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MODULATION OF GASTRIC MUCOSAL CALCIUM CHANNELS ACTIVITY BY PLATELET-DERIVED GROWTH FACTOR

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A dihydropyridine-sensitive gastric mucosal calcium channels were isolated from the solubilized epithelial cell membranes by affinity chromatography on wheat germ agglutinin. The channels following labeling the calcium antagonist receptor site with [^{3}H] PN200-100 were reconstituted into phospholipid vesicles which exhibited active $^{45}Ca^{2+}$ uptake as evidenced by La^{3+} displacement assays. The uptake of calcium was independent of sodium and potassium gradients indicating the electroneutral nature of the process. The channels responded in a dose dependent manner to dihydropyridine calcium antagonist, PN200-110, which at 0.5 μm exerted maximal inhibitory affect of 66% on $^{45}Ca^{2+}$ uptake, while a 52% enhacement in $^{45}Ca^{2+}$ uptake occurred with a specific calcium channel activator, BAY K8644. On platelet-derived growth factor (PDGF) binding in the presence of ATP, channel protein showed an increase in tyrosine phosphorylation of 55 and 170 kDa calcium channel proteins. Such phosphorylated channels following reconstitution into vesicles displayed a 78% greater $^{45}Ca^{2+}$ uptake. The results demonstrate the importance of PDGF in the regulation of gastric mucosal calcium uptake.

Key words: *Gastric mucosa, calcium channels, activation, PDGF, phosphorylation*

INTRODUCTION

The preservation of gastric mucosal integrity depends upon a delicate balance of factors which control the processes of mucosal repair and restitution. Primary among these factors is the maintenance of intracellular calcium levels. This important regulatory element is recognized for its participation in many cellular processes, including contraction, cell differentiation and secretion (1, 2), and its influx has been shown to potentiate cellular injury (3). Under normal physiological conditions, calcium entry in most excitatory and secretory cells occurs through carefully controlled process involving specific voltage and receptor-dependent channels (1). The channels present in majority

of different types of cells including those of secretory glands are known as L-type or dihydropyridine-sensitive calcium channels (1, 4, 6).

Therefore, factors capable of affecting the expression of calcium channels activity could be of significance to the processes of mucosal integrity maintenance and repair. Primary among these is platelet-derived growth factor (PDGF), a potent mitogen widely recognized for its influence on a variety of functions associated with cellular proliferation and secretion (7—9). The biological effects of PDGF are mediated by receptors located on the target cell surfaces (7—9). The ligand binding of the receptor results in activation of the intrinsic receptor tyrosine kinase activity and phosphorylation of the receptor on tyrosine residues (7—9). This autophosphorylation is a central event towards mediating the proliferative effects of PDGF.

Here we describe the isolation of calcium channel complex from rat gastric mucosal cell membranes and show that PDGF-stimulated channel protein phosphorylation affects the calcium uptake into vesicles containing reconstituted calcium channels.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (180—200 g) were obtained from Taconic Farms Inc., Germantown, NY, ([^{45}Ca]$CaCl_2$, (+) -[methyl-^{3}H]PN200-110 from New England Nuclear, Boston, MA, and [^{125}I]PDGF from Amersham Corp., Arlington Heights, IL. BAY K8644, egg-yolk phosphatidylcholine and human PDGF were supplied by Sigma. PN200-110 was generously given by Dr. Houlihan, Sandoz Research Institute, E. Hanover, NJ. 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 0-phospho-1-tyrosine were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, and anti-phosphotyrosine monoclonal IgG from Upstate Biotechnology Inc., Lake Placid, NY.

Membrane preparation

The dissected stomachs were 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. 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 fluoride (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 xg for 15 min at 4°C and $NaCl/MgSo_4$ 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 xg for 1h at 4°C, the pellet was resuspended n 0.1 mM sodium phosphate buffer (pH 7.2) and aliquots of such prepared gastric membrane samples were 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 xg at 4°C, and the pellet solubilized using a buffer containing 50 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, an 1 mM PMSF. After 1 h at 4°C, the mixture was centrifuged at 105,000 xg 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. 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-acetylglucosamine (11).

PDGF binding

PDGF binding assays were carried out by incubating the membrane samples (300 μg protein/assay) with [^{125}I] PDGF (0.016 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 PDGF was added to the incubations to form a final concentration of 0.66 nM in the experiments where nonspecific binding was being estimated. Incubates were maintained for 1 h 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] PDGF was separated from the unbound [^{125}I] PDGF by centrifugation at 10,000 xg 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 PDGF was assessed following preincubation of membrane preparation with PN200-110 (0—200 μM) at room temperature for 30 min.

