J. OSTROWSKI, W. ZYCH, K. DOŁOWY*, E. BUTRUK

THE RELATIONSHIP BETWEEN EXTRACELLULAR K+ AND Ca²+ ON AMINOPYRINE ACCUMULATION IN RAT PARIETAL CELLS

Department of Gastroenterology and Metabolism and *Department of Biophysics and Biomathematic, Medical Center of Postgraduate Education, Warsaw, Poland.

The effects of extracellular K+ in relation to extracellular Ca²+ on acid production were studied. Studies were performed in vitro using isolated cells from rat stomachs, and acid production was indirectly determined by ¹⁴C-aminopyrine (AP) accumulation. In the absence of K+ in the incubation medium histamine-stimulated AP accumulation ratios were significantly decreased independently in the presence or absence of extracellular Ca²+. Under basal conditions, in the absence of extracellular Ca²+, increasing concentrations of extracellular K+ enhanced AP accumulation ratios to significantly higher than those found in the presence of Ca²+. In histamine-, cAMP-, and carbachol-stimulated parietal cells, high K+ concentrations increased AP accumulation significantly less in Ca²+-free than in Ca²+-containing media. High K+ also induced significantly both an increase in cytosolic free Ca²+ concentration and ⁴⁵Ca²+ uptake. The present results confirmed the importance of K+ in gastric acid production and suggested a role for Ca²+ as a modulator of mechanisms of parietal cell stimulation.

Key words: *rat parietal cells; acid secretion; extracellular K+ and Ca²+*

INTRODUCTION

In parietal cells there are two separate cAMP- and Ca+-dependent pathways involved in the stimulation of acid secretion which are activated by histaminergic H_2, cholinergic, and gastrinergic receptors on the plasma membrane (for review see 1—5). Through undefined mechanisms, the subsequent actions of cAMP- and Ca²+-dependent protein kinases enhance pumping of protons by the K+-H+-ATPase.

Acid secretion depends on the extracellular K+ (6—9), and high extracellular K+ concentration stimulates acid production (10—13). Since K+-H+-ATP-ase activation requires a supply of K+ at its luminal surface (14—16), the increased concentration of extracellular K+ may stimulate acid production in a direct manner. On the other hand, high K+ can intro-

duce Ca²⁺ into the cell without receptor stimulation (17). Therefore, since Ca^{2+}-mediated mechanisms (synergistic with the cAMP-dependent system) are among second messengers, the stimulatory effect of high extracellular K^+ on acid secretion might also act via increased intracellular Ca^{2+} level, activating a Ca^{2+}-dependent pathway involved in acid production.

The aim of this study was to evaluate the relationship between extracellular potassium and calcium on acid accumulation in isolated parietal cells from rat stomachs.

MATERIALS AND METHODS

Reagents: [Dimethylamine-¹¹C]-aminopyrine and ⁴⁵CaCl₂ were obtained from New England Nuclear Research Products; dibutyl phthalate and bisphthalate were purchased from Kodak (Rochester, N. Y.); Fura-2 AM was from Molecular Probes and all other chemicals were from Sigma (St. Louis, Mo.).

Cells. Usually, two or three nonfasted female Wistar rats, weighing 240—300 g, were used per experiment. The isolation of cells was performed according to the method of Gespach et al (18) through the use of the stomachs transformed into „everted sacs". Each sac was filled with 3 ml of ice-cold isolating medium containing 2.5 mM EDTA in 0.25 M NaCl, pH = 7.5. The sacs were incubated in the same solution on ice for 30 min. Thereafter, the gastric glands were liberated from the mucosa by hand shaking for 10 sec. After shaking isolated glands were harvested by centrifugation (200x g for 2 min) and stomachs were shaken again in 25 ml of fresh isolating medium. This procedure was repeated several times until no more glands were obtained. The combined isolated gastric glands were washed and resuspended three times in the incubation medium containing (in mM): 0.5 NaH_2PO_4, 1 Na_2HPO_4, 20 $NaHCO_2$, 70 NaCl, 5 KCl, 11 glucose, 1 $CaCl_2$, 1.5 $MgCl_2$, 50 HEPES-NaOH, pH 7.4, and 0.1 mg bovine serum albumin/ml. The gastric glands were further mechanically dispersed into single cells by repeated aspiration into a Pasteur pipette with a fire-polished tip. The dissociated product, consisting of single cells and small clumps was then filtered through a 60-μm nylon mesh. Isolated cells were collected by centrifugation at 200 x g for 5 min. and finally suspended in the same incubation medium. The viability of isolated cells was determined by exclusion of 0.4% trypan blue, and was over 90% in all preparations. Each cell separation yielded 18—27% of parietal cells, as identified by examination with a light microscope.

