametric data were expressed as the mean $\pm$ S.E. Multiple comparisons were analyzed by ANOVA and Dunnett's post hoc test using a computer program (Super ANOVA, ABACUS Concepts). When appropriate, the paired *t*-test was also employed. The level of significance was uniformly set at $P < 0.05$, and no further calculation of *P* value was performed.

RESULTS

Figure 1A shows the aminopyrine ratios when the extracellular NaCl was replaced with equimolar KCl in the

presence or absence of the maximal dose of 0.1 mM carbachol or the submaximal dose of 0.1 mM *N*⁶,2'-*O*-dibutyryl adenosine 3':5'-cyclic monophosphate (dbcAMP). In order to eliminate the possible involvement of endogenous histamine, all the tubes contained 0.1 mM cimetidine. It is evident from the figure that K⁺ alone stimulated aminopyrine accumulation in a concentration-dependent manner. This result is similar to that by Berglinde et al. (12), except that they found a peak around 100 mM KCl. As obvious from the figure, the stimulatory effects of both carbachol and dbcAMP were markedly augmented as the concentration of KCl was increased up to 120 mM. Although the aminopyrine ratio at 138 mM was the highest, the net increase with the stimulants was found to be the highest at 120 mM KCl, because as the stimulatory effect of KCl alone steeply increased at 138 mM. Therefore, 120 mM KCl was employed as the optimal high K⁺ condition for the agonist-stimulated secretion in the following routine assay.

Figure 1B shows the similar experiments using sucrose instead of NaCl. In this series of experiments, NaCl was replaced with the same osmolality of sucrose, e.g., the medium of 120 mM KCl contained 18 mM NaCl in Fig. 1A while it contained 36 mM sucrose in Fig. 1B. The figure indicates that the effects of K⁺ on the resting and

carbachol- or dbcAMP-stimulated aminopyrine accumulation were essentially the same as those in Fig. 1A, except that the absolute values in the low K⁺ range were smaller in Fig. 1B.

In order to characterize the high K⁺ condition, we examined the effects of several inhibitors on the increment of aminopyrine accumulation by 120 mM KCl alone (plus 18 mM NaCl), as shown in Fig. 2. As it was suspected that either histamine or acetylcholine might be released by the depolarization of the neuronal cells, H₂ and muscarine antagonists were applied. Cimetidine at 0.1 mM tended to decrease the ratio. This means that either the effect of background histamine was potentiated by 120 mM K⁺ or that histamine was released by 120 mM K⁺, although its contribution to the whole reaction was relatively small. On the other hand, atropine was without effect, excluding the possible involvement of endogenous acetylcholine release in this response. The response to 120 mM K⁺ was abolished by 0.1 mM rotenone, and also by 10 μ M omeprazole, confirming that the increment of the ratio was dependent upon the H⁺,K⁺-ATPase utilizing mitochondria-derived energy.

We then followed the time course of the response to 0.1 mM carbachol employing 5.4 mM KCl for normal and 120 mM KCl for high K⁺ conditions. To exclude the in-

Fig. 1. Effects of replacement of NaCl with KCl, or with KCl and sucrose, on the aminopyrine accumulation of isolated rabbit gastric glands. The glands were incubated without stimulants, with 0.1 mM carbachol or with 0.1 mM dbcAMP in the presence of ¹⁴C-aminopyrine and 0.1 mM cimetidine for 30 min at 37°C, and the aminopyrine ratio was calculated. A: 5 mM K⁺ medium contained 133 mM NaCl, and NaCl was subsequently replaced with KCl as indicated. B: 5 mM K⁺ medium contained 266 mM sucrose, and sucrose was subsequently replaced with KCl in such a way that the osmolality was constant. The data are expressed as the mean $\pm$ S.E. of 4–6 experiments performed in duplicate.