PDGF-stimulated channel protein phosphorylation

The solubilized calcium channel preparations containing 200—300 μg protein were incubated with 50 mM HEPES buffer, pH 7.6, 10 mM $MgSO_4$, 1 mM PMSF and with or without 1.4 nM PDGF, or with 0.1 mM 0-phospho-1-tyrosine. After 30 min at 25°C, phosphorylation was 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, an 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. Ten percent BSA was used to block the membranes, mouse anti-phosphotyrosine monoclonal IgG (10 μg/ml, 1 h) 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).

Reconstitution of calcium channel

Liposomes were prepared by the method of Mimms et al. (13). Purified calcium channel samples in their intact form and following PDGF-stimulated phosphorylation were solubilized with a buffer containing 20 mM CHAPS, 5 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.5, 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 xg in a Beckman SW50 rotor (14). The purified liposomes containing [^{3}H] PN200-110 labeled channel protein were recovered as a white band at the top of 10% sucrose layer.

$^{45}Ca^{2+}$ *uptake*

For the measurements of $^{45}Ca^{2+}$ uptake into protein-free vesicles (control) and vesicles containing the reconstituted calcium channels, external divalent cations were removed by application of 200 μl of vesicle suspension to a 3 ml Sephadex G-50 column equilibrated with a 0.34 M sucrose, 10 mM 3-(N-morpholino)-propanesulfonic acid (MOPS)/tetramethylammonium, pH 7.0 (15). Uptake into vesicles (100 μl in sucrose medium was initiated by addition of 50 μl of sucrose medium containing 0.1 mM $CaCl_2$ plus 2 μCi of $^{45}Ca^{2+}$. The mixture was incubated at 37°C for various periods of time up to 1 h, and the reaction was terminated with 150 mM $MgCl_2$ in 10 mM HEPES-Tris buffer, pH 7.4.

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). The samples were then applied to a Chelex 100 column (0.5 x 5 cm) equilibrated with sucrose medium containing 1 mg/ml BSA. Vesicles were eluted from the column with 1 ml of sucrose-BSA medium, and the specific $^{45}Ca^{2+}$ uptake was determined by substracting the background uptake into the protein-free vesicles.

Effect of cationic gradient

The effect of cationic gradient on calcium uptake into phospholipid vesicles reconstituted channels was assessed using 100 μl aliquots of vesicles preincubated for 5 min at room temperature either with buffer consisting of 0.34 M sucrose, 10 mM MOPS/tetramethylammonium, pH 7.0, alone or with the buffer containing different concentrations of KCl (5—150 mM) and NaCl (145—50 mM). Calcium uptake was then initiated by addition of 0.1 mM $CaCl_2$ and 2 μCi $^{45}Ca^{2+}$. The uptake was terminated after 10 min by addition of 10 mM HEPES/Tris buffer, pH 7.4, containing 150 mM $MgCl_2$, loaded onto a Chelex-100 column, and the vesicles eluted with 0.34 M sucrose, 10 mM MOPS buffer, pH 7.0, containing 1 mg/ml BSA, were subjected to $^{45}Ca^{2+}$ measurement.

Statistical analysis

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

RESULTS

Gastric mucosal cell membranes prepared from rat gastric epithelium were used for the isolation of calcium channels. The solubilized membranes were prelabeled with [^{3}H]PN200-110, a dihydropyridine calcium channel receptor antagonist, and subjected to affinity chromatography on Sepharose-bound wheat germ agglutinin. Elution of the column with medium buffer containing 300 mM N-acetylglucosamine yielded the [^{3}H]PN200-100 labeled purified channels. On SDS-PAGE under reducing condition, the preparation gave four major protein bands which migrated at 170, 130, 90 and 55 kDa *(Fig. 1)*. Upon PDGF induced phosphorylation, an increase in protein tyrosine phosphorylation was observed of the proteins in the region of 170 and 55 kDa.

