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REGULATION OF GASTRIC MUCOSAL CALCIUM CHANNEL ACTIVITY BY AN ANTIULCER AGENT, EBROTIDINE

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Ebrotidine is a new H_2-receptor antagonist also known for its gastroprotective effect against ethanol-induced mucosal injury. In this study, we investigated the effect of ebrotidine on the activity of the gastric mucosal calcium channels. The channel complex was isolated from the solubilized gastric epithelial cell membranes by affinity chromatography on wheat germ agglutinin. The complex following labeling with [^{3}H] PN200-110 was reconstituted into phosphatidylcholine vesicles which exhibited active $^{45}Ca^{2+}$ uptake into intravesicular space and responded in a concentration-dependent manner to calcium chennel activator, BAY K8644, as well as to calcium channel antagonist, PN200-100. The $^{45}Ca^{2+}$ uptake was inhibited by ebrotidine which caused maximum inhibitory effect of 54.9% at 50 μg/ml. The gastric mucosal calcium channels on epidermal growth factor binding (EGF) in the presence of ATP responded by an increase in tyrosine phosphorylation of 55 and 170 kDa proteins, and the vesicles containing the phosphorylated channels displayed a 48% greater $^{45}Ca^{2+}$ uptake. This phosphorylation process was inhibited by ebrotidine which also interfered with the binding of EGF to calcium channel protein. The results point towards the importance of EGF in the maintenance of gastric mucosal calcium homeostasis, and suggest that ebrotidine has the ability to protect the cellular integrity from calcium imbalance by modulating the EGF-stimulated gastic mucosal calcium channel phosphorylation.

Key words: *Calcium channel, gastric mucosa, regulation, ebrotidine*

INTRODUCTION

Ebrotidine is a new H_2-receptor antagonist with antisecretory potency comparable to that of ranitidine and cimetidine (1, 2). Structurally, although the agent shares many common features with ranitidine and cimetidine, it contains N-sulfonyl formamidine group instead of cyanoguanidine group of cimetidine and 2-nitroethendiamine group of ranitidine, while the imidazole ring of cimetidine is substituted by guanidinothiazole (1). These modifications endow ebrotidine with diminished cytochrome P-450 binding and eliminate the potential for mutagenic nitrosamine formation (1). Furthermore, in contrast to ranitidine and cimetidine, the agent displays gastroprotection against ethanol-induced mucosal injury (2—4). This protective effect of ebrotidine is

maintained even in the presence of indomethacin, a potent prostaglandin synthesis inhibitor, and appears to stem from the drug's ability to enhance the physiochemical qualities of the gastric mucus gel and in particular its sulfomucin level (4). Quite recently, the evidence has also been obtained that ebrotidine is capable of affecting the level of epidermal growth factor (EGF) in the ulcer area (5), a phenonemon associated with the tissue repair and growth. Thus, ebrotidine seems to possess an apparent ability of initiating the events closely linked to cellular proliferation and the mucosal integrity maintenance.

Among the factors implicated in the preservation of gastric mucosal integrity is the maintenance of intracellular calcium level. Under normal physiological conditions, calcium entry in most excitatory and secretory cells occurs through carefully controlled process involving specific voltage and receptor dependent channels (6—8). In this report, we describe the reconstitution of gastric mucosal calcium channels into phospholipid vesicles and show the effect of ebrotidine on the vesicular calcium uptake.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats weighing 180—200 g were obtained from Taconic Farms Inc., Germantown, NY, [^{45}Ca]$CaCl_2$, (+)−[methyl[^{3}H]PN200-100 from New England Nuclear, Boston, MA, and [^{125}I] EGF from Amersham Corp., Arlington Heights, IL. BAY K8644, egg-yolk phosphatidylcholine and mouse EGF were supplied by Sigma. PN200-110 was generously given by Dr. Houlihan, Sandoz Research Institute, E. Hanover, NJ, and Whatman filter GF/C (0.22 μm) from Whatman Internatl., Ltd., Maidsone, England. Wheat germ agglutinin Sepharose was obtained from Pharmacia, Piscataway, NJ, Chelex 100 (50—100 mesh) and reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad, Rockville Centre, NY, and BCA protein assay kit from Pierce, Rockford, IL. 5-Bromo-4-chloro-3-indole phosphate and nitro blue tetrazolium were from Oncogene Science Inc., Manhasset, NY, goat anti-mouse alkaline phosphatase conjugated IgG and O-phospho-1-tyrosine were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, and anti-phosphotyrosine monoclonal IgG was from Upstate Biotechnology Inc., Lake Placid, NY.