Fig. 2. Effects of cimetidine (0.1 mM), atropine (10 μ M), rotenone (0.1 mM) and omeprazole (10 μ M) on the aminopyrine accumulation of isolated rabbit gastric glands in the medium containing 120 mM KCl. Stimulation of acid secretion was expressed as the increment of the aminopyrine ratio in the high K^+ medium containing 120 mM KCl above the normal medium, and the effects of drugs were expressed as the percentage of the control value. Therefore, when an aminopyrine ratio lower than that in the normal resting value was obtained with the drug treatment, the result had a negative value. The data are expressed as the mean $\pm$ S.E. of 3–4 experiments performed in duplicate. Inhibitions by rotenone and omeprazole were both statistically significant ($P < 0.05$).

volvement of histamine, 0.1 mM cimetidine was included in all the tubes. As shown in Fig. 3A, the stimulatory effect of 120 mM K^+ alone increased with time (aminopyrine ratio: 25.0 ± 5.8 , 26.1 ± 7.4 and 34.8 ± 7.4 , at 5, 15 and 30 min of incubation, respectively; mean $\pm$ S.E.M., $N=4$). As previously reported (9, 13, 14), the response to carbachol under the normal K^+ was a transient one, showing a blunt peak around 15 min of stimulation (aminopyrine ratio: 23.9 ± 3.6 , 29.1 ± 5.9 and 25.0 ± 6.6 , at 5, 15 and 30 min of stimulation, respectively; mean $\pm$ S.E.M., $N=4$). On the other hand, the response to carbachol in 120 mM K^+ was markedly augmented and continued to increase up to 30 min without decline (aminopyrine ratio: 39.5 ± 7.7 , 76.4 ± 17.5 and 108.7 ± 26.6 , at 5, 15 and 30 min of stimulation, respectively; mean $\pm$ S.E.M., $N=4$).

The role of extracellular Ca^{2+} on the carbachol-stimulated secretion was examined and the results are shown in Figure 3B. Extracellular Ca^{2+} was removed by the addition of 4 mM EGTA to the incubation medium containing 1 mM $CaCl_2$. The concentration of free Ca^{2+} in this condition was calculated to be 44 nM. Under the normal condition, the addition of EGTA abolished the responses

Fig. 3. Time course and extracellular Ca^{2+} dependency of carbachol (CCh)-stimulated aminopyrine accumulation of the isolated gastric glands under the normal and 120 mM K^+ conditions in the presence of 0.1 mM cimetidine. A: Isolated glands were incubated with or without 0.1 mM carbachol for 5, 15 and 30 min at 37°C in the normal and high K^+ (120 mM) conditions, and aminopyrine accumulation was quantified. B: Isolated glands were incubated with or without 0.1 mM carbachol for 5, 15 and 30 min at 37°C in the normal and high K^+ (120 mM) conditions with or without 4 mM EGTA. The aminopyrine accumulation was quantified in each point and normalized by the ratio obtained in the resting value in the normal medium. The data are expressed as the mean $\pm$ S.E. of 4 separate experiments performed in duplicate.

to carbachol at 5 and 15 min after stimulation (aminopyrine ratio: 19.2 ± 3.5 and 20.5 ± 3.6 , respectively; mean $\pm$ S.E.M., $N=4$) in accordance with the previous reports (14). In the 120 mM K⁺ condition, the removal of extracellular Ca²⁺ did not reduce, but rather tended to increase, the carbachol stimulation (aminopyrine ratio: 29.5 ± 7.4 , 59.5 ± 5.9 and 139.2 ± 45.6 , at 5, 15 and 30 min of stimulation, respectively; mean $\pm$ S.E.M., $N=4$).

Figure 4 shows the effect of 120 mM K⁺ on the histamine-stimulated secretion. The dose response curve of histamine shifted upwards under the condition of 120 mM K⁺ (Fig. 4). The augmentation of the effect of histamine was only evident at 10^{-7} and 10^{-6} M. In order to examine the possible involvement of phosphodiesterase in the potentiation under the high K⁺ condition, stimulation with histamine was performed in the presence of 50 μ M isobutylmethylxanthine (IBMX). However, IBMX alone elicited almost the maximal response and further increment by histamine was small under the normal condition. Raising the K⁺ concentration in the medium to 120 mM did not cause any further augmentation in the acid secretion (Fig. 4).

Fig. 4. Dose-response curves for histamine in the normal and the high K⁺ (120 mM) conditions with or without 50 μ M isobutylmethylxanthine (IBMX). Stimulation was performed for 30 min at 37°C. The data are expressed as the mean $\pm$ S.E. of 4 separate experiments performed in duplicate. *The net increases of aminopyrine ratio by 10^{-7} or 10^{-6} M histamine above the values without stimulant were significantly augmented by high K⁺ at $P < 0.05$.

In a similar way to the maximal concentration of histamine, the response to the maximal concentration of dbcAMP was not augmented by 120 mM K⁺. In the normal medium, the net increase of aminopyrine ratio with 1 mM dbcAMP was observed to be 252.9 ± 47.1 , and that in the high K⁺ condition was 255.0 ± 51.5 (mean $\pm$ S.E.M., $N=4$).