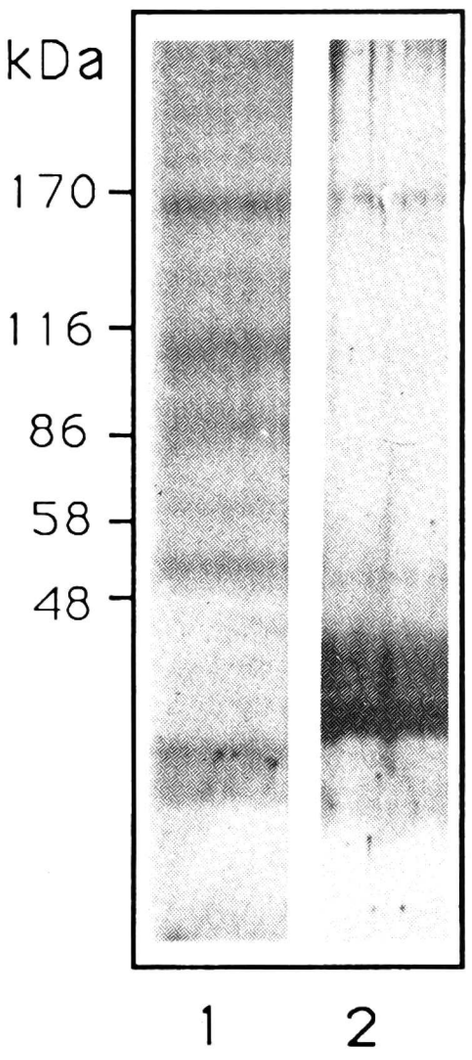

Fig. 1. Effect of PDGF on gastric mucosal calcium channel proteins tyrosine phosphorylation (2). The purified channel preparation was incubated with PDGF (1.4 nM) for 30 min and then ATP stubstrate (10 μM) was added, and the reaction stopped after 10 min. Following SDS-PAGE, the proteins were transferred onto nitrocellulose membranes and probed with mouse antiphosphotyrosine monoclonal IgG. Visualization was achieved using alkaline phosphatase conjugated goat anti-mouse IgG and 5-bromo-4-chloro-3-indole phosphate/nitro blue tetrazolium. 1, channel protein pattern under reducing conditions visualized by silver stain.

Figure 2 shows the effect of PN200-110 on the gastric mucosal receptor binding of PDGF. The binding assays gave a mean specific value of 1.46 fmol/mg membrane protein in the absence of PN200-110, and was not significantly different following preincubation with calcium channel receptor antagonist, PN200-110.

The function of the isolated calcium channels was evaluated following incorporation of the channel protein complex into phosphatidylcholine vesicles which conform the structure of membrane bound protein. Following purifica-