Enrichment of parietal cells was achieved by isopycnic centrifugation using a Percoll gradient according to Sonnenberg et al as described previously (19). The enriched cell population contained 72—80% parietal cells with 82% viability.

¹⁴C-aminopyrine accumulation was used as an index of acid secretory activity. Accumulation of ¹⁴C-aminopyrine was measured by the method of Berglindh et al (20) with some modifications as described in detail previously (19). Briefly, after a 60 minute preincubation at 37°C in incubation medium containing 0.1 mM IBMX gassed with 95% O_2—5% CO_2, the crude cell suspension was divided into suitable portions, then the cells were harvested by centrifugation (200 x g for 5 min.) and resuspended in the various media (specified in figure legends). These media were the same as incubation medium with the exception that different concentrations of KCl isotonically replaced NaCl.

The compositions of the Ca^{2+}-free media were the same as those in the normal incubation medium, except that they contained 2 mM EDTA (without $CaCl_2$ and $MgCl_2$), and in media with lower or higher concentrations of K^+, Na^+ concentrations increased or decreased equivalently to maintain isotonicity. Replaced media containing 0.1 mM IBMX and 0.05 μCi ^{14}C-aminopyrine per each 5×10^6 cells were previously gassed for 5 min. with 95% O_2—5% CO_2. Next, 5×10^6 cells were incubated in 1 ml of medium, without or with inducers of acid production in capped 1.5 ml Eppendorf tubes for 60 minutes more at 37°C.

All experiments were performed in triplicate. The ^{14}C-aminopyrine accumulation was calculated as an accumulation ratio of ^{14}C-aminopyrine radioactivity in the parietal cell to that in the medium (19).

Measurement of cytosolic Ca^{2+} was perfomed using a intracellulary trapped fluorescent indicator Fura-2. Briefly, 10^6 cells/ml (from the parietal cell enriched fraction) were incubated in incubation medium (same as described above) with 2 μM Fura 2-acetoxymethyl ester for 30 min. in a 95% O_2—5% CO_2 environment at 37°C. After loading, the cell suspension was rinsed and resuspended in incubation medium without Fura 2-AM and kept at 37°C until use. Immediately before use, a 1 ml cell suspension (2×10^6 cells) was harvested by centrifugation (8000 g for 5 sec) and the cells were washed twice with Hepes-NaOH-buffered EGTA containing saline that contained (in mM): 120 NaCl, 5 KCl, 10 glucose, 0.5 EGTA, and 20 HEPES-NaOH, pH = 7.4. In 70 mM K^+ saline, KCl isotonically replaced NaCl. The cell pellet was dispersed and diluted with either 5 or 70 mM KCl, Ca^{2+}-free saline and then transferred to a quartz cuvette with a magnetic stirrer and the cuvette was placed in the sample chamber of a SLM Aminco SPF-500 spectrofluorometer (Urbana, IL) maintained at 37°C. The intracellular free Ca^{2+} concentration was calculated from the fluorescent signals as described by Grynkiewicz et al (21).

Measurement of $^{45}Ca^{2+}$ *uptake.* 2×10^6 cells from the parietal cell enriched suspension were incubated for 60 minutes with 0.5 μCi $^{45}Ca^{2+}$ plus the indicated agents in 1 ml of incubation medium (same as for aminopyrine accumulation) gassed with 95% O_2—5% CO_2. At the end of the incubation, an aliquot of the cell suspension was centrifuged (12.000 x g for 2 min) through 0.3 ml dibutylphthalate-bisphthalate oil (1:1) in 1.5-ml Eppendorf tube. The medium was pipetted out and the wall of the tube was washed two times by adding and pipetting out deionized water. Next, the oil was discarded and the cell pellets were lysed with 200 μl of water and then boiled, vortexed, and centrifuged, and the radioactivity of the supernatant from the cell lysate was counted.

Data. Data are expressed as mean±S. D. Significance was determined by Student's *t* test.

RESULTS

In this study, the parietal cells from rat stomachs were isolated with hyperosmolar NaCl-EDTA solution and it seems that the method of gastric cell isolation without any enzyme digestion is superior to the pronase-EDTA method used in our laboratory previously. By using hyperosmotic NaCl-EDTA solution we have obtained cellular preparations with almost exclusively tubular glands which preserved the morphological

and functional characteristics of parietal cells in situ. Compared to our previously published results (19, 22), the response of parietal cells from this study to secretagogues, especially carbachol, has been much improved. The variability in the responsiveness of isolated gastric cell to histamine or dbcAMP was less than previously, and carbachol-stimulated AP accumulation was found in each cell preparation. Finally, the isolation of gastric glands by use of hyperosmolar NaCl-EDTA solution is faster, less expensive and easier than isolation of gastric cells by the pronase-EDTA method.