Figure 5 shows the influence of the 120 mM K⁺ condition on the effects of other agonists. The response to the sub-maximal dose of forskolin was also potentiated by high K⁺, although the extent of the augmentation was relatively small. It is well-known that the stimulatory effect of gastrin in rabbit gland preparation is minimal (15), and we could not observe any consistent stimulatory effects of gastrin even under the high K⁺ condition (data not shown). However, when gastrin stimulation was performed in the presence of 0.1 mM dbcAMP under the high K⁺ condition, a small but consistent increase of the aminopyrine ratio was observed. In the case of calyculin A, a phosphoprotein phosphatase inhibitor, which acts as a secretagogue in the normal K⁺ condition (10), there was no augmentation by high K⁺. Interestingly, 12-*o*-tetradecanoylphorbol-13-acetate (TPA), which is a very weak secretagogue under the normal K⁺ condition, showed a

Fig. 5. Stimulatory effects of Forskolin (Frk, 1 μ M), calyculin A (CLA, 0.3 μ M), 12-*o*-tetradecanoylphorbol-13-acetate (TPA, 1 μ M), dbcAMP (0.1 mM) and dbcAMP plus gastrin (Gast, 0.1 μ M) in the normal (filled column) and the high K⁺ (120 mM, open column) conditions. Values were the net increase in the aminopyrine ratio above the values without stimulant. The data are expressed as the mean $\pm$ S.E. of 4 to 5 separate experiments performed in duplicate. *High K⁺ significantly augmented the responses at $P < 0.05$ by the Dunnett's test. \$High K⁺ significantly augmented the responses at $P < 0.05$ by the paired *t*-test. #Gastrin significantly increased the aminopyrine ratio over corresponding dbcAMP-stimulated control at $P < 0.05$ by the paired *t*-test.

marked stimulatory effect under the high K^+ condition. To confirm that this effect of TPA was mediated by the activation of protein kinase (PK) C, bisindolylmaleimide I, a PKC inhibitor (16), was included, and we observed a complete inhibition of the aminopyrine ratio ($1 \mu M$ TPA in $120 \text{ mM } K^+$: 40.0 ± 5.0 ; $1 \mu M$ TPA plus $10 \mu M$ bisindolylmaleimide I in $120 \text{ mM } K^+$: 8.0 ± 2.0 , $N=4$; $P<0.05$).

As carbachol and TPA behaved similarly in the experiments described above, we further compared these two agonists. As shown in Fig. 6A, carbachol alone showed a small increase in aminopyrine ratio, whereas it elicited a large stimulation in combination with the sub-maximal concentration of dbcAMP (0.1 mM), exhibiting a marked synergistic effect. In a similar way, TPA had a small stimulatory effect by itself, while it showed a marked synergism with dbcAMP. When the stimulation was performed under the $120 \text{ mM } K^+$ condition, each response to dbcAMP, carbachol and TPA was augmented, whereas the responses to dbcAMP were potentiated by neither carbachol nor TPA (Fig. 6B). This means that the synergism between cAMP and carbachol or cAMP and TPA was lost under the high K^+ condition. However, it might be due to the fact that the combined responses (aminopyrine ratio about 200) approached the maximal ability (about 300) for the glands.

Although it is obvious from Fig. 1B that the stimulatory effect of high K^+ is not due to the low Na^+ , we examined the possibility that the effect of high K^+ shown above

could be surrogated by other cations, i.e., Na^+ ions were replaced with those other than K^+ . The results are summarized in Figure 7. Surprisingly, replacement of Na^+ with any other cations including Rb^+ , Cs^+ , Li^+ , tetramethylammonium (TMA) and choline caused obvious increases in aminopyrine ratio. In general, the aminopyrine ratio varies with the gland preparations, but the stimulatory effect of choline chloride was small but consistently observed. On the other hand, the stimulatory effects of $CsCl$ were not very reproducible, as is obvious from the standard error in the figure. In this series of data, the maximal value was 490, while the minimum was 7.0; the latter value was smaller than the resting value, 12.1, in this particular measurement. Furthermore, a large variation was sometimes observed between duplicate measurements. In the case of $LiCl$, stimulation was consistently observed, although the variation was also large. Figure 8 shows the representative concentration-response curve for KCl , $RbCl$ and $LiCl$. It is obvious that the shape of the curve for Rb resembled that for KCl , whereas for $LiCl$, the shape of the curve was different, i.e., the stimulatory effect was evident in the range of lower concentration.