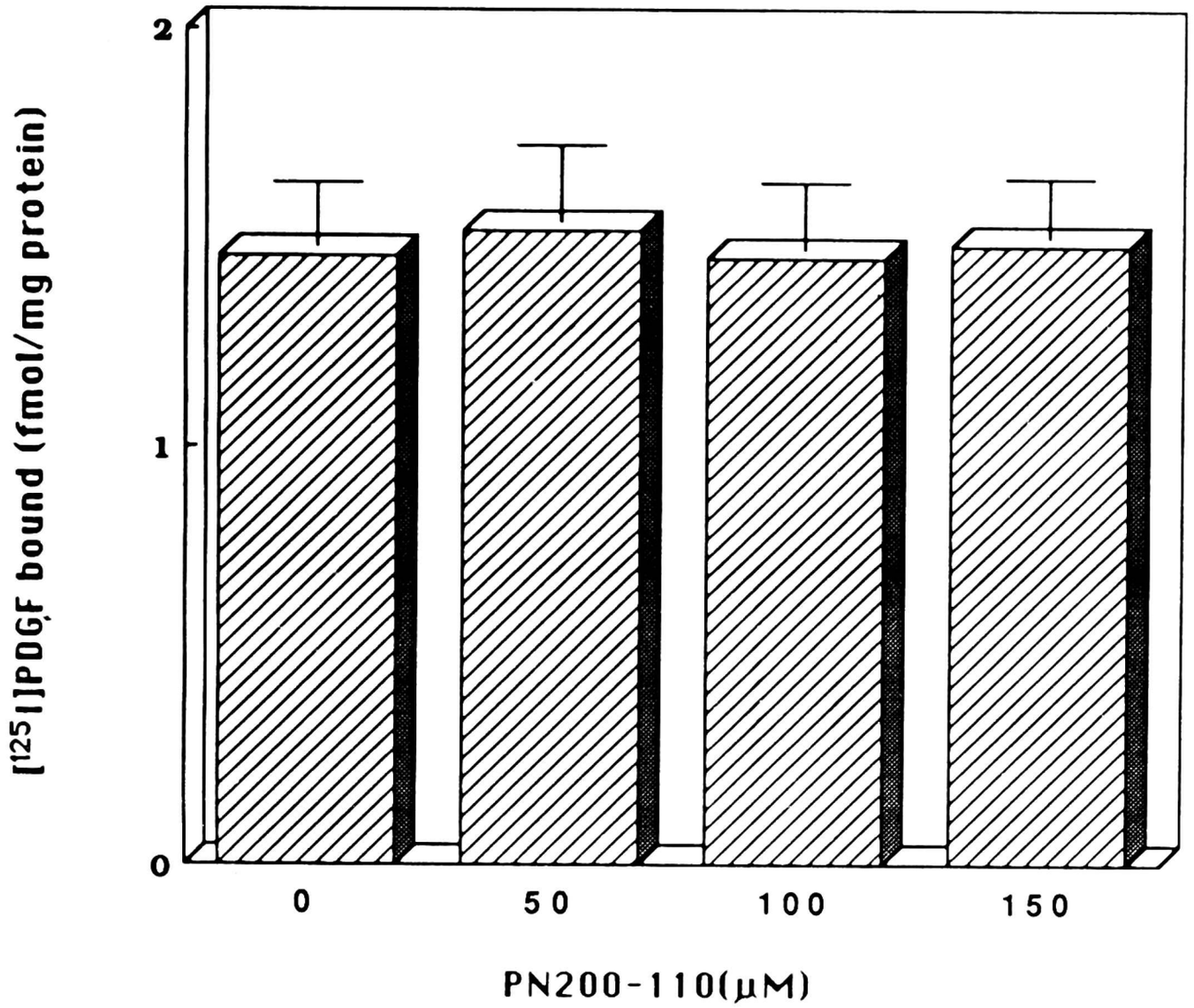

Fig. 2. Effect of PN200-110 on the PDGF binding to gastric mucosal calcium channel protein. Assays were conducted as described in Materials and Methods, except that membranes prior to binding assay were preincubated at room temperature for 30 min with 0—200 μM PN200-110. Values represent the means $\pm$ SD of five separate experiments performed in duplicate.

tion on sucrose gradient, the isolated vesicles were used to measure $^{45}Ca^{2+}$ uptake. Under the assay conditions, the reconstituted gastric mucosal calcium channel protein complex exhibited 6-fold greater $^{45}Ca^{2+}$ uptake into vesicles as compared to that of protein-free vesicles. The vesicles containing reconstituted calcium channels responded in a concentration dependent manner to PN200-110, a dihydropyridine calcium channel antagonist, as well as to BAY K8644, a specific calcium channel activator. The maximal inhibitory effect was attained at 0.5 μM PN200-110 at which concentration of antagonist a 66% decrease in $^{45}Ca^{2+}$ uptake occurred *(Fig. 3)*, while BAY K8644 caused maximal (52%) enhancement in $^{45}Ca^{2+}$ uptake at 6 μM *(Fig. 4)*.