In all experiments on acid production by indirect measurement with AP accumulation, crude cell fractions were used with saturating concentr-
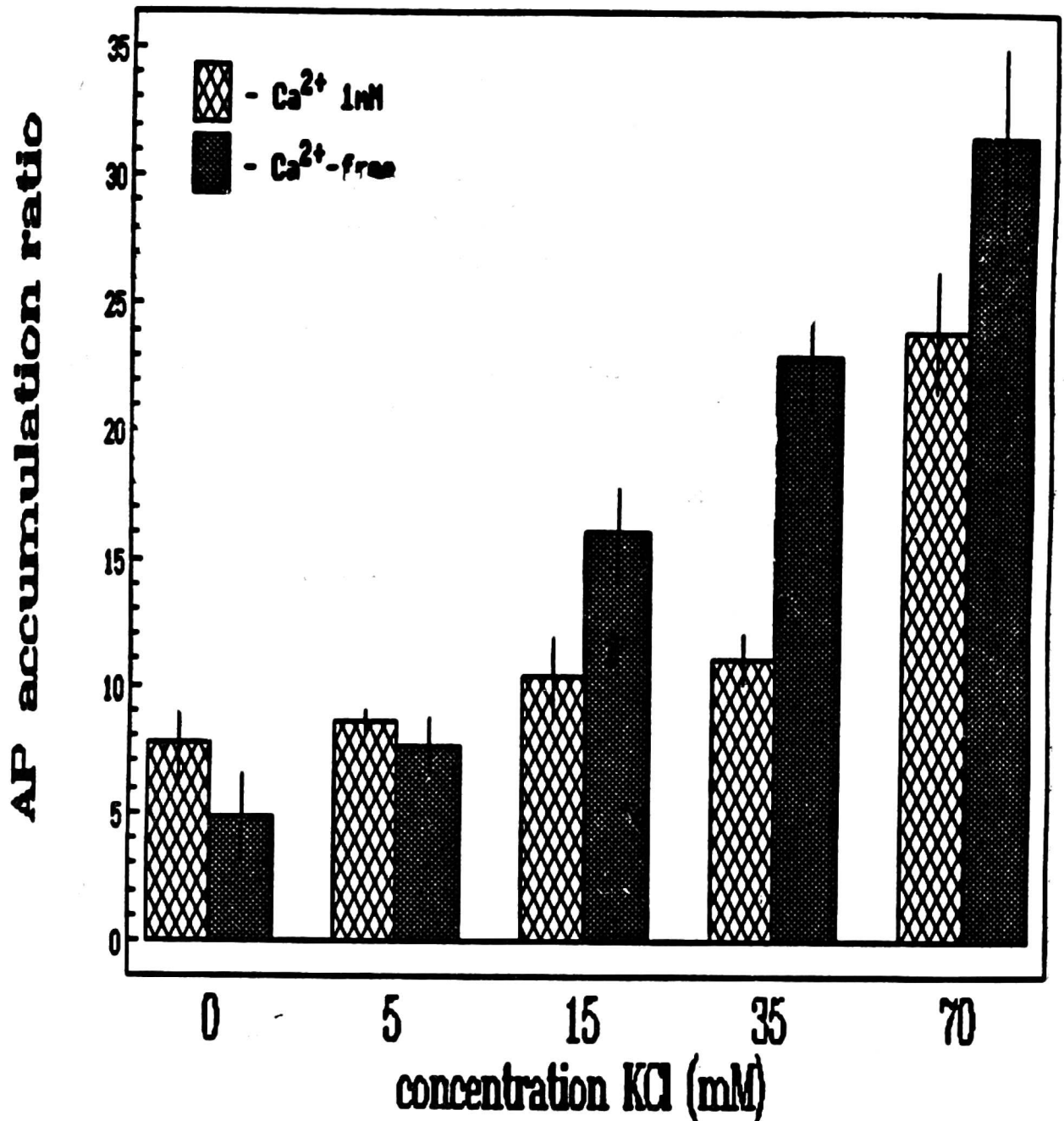

Fig. 1: Effects of extracellular calcium removal on aminopyrine (AP) accumulation in response to varying K+ concentrations in the incubation media. Data are expressed as the ratio of ^{14}C-aminopyrine activity in cells to that in medium. Results represent mean±SD of three experiments performed on different cell preparations.

ations of secretagogues (histamine and carbachol at 10^{-4}M and dbcAMP at 10^{-3}M) (19, 22). In all experiments to determine the response of parietal cells to histamine and dbcAMP, 0.1 mM IBMX was used to inhibit phosphodiesterase. At the concentration used, IBMX alone had no significant effect on basal AP accumulation.