The effects of Na^+ replacement could be explained by the inhibition of phosphodiesterase if one ignores the observation that high K^+ did not so obviously augment the response to histamine. Therefore, we measured the cAMP contents in the glands under the various conditions and results are summarized in Table 1. In order to

Fig. 6. Effects of combination of secretagogues on acid secretion in isolated rabbit gastric glands under the normal and high K^+ conditions in the presence of 0.1 mM cimetidine. Carbachol (CCh, 0.1 mM) and dbcAMP (cAMP, 0.1 mM), each alone or their mixture (cAMP+CCh), or 12-*o*-tetradecanoylphorbol-13-acetate (TPA, $1 \mu M$) and dbcAMP, each alone or their mixture (cAMP+TPA), added to the glands and incubated for 30 min at $37^\circ C$ in the normal (A) or $120 \text{ mM } KCl$ (B) medium. Aminopyrine ratios in the presence of cimetidine alone (R) were also measured and expressed as means $\pm$ S.E. of 4 separate experiments performed in duplicate. *Significantly higher than cAMP alone at $P<0.05$.

Fig. 7. Effects of replacement of NaCl with various salts or sucrose on the aminopyrine accumulation of isolated rabbit gastric glands. The replacement of the ions was performed within the sum of the concentrations of NaCl and KCl, 137.8 mM, in the incubation medium. The glands were incubated with ¹⁴C-aminopyrine and 0.1 mM cimetidine for 30 min at 37°C and the aminopyrine ratio was calculated. The data are expressed as the mean ± S.E. of 4 experiments performed in duplicate.

Fig. 8. Effects of replacement of NaCl with KCl, LiCl or RbCl on the aminopyrine accumulation of isolated rabbit gastric glands. The replacement of the ions was performed within the sum of the concentrations of NaCl and KCl, 137.8 mM, in the incubation medium. The glands were incubated with ¹⁴C-aminopyrine and 0.1 mM cimetidine for 30 min at 37°C and the aminopyrine ratio was calculated. As the absolute values of aminopyrine ratio largely varied, as shown in Fig. 7, a representative experiment performed in duplicate in the same lot of glands is depicted.

exclude the possible involvement of endogenous histamine, 0.1 mM cimetidine was included in all the tubes except those where histamine stimulation was performed. Histamine increased intracellular cAMP at 5 and 30 min of stimulation. IBMX alone caused a rapid and large increase in cAMP even in the presence of cimetidine. The addition of histamine in the presence of IBMX and absence of cimetidine further increased the cAMP contents.

On the other hand, any type of replacement of K⁺ did not affect the contents of cAMP either with or without IBMX, but tended to decrease the contents.

In order to further characterize the effects of cation replacement, we examined the effects of various ions on the stimulated secretion. At first, we compared the various concentrations of K⁺ on the stimulated secretion by

Table 1. Effects of histamine, IBMX and ion-replacement on cAMP content of isolated gastric glands

Without IBMX						
	Control	Histamine	KCl	RbCl	CsCl	LiCl
5 min	6.0 ± 0.9	13.6 ± 2.4*	6.3 ± 1.7	4.6 ± 0.7	4.3 ± 0.6	5.0 ± 0.9
30 min	6.3 ± 1.7	17.0 ± 2.9*	6.2 ± 2.1	4.7 ± 1.2	4.0 ± 0.8	4.2 ± 1.3
With IBMX						
	Control	Histamine	KCl	RbCl	CsCl	LiCl
5 min	11.9 ± 1.8	37.3 ± 9.5*	9.9 ± 1.4	10.8 ± 2.0	8.9 ± 1.3	10.6 ± 2.6
30 min	15.2 ± 3.9	52.3 ± 9.7*	10.6 ± 1.5	12.7 ± 2.3	9.4 ± 1.3	6.3 ± 1.8

Values (pmol/mg protein) are means ± S.E. from 4 separate experiments. Isolated rabbit gastric glands were incubated with histamine in normal medium or with the media in which NaCl was replaced by the indicated salts, for 5 or 30 min in the presence or absence of 50 μM isobutylmethylxanthine (IBMX). To exclude the possible involvement of endogenous histamine, 0.1 mM cimetidine was included in all of the tubes except when histamine stimulation was performed. The glands were harvested by a quick centrifugation and boiled in 0.1 N HCl for 5 min. After centrifugation, extracted cAMP was quantified by radioimmunoassay and normalized by protein content of the pellet. *Significantly different from the respective control at P < 0.05.