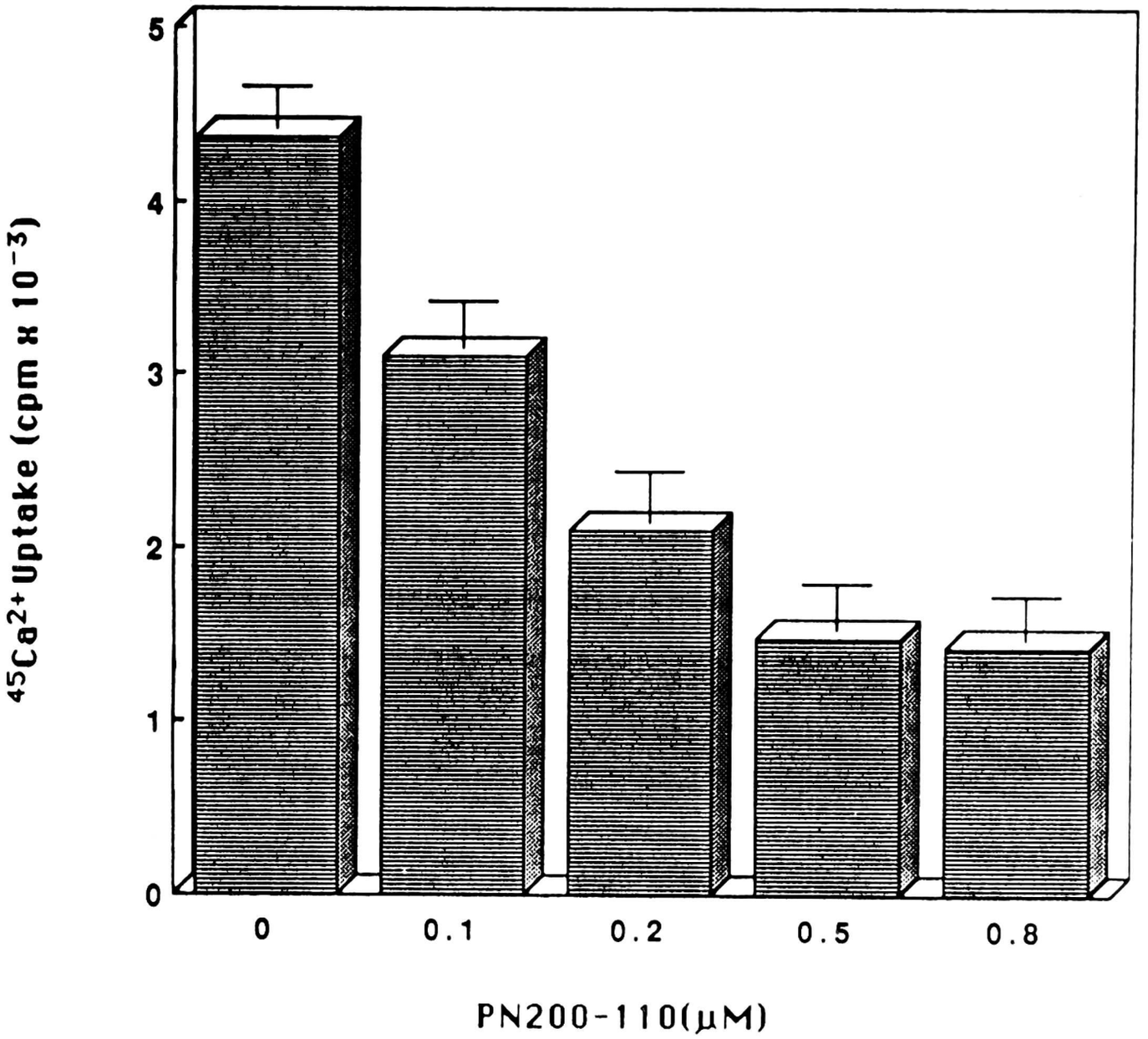

Fig. 3. Effect of PN200-110 on the uptake of $^{45}Ca^{2+}$ into vesicles containing the reconstituted gastric mucosal calcium antagonist receptor. Uptake assays were conducted as described in Materials and Methods using vesicles preincubated for 20 min at room temperature with different concentrations (0—1 μM) of PN200-110. Values represent the means $\pm$ SD of five separate experiments performed in duplicate.

To ascertain whether the calcium uptake by the vesicles represents transport into osmotically active space and not mere binding to the vesicular surface, the $^{45}Ca^{2+}$-preloaded vesicles were treated with La^{3+} to displace the calcium from the vesicle surface. The data revealed that lanthanum at its optimal concentration (0.7 mM/ml) caused only 11% $^{45}Ca^{2+}$ displacement, thus indicating that the majority of calcium was present in the intravesicular space and hence inaccessible to La^{3+} displacement. The results of calcium uptake assays in the presence of sodium and potassium gradients *(Fig. 5)* did not reveal significant differences in $^{45}Ca^{2+}$ uptake with cationic gradient change which suggests the electroneutral nature of the uptake process.