To examine the effect of increased extracellular K^+ on AP accumulation by isolated rat parietal cells, we used incubation media with different concentrations of K^+ in the presence or absence of calcium ions (as specified in *Figure 1 and 2 legends*). In the absence of K^+ in the medium (at the end of the incubation, 0.1 to 0.15 mM K^+ was detected in the medium by
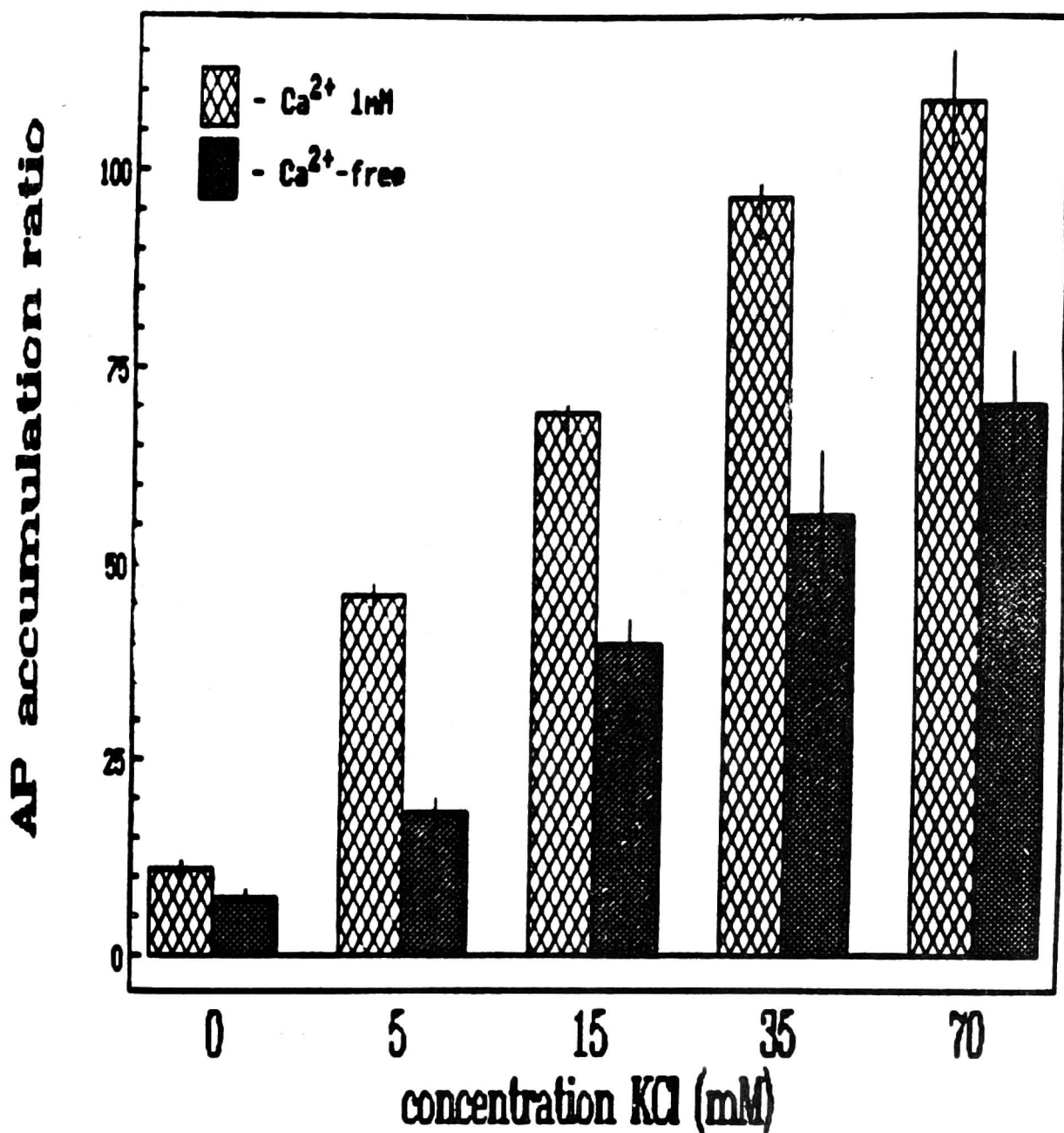

Fig. 2: Effects of extracellular calcium removal on aminopyrine (AP) accumulation by histamine-stimulated rat parietal cell in the presence of varying K^+ concentrations in the incubation media. Data are expressed as the ratio of ^{14}C-aminopyrine activity in cells to that in medium. Results represent mean $\pm$ SD of three experiments performed on different cell preparations.