0.1 mM dbcAMP or 0.1 mM carbachol in the medium where NaCl was replaced with choline-Cl. We chose choline for this purpose because the stimulatory effect of choline alone was small and with least variations as shown in Fig. 7. Replacement of 138 mM NaCl with 5 mM KCl plus 133 mM choline-Cl resulted in an aminopyrine ratio that was slightly larger than that in the normal medium. When the K^+ concentration was increased by the replacement with choline, the aminopyrine ratio was further increased (Fig. 9). When the glands were stimulated by 0.1 mM dbcAMP, the response was markedly augmented even in the normal K^+ condition, i.e., 5 mM KCl + 133 mM choline-Cl, and the increment of K^+ to 80 mM or more further increased the stimulatory response. On the other hand, the stimulatory effect of carbachol was only augmented by the increasing dose of K^+ in a way similar to that in Fig. 1. These results clearly indicate that low Na^+ increases basal and dbcAMP-stimulated aminopyrine accumulation, while high K^+ increases basal and stimulated aminopyrine ratio either by carbachol or by dbcAMP.

To confirm the conclusion above, we compared the effects of ion replacement with various cations. In this series of experiments, K^+ concentration was fixed to 5 mM, and 133 mM NaCl (normal) was replaced with equimolar KCl, RbCl, CsCl, choline-Cl or TMA. The glands

Fig. 9. Effects of KCl and choline-Cl on the aminopyrine accumulation of isolated rabbit gastric glands. The glands were incubated without stimulants, with 0.1 mM carbachol or with 0.1 mM dbcAMP in the presence of ^{14}C -aminopyrine and 0.1 mM cimetidine for 30 min at 37°C, and the aminopyrine ratio was calculated. Zero K^+ medium contained 138 mM of choline-Cl, and choline was subsequently replaced with K^+ as indicated. The data are expressed as the mean $\pm$ S.E. of 4 experiments performed in duplicate.

Fig. 10. Stimulatory effects of carbachol and dbcAMP in the normal and the high K^+ , Rb^+ , Cs^+ , TMA, choline and Li^+ -medium. Rabbit isolated glands were incubated with 0.1 mM dbcAMP (A) or 0.1 mM carbachol (B) for 30 min at 37°C in the normal medium or in the medium in which NaCl+KCl (138 mM total) was replaced with equimolar of KCl, RbCl, CsCl, TMA-Cl, choline-Cl and LiCl. To exclude the possible involvement of endogenous histamine, 0.1 mM cimetidine was included in all the tubes. The net increase of the aminopyrine ratio above the values without stimulant is shown. The data are expressed as the mean $\pm$ S.E. of 4–6 separate experiments performed in duplicate. *Significantly different from the value under the normal condition at $P < 0.05$. #Significantly different from the value without carbachol at $P < 0.05$.

were stimulated by 0.1 mM dbcAMP or carbachol, and the increment in the aminopyrine ratio over the value under the stimulant-free condition was calculated and depicted in Fig. 10. The stimulatory effect of dbcAMP was again markedly augmented by K⁺ (138 mM), to 6- to 7-fold of the normal value (Fig. 10A). Although weaker than that by K⁺, other ions including Rb⁺, Li⁺, choline and TMA were also able to augment the effect, except Cs⁺, which failed to cause statistically significant augmentation. In the case of carbachol stimulation, the augmentation was only evident in the high K⁺ medium (Fig. 10B). The stimulatory effects of carbachol in the Rb⁺ medium were the same extent as those in the normal condition, whereas those in the media containing Cs⁺, choline and TMA were all reduced. When carbachol stimulation was performed in Li⁺ medium, a lower aminopyrine ratio than the value without stimulant was consistently obtained, i.e., the effect of carbachol became inhibitory.