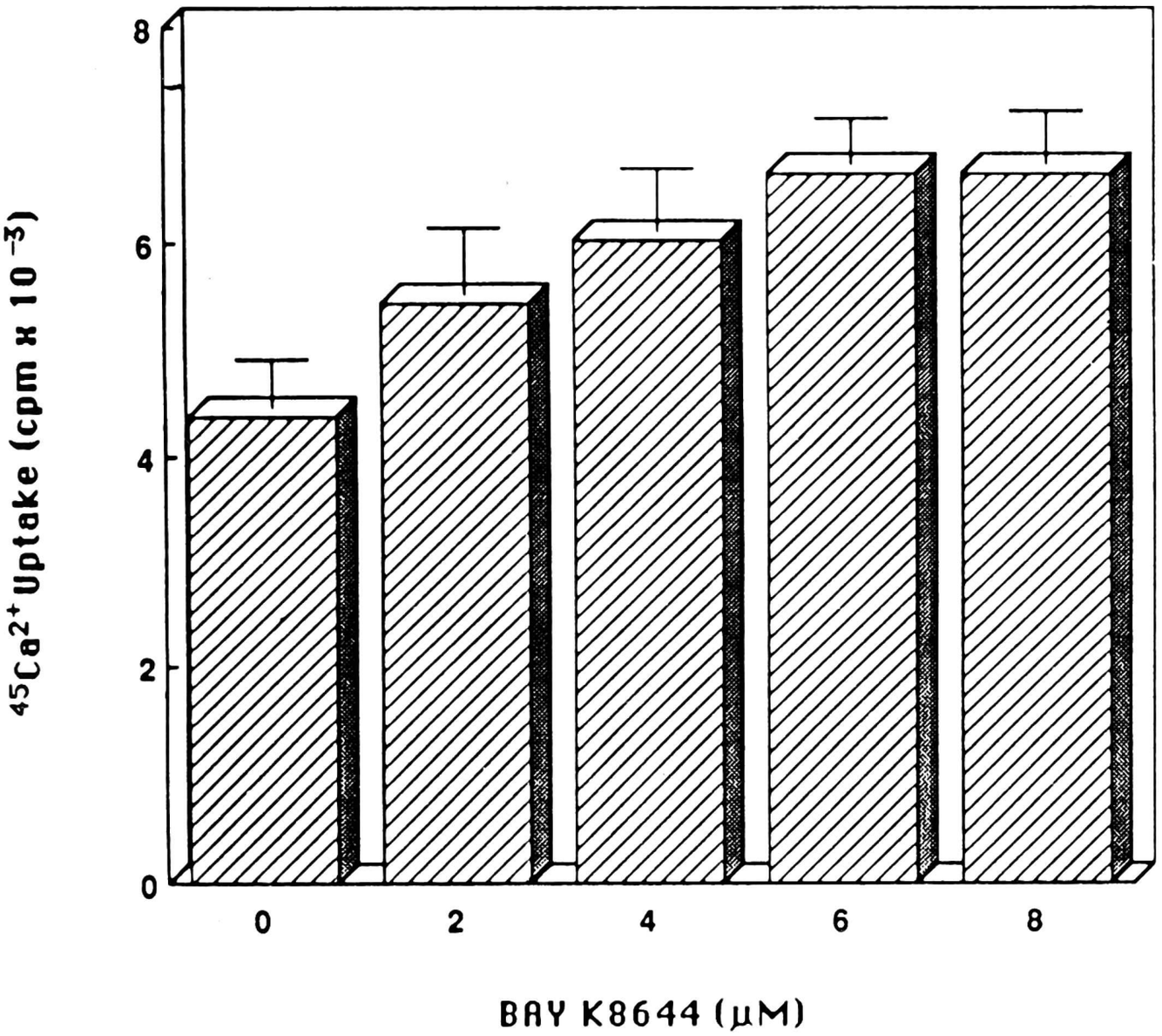

Fig. 4. Effect of BAY K8644 on the uptake of $^{45}Ca^{2+}$ into vesicles containing the reconstituted gastric mucosal calcium channels. Uptake assays were conducted as described in Materials and Methods using vesicles preincubated for 20 min at room temperature with different concentrations of BAY K8644 (0—10 μM). Values represent the means $\pm$ SD of five separate experiments performed in duplicate.

The $^{45}Ca^{2+}$ uptake into vesicles containing the intact and phosphorylated purified gastric mucosal calcium channel protein is shown in *Figure 6*. In both types of vesicles, the uptake of $^{45}Ca^{2+}$ was proportional to the time of incubation and reached maximum at 30 min. However, the extent of uptake of $^{45}Ca^{2+}$ by vesicles containing PDGF-induced phosphorylated calcium channels was 78% greater as compared to that of the controls.

DISCUSSION

Platelet-derived growth factor is a potent mitogen capable of stimulation of epithelial call secretory responses (7—9). The biological effects of PDGF like those of epidermal growth factor (10, 16), are mediated by receptors located on