a Beckman system E2A™ electrolyte analyzer) the basal AP accumulation was not significantly different from AP accumulation found in media containing 5 mM K$^+$ both in the presence and absence of Ca^{2+}. On the other hand, the basal AP accumulation appeared to be activated by high K$^+$ concentrations (*Fig. 1*). In the presence of extracellular Ca^{2+} and 15 and 35 mM K$^+$, the AP accumulation rose slightly but it increased as much as 3-fold at 70 mM K$^+$ compared with AP accumulation in the standard (5 mM KCl) medium. In Ca^{2+}-free standard incubation medium, basal AP accumulation was significantly lower (p < 0.01) than in standard medium containing Ca^{2+}. Contrary to expectation, the increasing K$^+$ concentrations in Ca^{2+}-free medium stimulated acid accumulation in a concentration-dependent manner to levels significantly higher than those found in the presence of extracellular calcium ions.

When K$^+$ was absent from the incubation media, histamine-stimulated AP accumulation was decreased almost to the basal acid accumulation independent of the presence or absence of extracellular Ca^{2+}. When the effects of high K$^+$ on the stimulated secretory response of parietal cells were studied, potentiating interactions between histamine and the increasing extracellular K$^+$ concentrations were found (*Fig. 2*). Beginning from 15 mM K$^+$, the responses to the combinations of histamine and high K$^+$ were significantly greater than the sum of responses to high K$^+$--stimulated and histamine-stimulated AP accumulation by parietal cells in medium containing 5 mM KCl. These potentiating effects of increased K$^+$ concentrations were independent of extracellular Ca^{2+}. However, in contrast to basal AP accumulation, the increased AP accumulation stimulated by the combination of histamine and high K$^+$ was significantly lower in Ca^{2+}-free media than in the correspondingly incubation media containing 1 mM CaCl$_2$. As shown in *Figure 3* such potentiating interactions were found not only between histamine and high extracellular K$^+$ but also between dbcAMP or carbachol and K$^+$ stimulation of AP accumulation.

Although in most experiments calcium-free media were also free of magnesium ions, our results can be related to interaction of extracellular K$^+$ and Ca^{2+}, since solely removal of extracellular Mg^{2+} revealed no effect on potassium-stimulated AP accumulation in rat parietal cells (data not shown).

Carbachol at a concentration of 10^{-4}M and the calcium ionophore A23187 at a concentration of 5X10^{-4}M, but not histamine (10^{-4}M) significantly increased ^{45}Ca^{2+} uptake by parietal cells. On the other hand, high K$^+$ significantly increased both the basal and histamine-, carbachol-, and ionophore-stimulated ^{45}Ca^{2+} influx, as shown in *Figure 4.* These results indicate that the high K$^+$ level introduces calcium ions into parietal cells probably directly without receptor activation. To determine whether
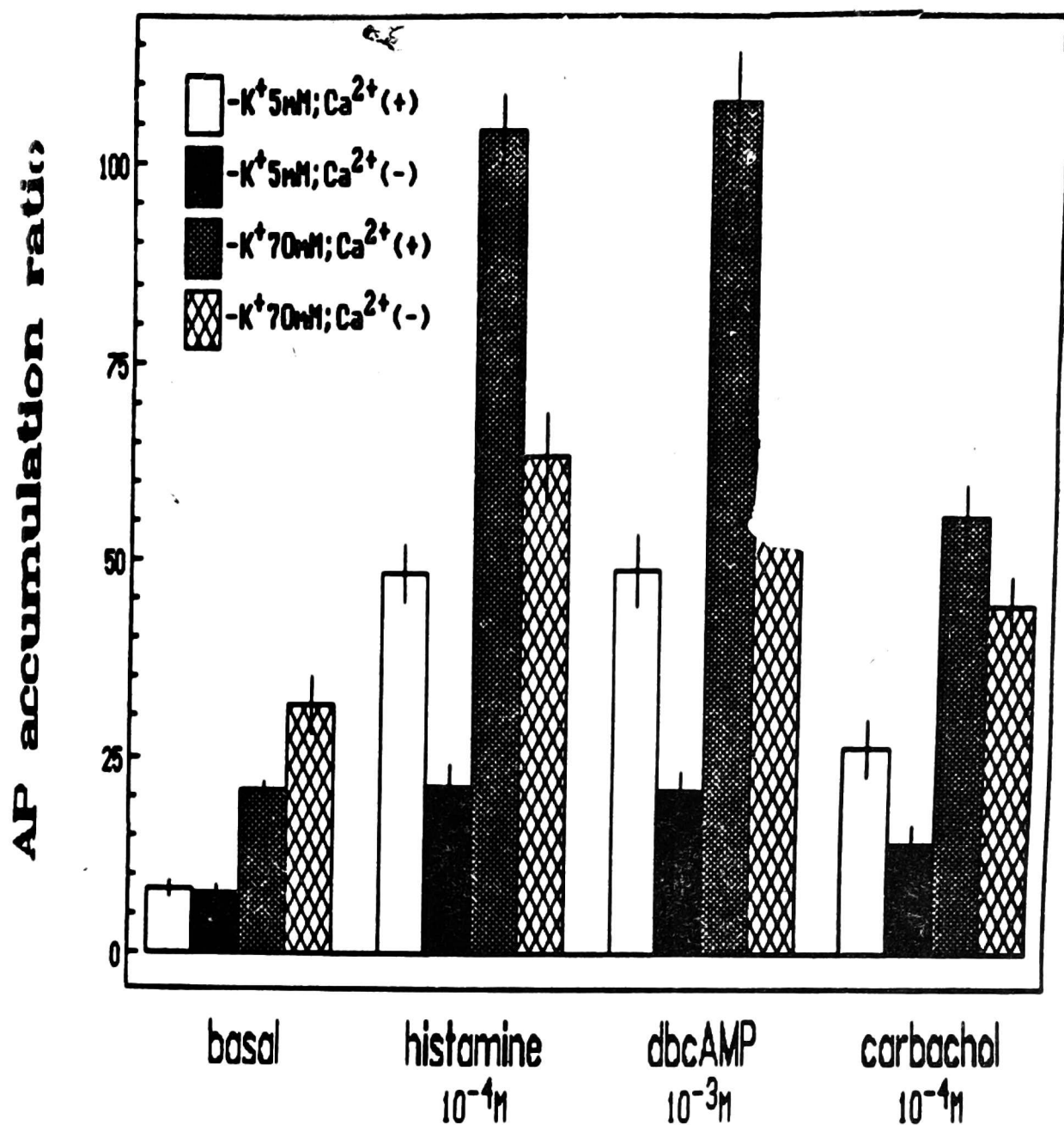
Fig. 3: Effects of extracellular calcium removal on changes in basal and histamine-, dbcAMP-, and carbachol-stimulated aminopyrine (AP) accumulation as a function of extracellular K+ concentrations. Data are expressed as ratio of ^{14}C-aminopyrine activity in cells to that in medium. Results represent mean±SD of four experiments performed on different cell preparations.