In the present study, the effect of cation-replacement was assessed by the accumulation of a weak base, aminopyrine, in the acidic space. Although rather unlikely, it might be possible that the physicochemical interactions between the ions and the bases resulted in an apparent stimulatory effect on the accumulation in the acid space. We therefore measured the actually secreted acid using isolated mouse whole stomach. Figure 11 depicts

the typical responses of this preparation to the electrical vagal stimulation (Fig. 11A), histamine (Fig. 11B), and bethanechol (Fig. 11C). When the serosal Na⁺ was replaced with K⁺, a large secretory response was observed (Fig. 12A). This response declined with time even though the serosal side was kept high in K⁺. The replacement with Rb⁺ also caused a transient secretion (Fig. 12B). Cs⁺ also caused an obvious secretory response (Fig. 12C), although its duration was smaller than those by K⁺ or Rb⁺. In contrast to these ions, Li⁺ showed different effects. Replacement of Na⁺ with Li⁺ did not cause a secretory stimulus by itself (data not shown), while it did cause an obvious secretory response after the stomach had previously received electrical vagal stimulation (Fig. 12D). This response to Li⁺ was also transient and turned into an inhibitory response, as the response to histamine disappeared in the presence of Li⁺. It was practically impossible to make a precise assessment of the drug effects on these responses, since the transient response to the ion replacement could not be repeated in the same preparation. In the preliminary experiments, it appeared that at least part of the effects of the ion replacement were independent of the endogenous histamine, since definite responses were observed in the presence of 10 μ M famotidine, an H₂-antagonist, although the responses did tend to be somewhat smaller than the control.

Fig. 11. Typical pattern of acid secretory responses in the isolated mouse whole stomach preparation. The isolated stomach was placed in a 20-ml organ bath containing a serosal side nutrient solution of the same composition as that for isolated glands, and the stomach lumen was perfused at the rate of 1 ml/min with a mucosal side nutrient solution without buffering components. Acid output was measured by titrating hydrogen ion with 1/500 N NaOH to the end point of pH 5.0 with an automatic titrator and shown in the ordinate. A: Effects of vagal stimulation (5 Hz, 0.3 msec, 10 V for 5 min; VS) applied 3 times with a 30-min interval. B: Effect of the maximal dose of histamine (500 μ M) applied to the serosal solution. C: Effect of the maximal dose of bethanechol (100 μ M) applied to the serosal solution.

Fig. 12. Typical pattern of acid secretory responses in the isolated mouse whole stomach preparation as described in Fig. 10. A: Effects of vagal stimulation and serosal high K^+ medium (138 mM). B: Effect of serosal high Rb^+ medium (138 mM). C: Effect of serosal high Cs^+ medium (138 mM). D: Effects of vagal stimulation and serosal high Li^+ (138 mM) medium. Note that all the responses by ion replacement are transient. This figure that 500 μM histamine, which elicits the maximal acid secretory response (Fig. 11), failed to stimulate acid secretion under the high Li^+ condition.

DISCUSSION

There have been a few riddles in the relationship between the activation of acid secretion and the K^+ concentration. The first is that high K^+ stimulates aminopyrine accumulation without any increase in oxygen consumption (1). The second is that morphological changes, typical to the secreting parietal cell, are not associated with high K^+ stimulation (12). The third is that the removal of K^+ from the medium abolishes the stimulation of acid secretion, while the replacement of Na^+ with choline does not inhibit acid secretion in spite of the fact that Na^+ removal causes a decrease of intracellular K^+ to a level lower than that attained by the K^+ removal (1, 17).

For the first question, there are several examples in which the rate of respiration is not closely related to the rate of acid secretion (13). However, these are not cases

where acid secretion occurs without oxygen consumption, but those in which oxygen consumption increases without acid secretion. The data from the isolated mouse whole stomach in the present study suggest that the stimulatory effects of ion replacement may possibly be transient. Because the measurement of oxygen consumption is not as sensitive as determining the aminopyrine accumulation and because the precise measurement is only possible after some equilibrium period (9), the increase in the energy demand for a transient stimulation of acid secretion might be lower than the detection limit. If the ion replacement acts in maintaining the acid space formed in the intraglandular space, an increase of aminopyrine accumulation could be observed. Concerning this possibility, we first hypothesized that the removal of Na^+ from the basolateral side would suppress the Na^+/H^+ -exchanger, which has been proven to be important in main-

taining the cytosolic pH (18). Although the lowest pH attained by the inhibition of the exchanger, about 6.5 (18, 19), would not be enough to cause any accumulation of aminopyrine, considering its pK_a value (9), it could be possible that the intracellular acidification works to restrict the passive leak of the intraluminal protons. However, such a mechanism is inconsistent with the reports that amiloride, an inhibitor of the Na⁺/H⁺-exchanger, does not affect the acid secretion in the rat gastric mucosa (20) and that another inhibitor, dimethylamiloride, inhibits acid secretion in the rabbit parietal cell (19). This of course does not entirely exclude the possibility that the ion-replacement indirectly increases any acid space within the glands, since the intracellular pH of the parietal cell has been proven to be controlled by multiple factors (18, 21).