Antiulcer Drug

Ebrotidine, p-bromo-N-[[[2-[[[2-[(diaminomethylene)-amino] 4-thiazolyl]-methyl]-thiol]-ethyl]-amino]-methylene]-benzene sulfonamide, lot No. D-6 was kindly donated by Ferrer Internacional, S.A., Barcelona, Spain. The drug was stored at 4°C in the dark.

Membrane preparation

The stomachs were dissected, opened along the greater curvature, rinsed with ice-cold saline in 0.05 M phosphate buffer pH 7.2, and the mucosal cells were collected by scraping the mucosa with a blunt spatula (9). Scrapings were placed in ice-cold buffer (2.5 mM Tris-HCl, pH 7.0, 250 mM

sucrose, 2.5 mM EDTA, 1 mM phenyl methyl sulfonyl fluride (PMSF), 100 TIU/ml aprotinin and 1 μg/ml leupeptin, and homogenized for 1 min in a polytron tissuemizer. The homogenate was centrifuged at 400 × g for 15 min at 4°C and $NaCl/MgSO_4$ was added to the supernatant to form final concentrations of 0.1 mM and 0.2 mM, respectively (10). After centrifugation of the supernatant at 40,000 × g for 1 h at 4°C, the pellet was resuspended in 0.1 M sodium phosphate buffer (pH 7.2), and aliquots stored at −70°C until use. Protein concentration of the resuspended pellet was estimated using the BCA protein assay kit.

Calcium channel isolation

The membrane preparation was centrifuged for 20 min at 10,000 × g at 4°C, and the pellet solubilized using a buffer containing 40 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 20 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 10% glycerol, 100 TIU/ml aprotinin, 1 μg/ml leupeptin, and 1 mM PMSF. After 1h at 4°C, the mixture was centrifuged at 105,000 × g for 60 min and the resulting supernatant collected, 1 nM of [^{3}H]PN200-110 was added and incubated at 4°C for 30 min (11). The preparation was then applied to a column of Sepharose-bound wheat germ agglutinin. The column was washed with 3 ml of a buffer consisting of 50 mM Tris-HCl, pH 7.4, containing 20 mM NaF and 0.5 M NaCl, and the [^{3}H]PN200-110 labeled channel protein was eluted with buffer containing 300 mM N-acetyl-glucosamine (12).

Effect of Ebrotidine on EGF binding

The effect of ebrotidine on the binding of EGF was assessed following preincubation of 200 μl aliquots of membrane preparation (250 μg protein/assay) with the drug (0—200 μg) at room temperature for 30 min. EGF binding assays were carried out by incubating the membrane samples (300 μg protein/assay) with [^{125}I]EGF (0.18 nM) and 5 mM Tris/HCl (pH 7.0), 125 mM sucrose, 75 mM NaCl, 0.5 mM $CaCl_2$, 0.5% bovine serum albumin (BSA) in a final volume of 200 μl 10)). Unlabeled EGF was added to the incubation to form a final concentration of 0.25 μM in the experiments where nonspecific binding was being estimated. Incubates were maintained for 1h at room temperature, and then stopped by addition of 1 mM ice-cold buffer containing 10 mM Tris/HCl (pH 7.0) and 0.5% BSA. Membrane-bound [^{125}I] EGF was separated from the unbound [^{125}I] EGF by centrifugation at 10,000 × g for 10 min at 4°C . The pellet was washed with 1 ml of ice-cold buffer, centrifuged, and counted in a gamma counter. The effect of dihydropyridine calcium antagonist, PN200-110, on the binding of EGF was assessed following preincubation of membrane preparation at room temperature for 30 min with 0-300 nM PN200-100.