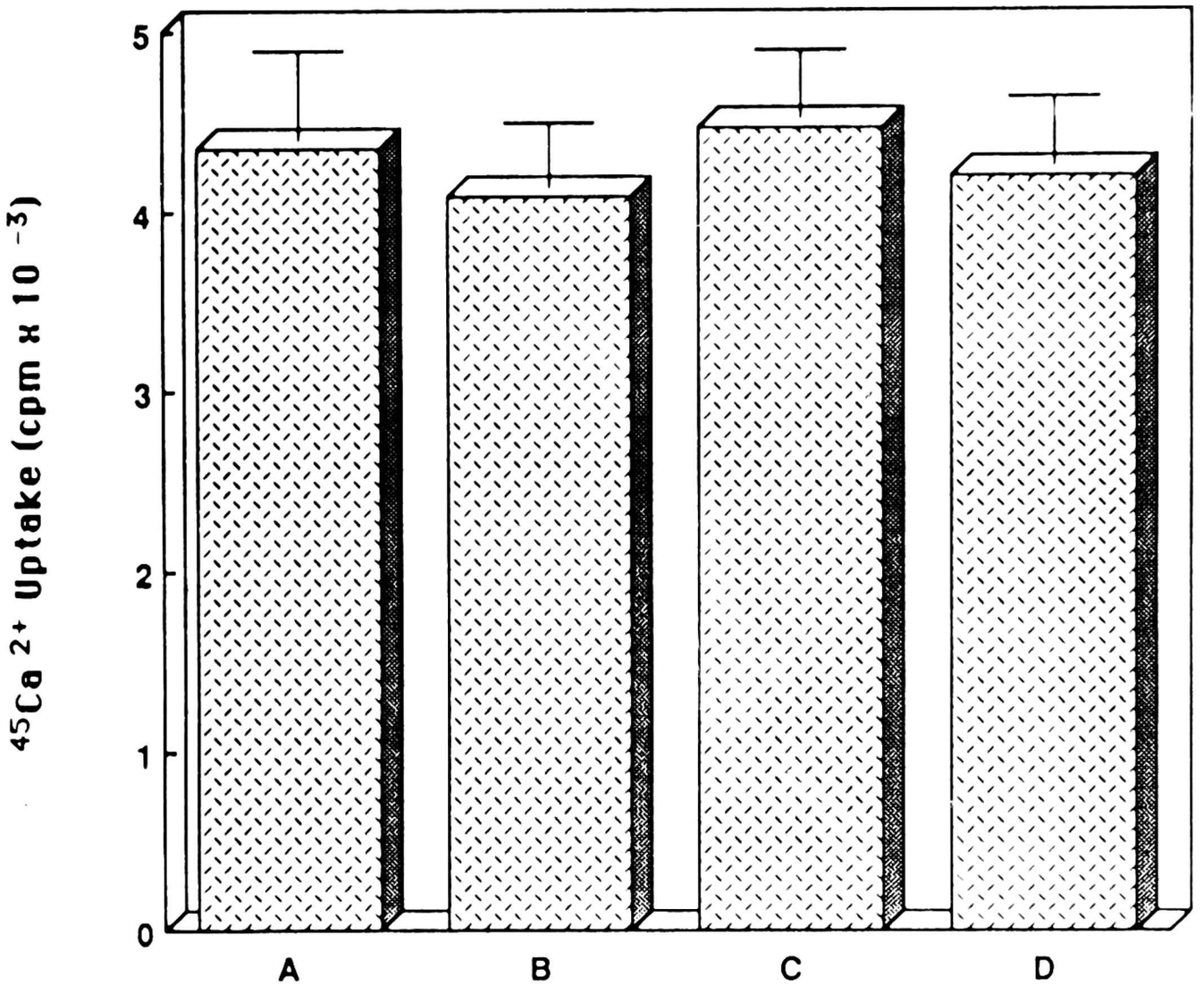

Fig. 5. Effect of cationic gradient on the uptake of $^{45}Ca^{2+}$ into phospholipid vesicles containing the reconstituted gastric mucosal calcium channels. The vesicle aliquots were preincubated for 5 min at room temperature in a buffer (pH 7.0) containing 0.34 M sucrose, 10 mM MOPS/tetramethylammonium, 5—150 mM KCl, 145—50 mM NaCl, and then subjected to $^{45}Ca^{2+}$ uptake assay. A, control (5 mM KCl/145 mM NaCl); B, 50 mM KCl/100 mM NaCl; C, 75 mM KCl/75 mM NaCl; D, 100 mM KCl/50 mM NaCl. Values represent the means ± SD of five separate experiments performed in duplicate.

the target cell surfaces and involve the activation of the intrinsic tyrosine kinase, an event essential for further signal transduction (9). While the majority of proliferative effects of EGF and PDGF are considered to be a direct consequence of phospholipase C activation for polyphosphoinositide hydrolysis and the release of calcium from intracellular stores (17, 18), there are strong indications that the earliest responses to the activation involve the influx of calcium (19).

Indeed, recent patch clamp recording data obtained with A431 cells suggest that activation of the EGF receptor triggers the receptor-operated calcium channel response (19). The results of studies presented herein provide evidence for the presence of voltage independent calcium channels in gastric epithelial cell membrane, and show that phosphorylation on the tyrosine residues