For the second question, it could be proposed that morphological change is not always necessary if H⁺,K⁺-ATPase, which is known to exist in the apical membrane as well as in the tubulovesicle membrane even in the resting state (22), can be directly activated and the acid space formed either in the glandular lumen or in the tubulovesicles. The morphological transformation of the parietal cell by secretagogue occurs when actual acid secretion is inhibited (13, 23), and a definite acid secretion without morphological transformation can sometimes occur (13). It could be concluded that acid secretion without morphological change would be possible under certain artificial conditions.

The third question means that the proton pump activity is correlated not with the intracellular K⁺ concentration but with the extracellular one. Koelz et al. (17) attributed this to the inhibitory effect of Na⁺ on the cytosolic side of H⁺,K⁺-ATPase. They examined the effects of K⁺ and Na⁺ on the aminopyrine accumulation of the amphotericin B-permeabilized glands and found the stimulation by K⁺, with a K_m about 16 mM, and the potent inhibition by Na⁺. Therefore, the increase in aminopyrine ratio by low Na⁺, as well as the decrease in aminopyrine ratio by low K⁺, could be all attributed to the inhibitory effect of intracellular Na⁺. On the other hand, the mechanism of how high K⁺ alone stimulates acid secretion has not been clearly explained. They postulated that K⁺ in the nutrient solution is accessible to the H⁺,K⁺-ATPase from the luminal side. However, this seems unlikely to us because if the ions in the nutrient solution are directly accessible to the luminal side, the proton gradient cannot be maintained, and subsequently the accumulation of aminopyrine would not be observed.

In the present study, the stimulatory effects of replacement of NaCl with KCl in the nutrient solution appear to consist of two components; namely, the effect of low Na⁺ and the effect of high K⁺. Low Na⁺, which does not

necessarily mean high K⁺, increases the basal aminopyrine accumulation as well as stimulation of secretion by dbcAMP but not carbachol, whereas high K⁺ increases the basal and stimulated secretion by any agonist except calyculin A. Replacement of Na⁺ with sucrose failed to augment aminopyrine accumulation, and this could be due to the reduction of the Cl⁻ concentration, which is known to be essential for acid secretion (13).

In previous reports (1, 12), the stimulation by histamine was not augmented by high K⁺. However, they used histamine at the maximal concentration (0.1 mM). In the present study, we observed that the potentiating effect of high K⁺ on the histamine-stimulated response was only evident in the range of sub-maximal concentration, although its degree was much less than that of cholinergic stimulation. In scrutinizing the original paper by Berglinth, he observed that the Na⁺-free (replaced with choline chloride) condition caused a small but significant increase in the aminopyrine ratio, as well as enhancing the dbcAMP-stimulated response, although he did not discuss these particular points in the paper (1). The simplest interpretation is that the replacement of Na⁺ in the nutrient solution with other ions decreases intracellular Na⁺, which is inhibitory to H⁺,K⁺-ATPase (17). However, this cannot explain the reason why the effects of the cation replacement varied with the secretagogues. It is also unsolved why some cations stimulated aminopyrine accumulation more potently than K⁺. Hofer and Machen (21) reported that 72 mM LiCl stimulated aminopyrine accumulation of rabbit glands. They attributed the effect to endogenously released histamine, since cimetidine attenuated but did not abolish the response. In the present study, we performed the ion replacement in the presence of 0.1 mM cimetidine and consistently observed an increase in the aminopyrine ratio by Li⁺. This discrepancy could be due to a difference in the experimental conditions, i.e., they pretreated the glands with amiloride to cause intracellular acidification, and this has been known to attenuate the secretory response (19).

The most interesting finding is that the augmentation of carbachol-stimulated secretion by high K⁺ is not surrogated by other cations. This means that carbachol-stimulated secretion is not subject to inhibition by intracellular Na⁺, if the result of cation replacement is explained by a "low Na⁺ effect". Based on the consensus that the activation of M3-receptor results in the activation of phospholipase C β via Gq (24), it should be reasonable to conclude that high K⁺ affects either the IP₃/Ca²⁺ pathway or the PKC pathway. Tsunoda and Mizuno (25) reported that the reduction of Na⁺ in the nutrient solution augmented the Ca²⁺-transient in the parietal cell because of attenuation of Ca²⁺ extrusion via the Na⁺/Ca²⁺ exchanger.