Effect of ebrotidine on EGF-stimulated channels phosphorylation

The solubilized gastric mucosal calcium channel preparations containing 200—300 μg protein were incubated at room temperature for 30 min in 50 mM HEPES buffer, pH 7.6, 10 mM $MgSO_4$ 1 mM PMSF with 0 or 5 μg ebrotidine and 2 μM EGF. The phosphorylation was then initiated by the addition of a solution containing 10 μM ATP, 1 mM CTP, 8 mM $MnCl_2$, 20 mM $MgCl_2$, and 2 mM sodium vanadate (10). Incubations were maintained for 10 min at 4°C after which the calcium channel preparation was recovered using wheat germ agglutinin affinity chromatography. For SDS-PAGE, the reaction mixture was treated with 170 mM Tris-HCl buffer, pH 6.8, containing 10% SDS and 100 mM dithiothreitol, and heated at 100°C for 8 min. Samples were then run on a 7.5% gel. Following electrophoresis, the proteins were electrophoretically transferred onto 0.2 μm nitrocellulose membranes. Mouse antiphosphotyrosine monoclonal IgG (10 μg/ml,

1 h) was used as detecting antibody, and goat anti-mouse alkaline phosphatase conjugated IgG (0.1 μg/ml, 2 h) as secondary antibody. Visualization was achieved using 5-bromo-4-chloro-3-indole phosphate and nitro blue tetrazolium (12).

Liposomal reconstitution of calcium channels

Liposomes were prepared by the method of Mimms et al. (13). Purified [^{3}H]PN200-110 labeled, calcium channel samples in their intact form and following EGF-stimulated phosphorylation were solubilized with a buffer containing 20 mM CHAPS, 5 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.4, and 10% glycerol, and mixed with 1 ml of octylglucoside containing 1% egg yolk phosphatidylcholine. Detergent was removed by dialysis against buffered saline for 24 h at 4°C, thus yielding liposomes. For further purification, a suspension of liposomes was made with 45% sucrose, overlaid with 2 ml of 30% sucrose and 1 ml of 10% sucrose, and then centrifuged at 4°C for 18 h at 45,000 × g in a Beckman SW50 rotor (14). The liposomes containing [^{3}H]PN200-110 labeled channel protein were recovered as a white band at the top of 10% sucrose layer.

Ebrotidine effect on $^{45}Ca^{2+}$ uptake into vesicles

The effect of ebrotidine on the $^{45}Ca^{2+}$ uptake into vesicles containing the reconstituted, [^{3}H]PN200-100 labelled, calcium channel was measured following vesicles (200 μl) preincubation for 30 min at room temperature with different concentrations of ebrotidine (0—150μg). The external divalent cations were removed on Sephadex G-50 column (11), the vesicles (100 μl) were suspended in 0.34 M sucrose, 10 mM MOPS/tetramethylammonium, pH 7.0, and the calcium uptake was initiated by addition of 50 μl of 0.1 M $CaCl_2$ plus 2 μCi of $^{45}Ca^{2+}$. After 20 min incubation at 37°C, the reaction was terminated with 150 mM $MgCl_2$ in 10 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES)-Tris buffer, pH 7,4 and the samples were applied into GF/C Whatman filter (0.22 μm). Following filtration and washing with 5 mM HEPES-Tris buffer, pH 7.4, containing 0.3 M glucose and 5 mM lanthanum oxide, the filter was subjected to $^{45}Ca^{2+}$ measurement. Binding of $^{45}Ca^{2+}$ to the filter was determined by filtration of incubation media without vesicle protein (15).

The effect of calcium channel receptor antagonist, PN200-110 and that of calcium channel activator, BAY K8644 on the $^{45}Ca^{2+}$ uptake was measured following vesicles preincubation at room temperature for 20 min with PN200-110 (0–1 μM) or BAY K8644 (0–10 μM) (11).

Statistical Analysis

All experiments were carried out in duplicate, and the results are expressed as mean ± SD. Student's t-test was used to determine significance, and p values of 0.05 or less were considered significant.

RESULTS

Gastric mucosal calcium channels were solubilized from epithelial cell membranes, labeled with a dihydropyridine calcium channel receptor antagonist, [^{3}H]PN200-110, and purified by affinity chromatography on Sepharose-bound wheat germ agglutinin. The labeled calcium channel protein

complex, eluted with 300 mM N-acetylglucosamine, displayed on SDS-gel under reducing conditions four major protein bands migrating in the region of 55, 90, 130 and 170 kDa. The functional performance of the isolated channels was assessed following incorporation of the channel protein into phosphatidylcholine vesicles which conform the structure of membrane bound protein.