high K+ can also increase cytosolic Ca^{2+} concentration by mobilization of intracellular calcium ions from endoplasmic reticulum, cytosolic free Ca^{2+} level was measured by use of intracellulary trapped fluorescent probe Fura-2. In parietal cells incubated in Ca^{2+}-free EGTA containing standard medium (K+ = 5 mM), the mean (±SD) resting free Ca^{2+} concentration was 174±13 nM, and the increase in K+ to 70 mM produced a corresponding increase in the cytosolic free calcium concentration to a mean (±SD) of 464±51 nM. The data present 12 measurements from six different cell preparations.
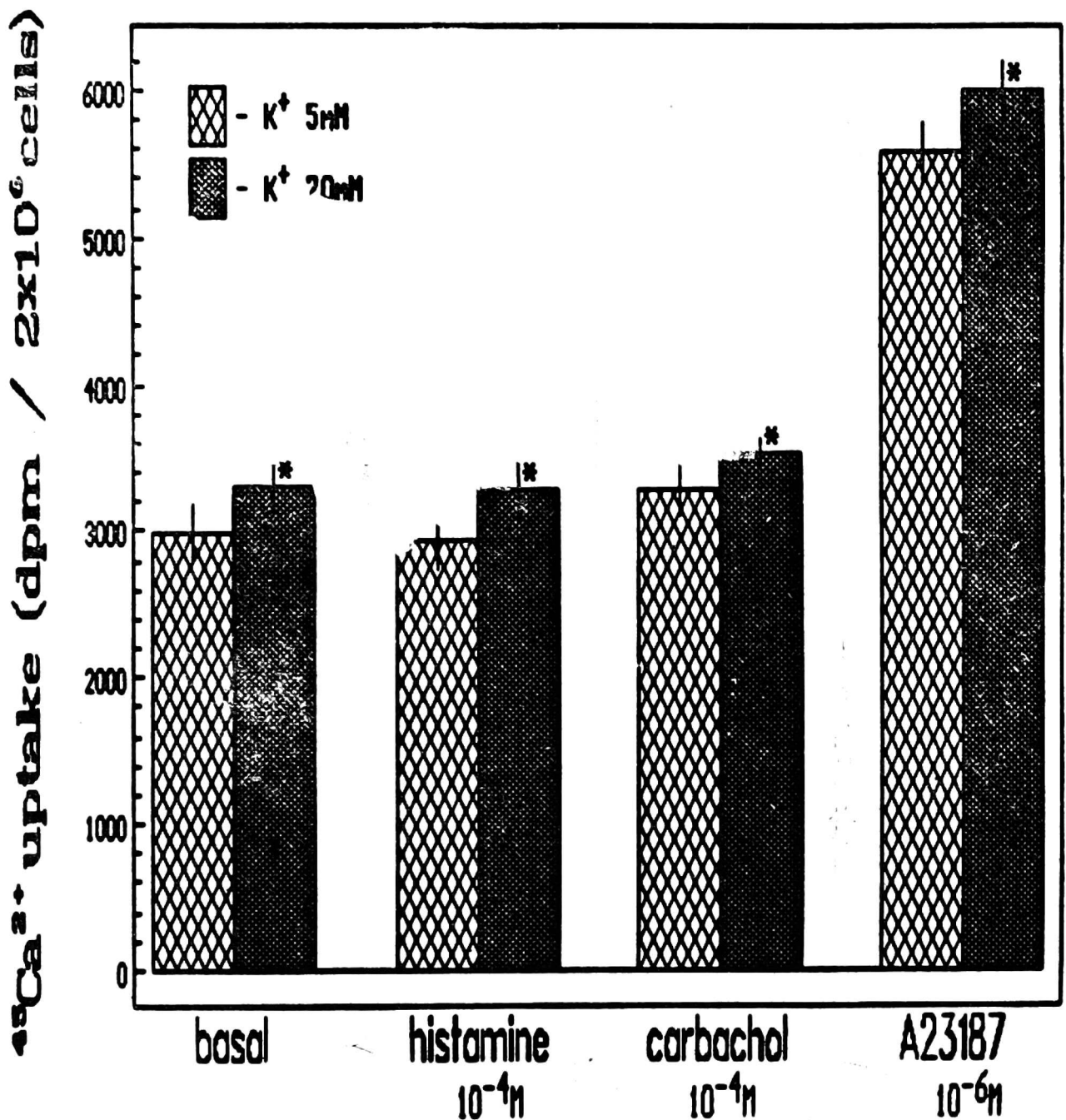
Fig. 4: $^{45}Ca^{2+}$ uptake responses to histamine, carbachol, and the calcium ionophore A 23187 by parietal cells in the presence of 5 and 70 mM of extracellular K^+. Results represent mean$\pm$SD of six experiments performed on different cell preparations. Asterisks indicate significant increase ($p < 0.05$) in $^{45}Ca^{2+}$ uptakes from high extracellular K^+ medium compared to those found in the presence of 5 mM K^+.