However, from the observation that the acid secretory response to carbachol under the high K^+ condition was not inhibited by the removal of extracellular Ca^{2+} , we consider that the involvement of Ca^{2+} is minimal. In our preliminary experiments, high K^+ did not affect the rise of the first phase of intracellular Ca^{2+} by carbachol, which has been reported to be more closely correlated with acid secretion than the second plateau phase (14).

The observation that TPA, an activator of PKC, which has little stimulatory effect in the normal medium, showed a potent stimulatory effect in the high K^+ medium strongly suggests that the PKC pathway is involved in the augmentation of the cholinergic pathway. The involvement of PKC in the acid secretion is complex: both stimulatory and inhibitory pathways have been reported. The activation of PKC by TPA shows a weak stimulatory effect by itself (26). However, it inhibits secretion stimulated by other agonists (27). PKC inhibitors usually suppress carbachol-stimulated secretion, but some of them augment the secretion stimulated by various agonists in certain concentration ranges (27). Therefore, it should be reasonable to conclude that high K^+ either augments the stimulatory part, or inhibits the inhibitory part, of the PKC pathway. In the present study, the activation of PKA and that of PKC work synergistically for acid secretion in the normal condition, while the synergism disappears under the high K^+ condition, suggesting that high K^+ plays a role similar to the crosstalk between PKA and PKC. Based on the observations that high K^+ augmented the response to sub-maximal activation of PKA as well as the response to PKC and that high K^+ failed to augment the response to calyculin A, a phosphoprotein phosphatase inhibitor, it could be postulated that the augmentation is due to inhibition of the dephosphorylation of some key proteins. Although there remain too many issues to be examined, the high K^+ condition may serve as a powerful tool for analyzing the activation process via PKC, which has been difficult to analyze because of its too small secretory response.

In the high Li^+ condition, the effect of carbachol turned out to be inhibitory. It has been suggested that the signaling pathway of cholinergic stimulation contains an inhibitory signal (14), although the precise mechanism is unclear. As the inhibitory effect of carbachol was most prominent and reproducible in the high Li^+ condition, this might be also useful for analyzing the putative inhibitory mechanism mediated by cholinergic transmission. It is at least evident from the present data that the stimulatory effect of Li^+ alone is not due to its well known property of inhibiting the degradation of phosphoinositides (28), since this ion did not augment, but inhibited cholinergic stimulation, which is considered to be involved in inositol phosphate metabolism.

Acid secretory activity of isolated gastric glands was monitored by an indirect measurement, i.e., the accumulation of a weak base, aminopyrine, in the acid space. Therefore, any change in the distribution of the base by non-physiological effects can be interpreted as a change in the acid secretion. Another disadvantage of the measurement with a weak base is that the actual secretory rate could not be estimated. We therefore measured the actually secreted acid using isolated whole mouse stomach preparation. This method employs a titration of the intragastric perfusate up to $pH=5.0$, excluding the possible interference of lactate and bicarbonate, both of which are usually problematic in case of pH measurement. This model also has an advantage in that it responds to the vagal nerve stimulation, which is considered to be more of a physiological stimulus than exogenously added agonists. Using this preparation, we observed transient but obvious secretory responses to the replacement with K^+ , Rb^+ and Cs^+ . On the other hand, Li^+ elicited acid secretion by an apparently different mechanism, i.e., it did not stimulate secretion by itself, but caused a secretion after the vagal stimulation. Although these results in the mouse stomach were not entirely consistent with those obtained in the rabbit glands, it was at least confirmed that the increase in aminopyrine ratio reflected the actual production of gastric acid and also that the effect might be transient.

In summary, we demonstrated in the present study that the replacement of Na^+ with other cations stimulates acid secretion or augments secretion stimulated by dbcAMP, possibly due to reduction of the inhibitory effects from intracellular Na^+ . High K^+ , on the other hand, augments basal and stimulated secretion by any agonists except for protein phosphatase inhibitor. The present study provides a useful tool for analyzing the activation pathway via PKC, which has been difficult to study because of its small response. We also propose that a high K^+ condition is not simply working to activate H^+ , K^+ -ATPase at the final step of the activation process of acid secretion, but also affects some of the steps related to protein phosphorylation.

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