Under the assay conditions, the reconstituted channels exhibited a 6-fold greater $^{45}Ca^{2+}$ uptake over that of protein-free vesicles, and responded in a concentration dependent manner to dihydropyridine calcium antagonist (PN200-110) as well as to BAY K8644, a specific calcium channel activator (*Fig. 1*). The maximal inhibitory effect was attained at 0.4 μM PN200-110 which gave 66% decrease in calcium uptake. In the case of BAY K8644, maximal enhancement (52%) in $^{45}Ca^{2+}$ uptake occurred at 6 μM.

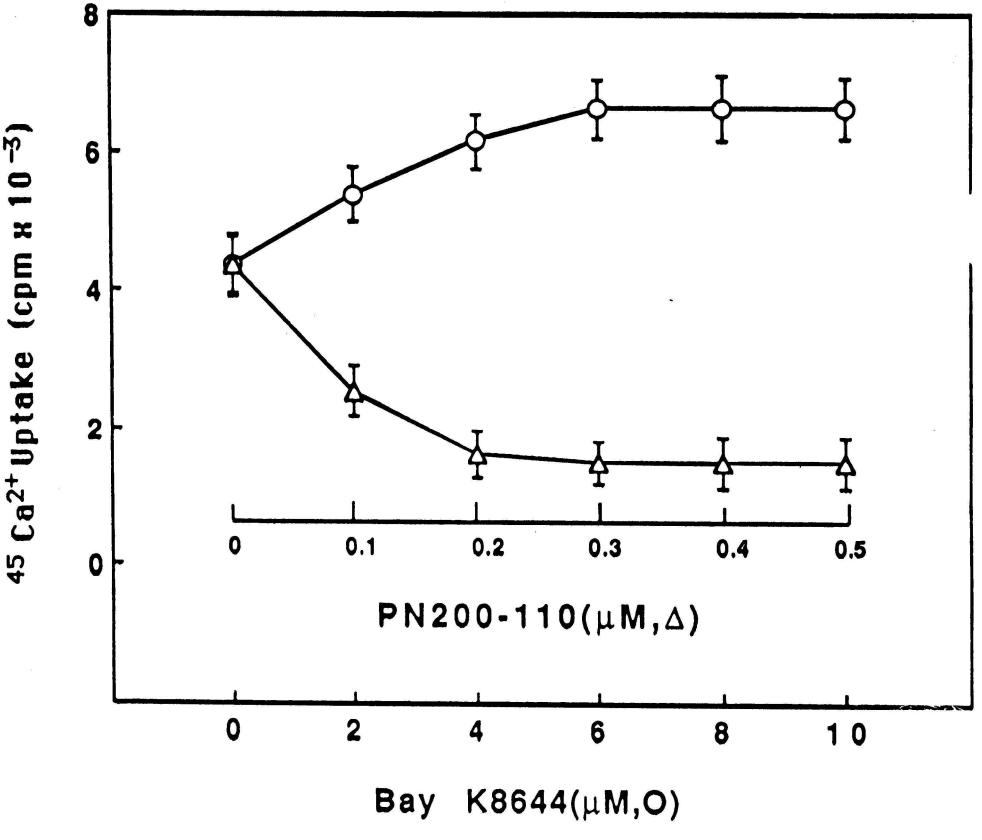

Fig. 1. Effect of PN200-110 and BAY K8644 on the uptake of $^{45}Ca^{2+}$ into vesicles containing the reconstituted gastric mucosal calcium channels. Uptake assays were conducted using vesicles preincubated for 20 min at room temperature with different concentrations of PN200-110 (0—0.5 μM) or BAY K8644 (0—10 μM). Values represent the means ± SD of five separate experiments performed in duplicate.