DISCUSSION

Presented work is concerned with interaction of extracellular K^+ and Ca^{2+} on acid secretion.

External signals detected by surface receptors on the parietal cell are transmitted across the plasma membrane by mechanisms utilizing the molecules of the three second messengers, namely cyclic nucleotides, inositol lipid derivatives, and calcium ion (23, 24). The main route for stimulation of acid secretion is connected with the activation of H_2-histam-

ine receptor which causes the increased activity of adenylate cyclase. This activation results in cAMP accumulation which activates cAMP-dependent protein kinases which, in turn, phosphorylate specific cellular protein. Other stimulants, such as acetylcholine and gastrin activate acid secretion by Ca^{2+}-mediated mechanisms (1—4). These two signal transduction pathways stimulate activity of $(H^+$-$K^+)$-ATPase bound to the tubulovesicle membranes of the resting parietal cells and to the secretory canalicular membranes of the secreting cells, across which a proton gradient is generated.

As described previously, omission of extracellular calcium from the incubation medium or depletion of intracellular Ca^{2+} by use of intracellular chelator, BAPTA/AM, resulted in inhibition of the initial transient secretory response to carbachol. On the other hand, histamine-stimulated acid production were not affected when extracellular Ca^{2+} was omitted (25, 26), whereas using the intracellular Ca chelator demonstated either suppressed (27) or slightly enhanced response parietal cell to histamine (28). Contrary to others we found that the removal of extracellular calcium significantly suppressed AP accumulation by rat parietal cells not only in response to carbachol but also to histamine and dbcAMP (*Fig. 3*).

The results of this study confirmed the earlier observations (11) that in the absence of K^+, both basal and histamine-stimulated AP accumulation were lowered independent of the presence or absence of extracellular Ca^{2+}. Under basal conditions, increasing the concentration of extracellular K^+ enhanced AP accumulation ratios which, surprisingly, were significantly higher in the absence of extracellular calcium than in its presence (*Fig. 1*). On the other hand, in the presence of secretagogues high K^+ increased AP accumulation by parietal cells from Ca^{2+}-free media to a significantly lesser degree than from corresponding media that contained Ca^{2+} (*Fig. 2*).