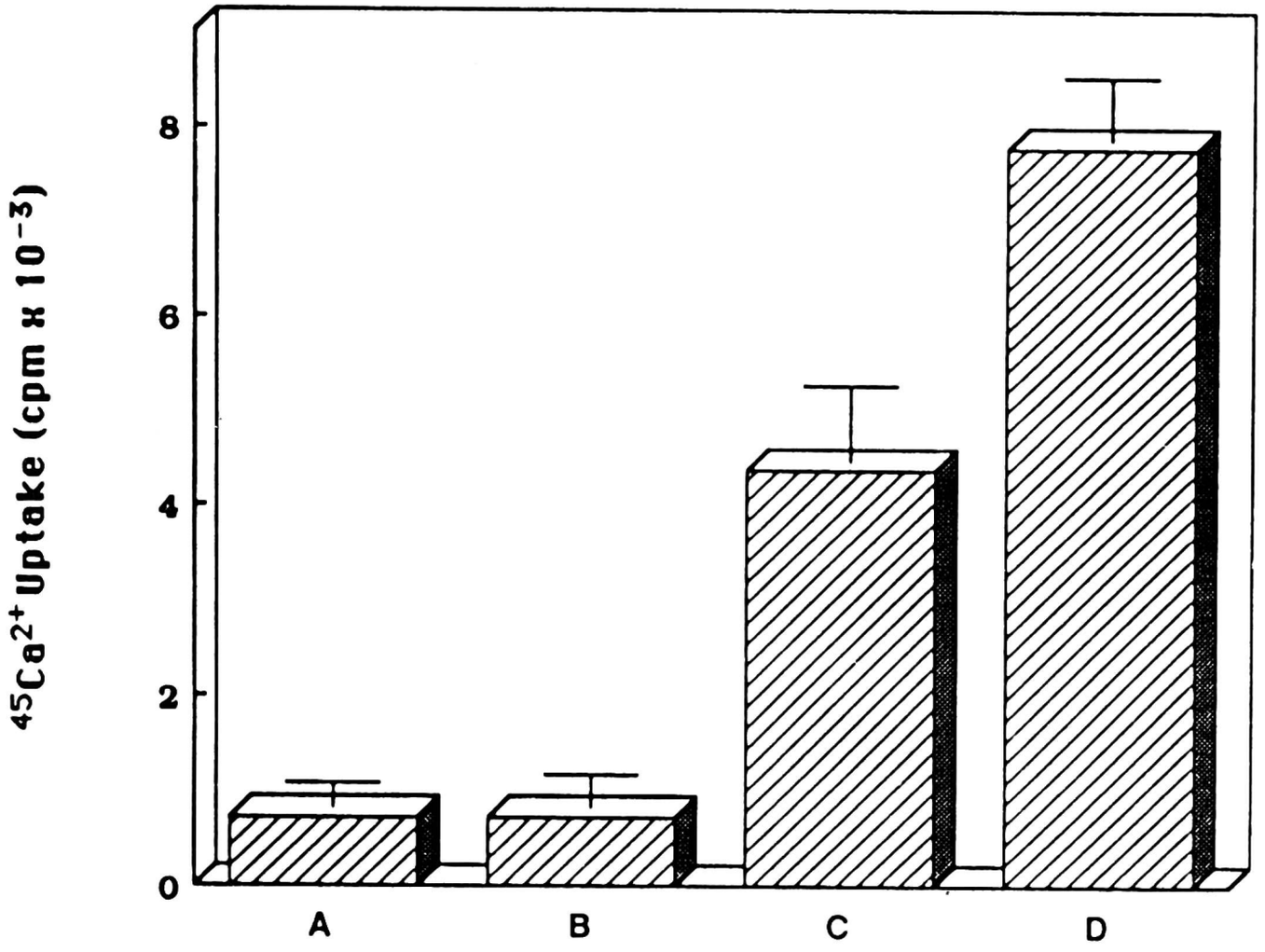

Fig. 6. Effect of PDGF-stimulated calcium channel protein phosphorylation on $^{45}Ca^{2+}$ uptake into vesicles containing the reconstituted gastric mucosal calcium channels. The purified channel protein was reconstituted into phospholipid vesicles either in its intact form (C) or following PDGF-induced phosphorylation (D). A and B, vesicles free of membrane protein in the absence (A) and the presence (B) of PDGF. Values represent the means ± SD of five experiments performed in duplicate.

through the activation of PDGF receptor leads to stimulation of calcium channel activity.

The solubilized calcium channels, labeled with [^{3}H] PN200-110 and purified by affinity chromatography on wheat germ agglutinin, 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, as well as to BAY K8644, a specific calcium channel activator. Upon SDS-PAGE examination under reducing conditions, the gastric mucosal calcium channel preparation yielded 170, 130, 90 and 55 kDa protein bands corresponding to those of calcium channels from other tissues (1, 15, 20).

On PDGF binding in the presence of ATP, the gastric mucosal calcium channels responded by an increase in protein tyrosine phosphorylation. Examination of the protein patterns using antiphosphotyrosine antibody revealed

that this increase in phosphorylation was reflected mainly in 55 and 170 kDa proteins. The vesicles containing phosphorylated channel protein displayed a 78% greater calcium uptake, thus indicating the tyrosine kinase involvement in PDGF dependent calcium channel activation. This finding together with the data from patch clamp recording at the level of single ion channels (19) suggest that the expression of calcium channel activity depends not only on the phosphorylation events controlled by protein kinase A and C (20, 21), but also on the phosphorylation triggered by PDGF receptor activation.

The fact that the activation of calcium channels in gastric mucosa is dependent on the PDGF receptor activation points towards the importance of this mitogen in the regulation of calcium influx, an event of significance to the processes of mucosal repair and integrity maintenance. Thus PDGF, along with EGF, (16, 18) appears to play a major role in gastric mucosal integrity maintenance.

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