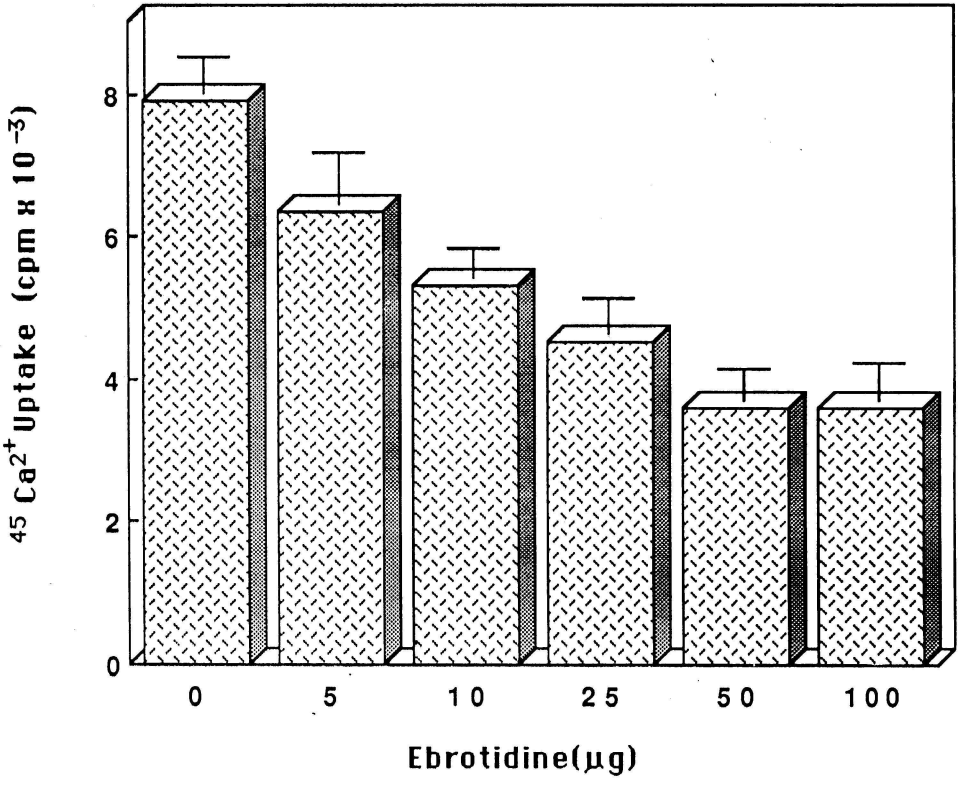

Fig. 2. Effect of ebrotidine on $^{45}Ca^{2+}$ uptake into vesicles containing the reconstituted gastric mucosal calcium channels. Uptake assays were conducted using vehicle preincubated for 30 min at room temperature with different concentrations (0—100 μg/ml) of ebrotidine. Values represent the means ± SD of six experiments performed in duplicate.

The effect of ebrotidine on $^{45}Ca^{2+}$ uptake into vasicles containing the reconstituted gastric mucosal calcium channels is depicted in *Fig. 2.* Preincubation of the vesicles containing channel protein with ebrotidine led to a concentration-dependent inhibition of calcium uptake. At the same time, the lanthanum displacement assays indicated that ebrotidine affected the intravesicular calcium level. The inhibitory effect was proportional to the drug concentration up to 50 μg/ml attaining maximum inhibition of 54.9%.

Fig. 3. presents the data on the effect of ebrotidine and PN200-110 on the EGF binding to gastric mucosal calcium channels. The results indicated that while calcium channel receptor antagonist, PN200-110, had bo effect on the receptor binding of EGF, ebrotidine exerted inhibitory effect. A decrease in EGF binding with ebrotidine was proportional to the drug concentration up to 100 μg/assay at which point a 40.1% reduction in the binding was attained.

Examination of the protein tyrosine phosphorylation patterns using anti-phosphotyrosine antibody revealed that ebrotidine caused inhibition in protein phosphorylation. This effect of ebritidine was particularly evident with the proteins in the region of 55 and 170 kDa.