As shown, high K^+ increases Ca^{2+} uptake by parietal cells and releases Ca^{2+} from the intracellular stores. However, although calcium ions are involved in acid secretion by parietal cells as one of the second messengers, the increased level of intracellular Ca^{2+} alone is not enough for a secretory response. In this study, performed on rat parietal cells, we found that extracellular Ca^{2+} is required for maximum acid accumulation stimulated by carbachol, as well as histamine and dbcAMP in the presence of standard and high K^+ concentration. On the other hand, the extracellular Ca^{2+} decreased AP accumulation stimulated by high extracellular K^+.

Intracellular ionized calcium is vital to regulation of cell functions, and cell responses to stimuli. One potential source of the cytoplasmic Ca^{2+} is the extracellular Ca^{2+} which leaks into the cell across the membrane upon depolarization or addition of specific chemical ligands. An alternative

source of Ca^{2+} exists within the cell, the endoplasmic reticulum and mitochondia from which it can be relased to the cytoplasm (29, 30).

It is apparent from our experiments that Ca^{2+} ion affects aminopyrine accumulation in tubulovesicle by at least two mechanisms. Extracellular Ca^{2+} is required for maximal acid accumulation stimulated by secretagogues independently of extracellular K^+ concentration. On the other hand, acid accumulation stimulated by high extracellular K^+ is decreased by extracellular Ca^{2+}. The mechanisms of this biological antagonism between Ca^{2+} and high K^+ are unclear. We found that the elevation of K^+ concentration in the medium leads to an increase in the cytoplasmic concentration of Ca^{2+} ion due to both leakage from the medium and release from intracellular sources. The increase of Ca^{2+} concentration in the cytoplasm may lead to activation of the mechanisms removing Ca^{2+} ion from the cytoplasm into the medium and into mitochondria at the expense of cytoplasmic ATP. It can only be speculated that this decrease in ATP concentration in the cytoplasm might be the reason for the Ca^{2+}-dependent decrease of aminopyrine accumulation stimulated by high extracellular K^+. While the same mechanism is operating in the secretagogue stimulated parietal cell, the elevated intracellular calcium acts in the system of the second messenger activating acid production. However, the mechanisms resulted from the activation of the second messengers which are involved in the stimulation of proton pump, are still unclear.

The mechanism of acid secretion in tubulovesicle is well established (3, 4, 31). In the resting state in tubulovesicle membrane there is an internal potassium for external proton exchanging ATP-ase (K^+-H^+-ATP--ase) and a route (channel?) for K^+ transport across the vesicle membrane. The internal K^+ is thus exchanged for H^+ ion, but only a very limited amount of additional K^+ ions can cross the tubulovesicle membrane because there is a positive membrane potential inside the vesicle, which increases with each additional cation transported. The activation of the tubulovesicle membrane consists of an opening of an additional route (channel ?) for Cl^- anion. In an activated tubulovesicle the inward transport of K^+ ions is not stoped by high positive membrane potential since it is short circuited by the chloride anions. Thus, a much higher number of K^+ can be exchanged for H^+ ion and, therefore, HCl concentration in the activated vesicle is much higher then in the resting one.

The experimental data show that the higher the K^+ concentration in the medium, the higher the aminopyrine accumulation in tubulovesicles becomes. Since high K^+ potentiated the response to histamine, dbcAMP, as well carbachol (each secretagogue activates parietal cell in different manner) it seems likely that these potentiating interactions are at the level of tubulovesicle membranes.

It is assumed that higher external K^+ concentration produces elevation of free cytoplasmic K^+ concentration; higher cytoplasmic K^+ concentration allows more K^+ to be transported into the tubulovesicle; and, finally, the more K^+ in the tubulovesicle the more is exchanged for H^+ ion. There are no data, however, relating external K^+ ion concentration to the activity of free cytoplasmic K^+. The data obtained so far show relative stability of total K^+ content in parietal cells incubated in media of different K^+ concentrations (with the exception of the lowest K^+ concentrations in the medium) (11). It should be expected, however, that cell organelles like mitochondria would store considerable part of the cellular K^+, and the free K^+ concentration in the cytoplasm might vary with the change of medium K^+. If that is not the case, one has to look for other mechanisms to explain the effect of the K^+ concentration in the medium on amino-pyrine accumulation. Such a mechanism will probably be membrane potential dependent and will change either K^+ permeability of tubulovesicle membrane or the number of tubulovesicles in the parietal cell.

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Author's address: Doc. dr hab. Jerzy Ostrowski, Department of Gastoenterology and Metabolism, Medical Center of Postgraduate Education, 02-616 Warszawa, Goszczyńskiego 1, Poland