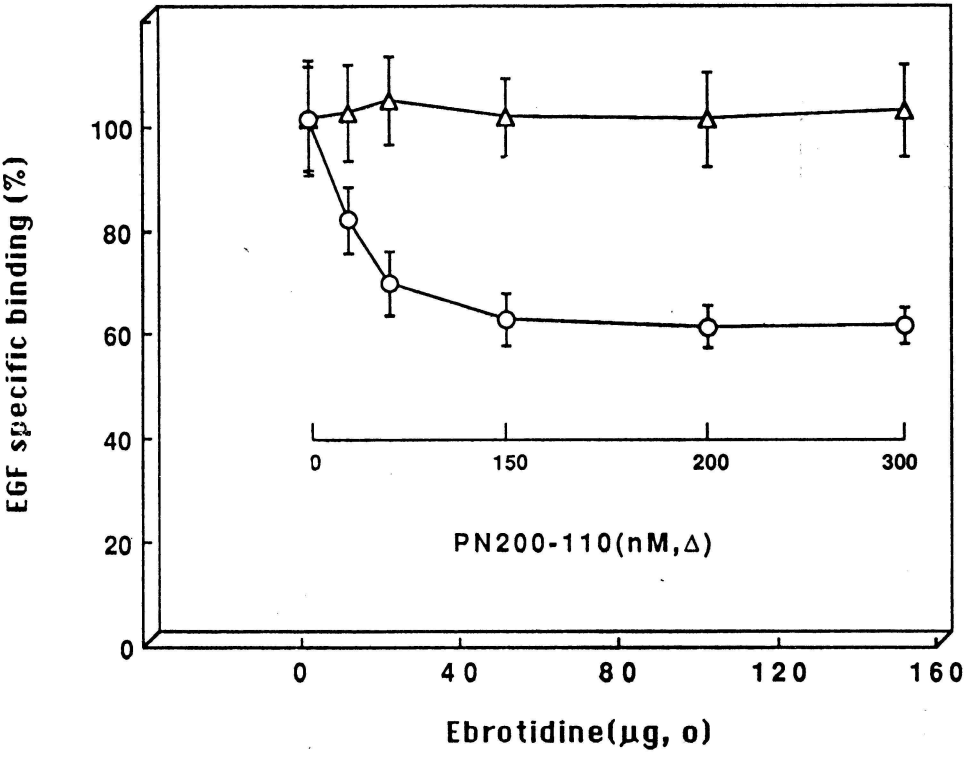

Fig. 3. Effect of ebrotidine on the EGF binding to gastric mucosal calcium channel protein. Uptake assays were conducted using vesicles preincubated for 30 min at room temperature with 0—150 μg ebrotidine or 0—200 nM PN200-110. Values represent the means ± SD of six experiments performed in duplicate.

The calcium uptake into vesicles containing the reconstituted intact and phosphorylated gastric mucosal calcium channels is illustrated in *Fig. 4*. The extent of uptake of $^{45}Ca^{2+}$ by vesicles containing EGF-induced phosphorylated calcium channels was 48% greater than that of the controls.

DISCUSSION

The maintenance of gastric mucosal integrity under the adverse luminal environment depends upon a delicate balance of a number of factors which control the process of mucosal repair and restitution. Primary among these is

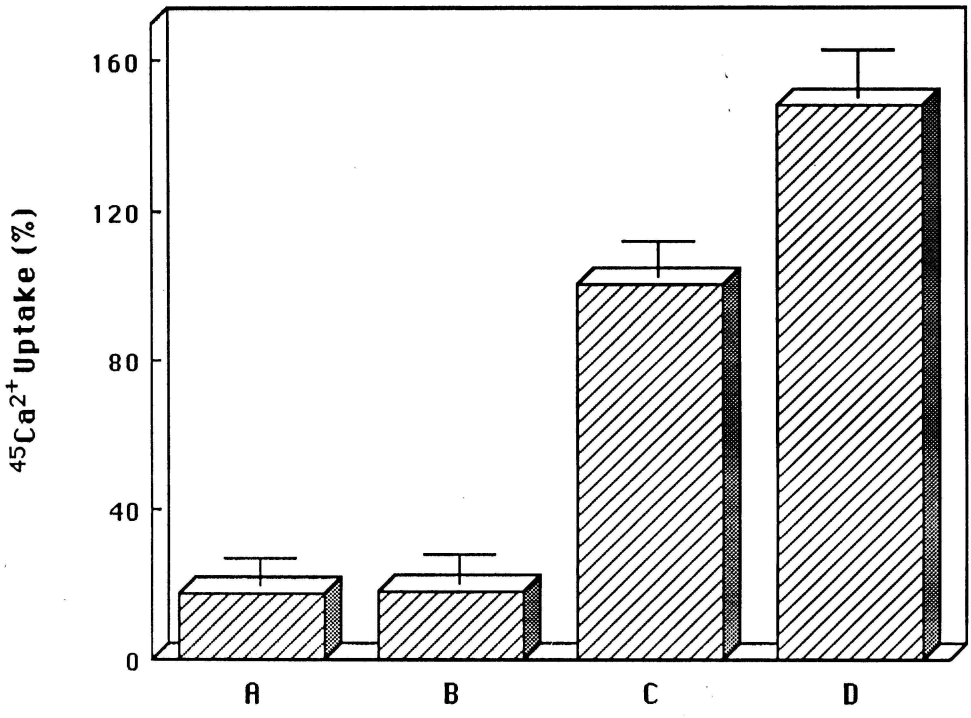

Fig. 4. Effect of EGF-stimulated calcium channel protein phosphorylation on $^{45}Ca^{2+}$ uptake into vesicles containing the reconstituted gastric mucosal calcium channels. Purified channel protein was reconstituted into phospholipid vesicles either in its intact form (C) or following EGF-induced phosphorylation (D). Vesicles free of membrane protein in the absence (A) and the presence (B) of EGF. Values represent the means ± SD of six experiments performed in duplicate.

epidermal growth factor (EGF), a potent mitogen recognized for its effectiveness in preventing the developing of gastric and duodenal lesions through stimulation of epithelial cells proliferation (16, 17). The biological effects of EGF are mediated by receptors located on the target cell surfaces and involve the activation of the intrinsic tyrosine kinase, an event essential for further signal transduction (10, 19—20). The earlier responses to the activation of the EGF receptor, furthermore, involve the influx of calcium (19, 20). Indeed, recent data with A431 cells suggest that activation of the EGF receptor triggers the receptor-operated calcium channel response (19). The results presented in this study show that ebrotidine is capable of modulation of gastric mucosal calcium channel activity. This effect of ebrotidine can be attributed to the drug's ability to affect the EGF-induced calcium channel protein phosphorylation.

The calcium channel complex was solubilized from gastric mucosal cell membranes, labeled with [^{3}H]PN200-110, and purified by affinity chromatography on wheat germ agglutinin. The labeled calcium antagonist-receptor

complex following incorporation into the phosphatidylcholine vesicles, exhibited active $^{45}Ca^{2+}$ uptake and responded in a concentration dependent manner to PN200-110, a dihydropyridine calcium antagonist. Furthermore, like calcium channel receptors from other tissues (7, 21, 22), the vesicles containing gastric mucosal receptor showed significantly greater rate of $^{45}Ca^{2+}$ uptake than that into protein-free vesicles. Upon SDS-PAGE examination, the gastric mucosal preparation yielded protein bands (55, 90, 130, and 170 kDa) corresponding to that of calcium channel preparations from other sources (10). Hence, the isolated gastric calcium mucosal channel complex has properties typical of membrane embedded proteins and displays close similarity to other known calcium channel antagonist receptors (21, 22).

The gastric mucosal calcium channels on EGF binding in the presence of ATP responded by an increase in protein tyrosine phosphorylation, reflected mainly in 55 and 170 kDa proteins, as revealed with phosphotyrosine antibody. The vesicles containing such phosphorylated channel proteins showed a 48% higher calcium uptake, thus point towards tyrosine kinase involvement in EGF-mediated calcium channel activation in gastric mucosa. These results along with the data from patch clamp recordings at the level of single ion channels obtained with A431 cells (19), indicate that the expression of calcium channel activity depends not only on the phosphorylation events controlled by protein kinase A and C (22, 23), but also on the phosphorylation triggered by EGF receptor activation.

As ebrotidine is recognized as a potent gastroprotective and ulcer healing agent capable of prevention of the mucosal damage by a variety of noxious substances including ethanol (2—5), and since calcium influx is known to potentiate the ethanol-induced mucosal injury (24), we investigated further the effect of ebrotidine on gastric mucosal calcium channel activity. The results revealed that ebrotidine caused inhibition of the uptake of $^{45}Ca^{2+}$ into vesicles containing the reconstituted calcium channels. This inhibitory effect was proportional to ebrotidine concentration up to 50 μg/ml at which point it attained maximal inhibition of 54%.

The effect of ebrotidine on calcium uptake apparently stems from the drug's ability to interfere with EGF binding, as our data revealed a concentration dependent inhibition by ebrotidine of the EGF-receptor interaction. Furthermore, preincubation with ebrotidine prior to EGF-stimulated calcium channel protein phosphorylation, caused a decrease in 55 and 170 kDa protein phosphotyrosine, thus suggesting the inhibitory effect of ebrotidine on tyrosine kinase activity. The finding that the activation of calcium channels in gastric mucosa is dependent on the EGF receptor activation points toward the importance of EGF in the regulation of gastric mucosal calcium homeostasis, a phenomenon of significance to the processes of mucosal repair and integrity

maintenance. Apparently, ebrotidine has the ability to protect this cellular integrity from calcium imbalance by modulating the EGF-stimulated gastric mucosal calcium channels phosphorylation.

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