JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 1994, 45, 2, 299—308

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EFFECTS OF PROSTAGLANDINS ON $[Ca^{2+}]_i$ AND ADENYLATE CYCLASE ACTIVITY IN ISOLATED PORCINE GASTRIC MUCOUS CELLS

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In porcine gastric mucous cells, isolated enzymatically from the fundic mucosa and enriched by counterflow centrifugation, PGE_2 (1 μM) increased adenylate cyclase activity to 225% and, distinct from that documented for other species, also $[Ca^{2+}]_i$, measured fluorimetrically with Fura2/AM, in Ca^{2+}-containing and Ca^{2+}-free incubation medium to 182% and 165% of control values, respectively. $PGF_{2\alpha}$, PGD_2, the stable prostacyclin analogue iloprost and the thromboxane-mimetic U46619 had no significant effects on adenylate cyclase activity and $[Ca^{2+}]_i$. Histamine (10 μM) stimulated adenylate cyclase activity to 236% of control value, an effect which could be blocked by the H_2-receptor antagonist ranitidine. However, histamine and the activators of the cAMP system forskolin and dibutyryl cAMP had no significant effect on $[Ca^{2+}]_i$, indicating that an activation of the adenylate cyclase/cAMP system per se does not result in an increase in $[Ca^{2+}]_i$. These data suggest that prostanoids stimulate adenylate cyclase activity and $[Ca^{2+}]_i$ in gastric mucous cells via activation of EP-receptors linked to both second messenger systems.

Key words: *Prostaglandins, dinoprostone, adenylate cyclase, calcium, mucous cells, gastric mucosa*

INTRODUCTION

Prostaglandins and histamine are considered as regulators of secretory functions of gastric mucous cells (1–3). We have previously shown that in guinea-pig gastric mucous cells PGE_2 and histamine stimulate adenylate cyclase activity, but have no efect on $[Ca^{2+}]$ i (4, 5). PGE_2 also had no effect on $[Ca^{2+}]_i$ in rabbit gastric mucous cells (6). However, in some other secretory cell systems, e.g. hepatocytes, luteal cells and adrenal chromaffin cells, effects of PGE_2 appear to be calcium-dependent (7–9). Since effects of prostaglandins on gastric chief cells are species-dependent (10, 11), the question arises, whether the unresponsiveness of gastric mucous cell $[Ca^{2+}]_i$ to prostaglandins is a general or a species-dependent phenomenon. To investigate this we worked

with porcine gastric mucous cells, which have not been used for this purpose till now. In detail we determined effects of different natural and synthetic prostanoids and, for comparison, of histamine, the receptor-independent adenylate cyclase activator forskolin (12), the activator of cAMP-dependent protein kinases dibutyryl cAMP (dB-cAMP) and the m-cholinceptor agonist carbachol on $[Ca^{2+}]_i$ and/or adenylate cyclase activity in these cells.

METHODS

Cell isolation

The techniques used have been described in detail previously (13). Briefly, the following buffers were used: buffer A consisted of (mM) NaCl 74, $NaHCO_3$ 20, KCl 5, NaH_2PO_4 0.5, Na_2HPO_4 1, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) 50, glucose 11 and indometacin 0.02. Buffer B had the same composition but contained additionally $CaCl_2$ 1, $MgCl_2$ 1.5 and bovine serum albumin (BSA) 2 mg/ml. Corpus mucosa of slaughterhouse pigs was cut into squares of about 0.25 cm^2 and incubated for 30 min at 37°C in 150 ml buffer A containing 187.5 mg of pronase E. The supernatant was discarded and the remaining tissue pieces were minced with scissors and incubated for 45 min in 100 ml of buffer B, containing 11600 units of collagenase. The suspension was filtered twice through a nylon mesh (pore size 200 µm), the remainder treated again with collagenase, and the combined filtrates were centrifuged at 580 g for 3 min and stored in 20 ml of buffer B.

Cell separation

Cell separation was achieved by counterflow centrifugation, using a Beckman elutriator rotor on a Beckman J-6 centrifuge. Cells (up to 10^9) were eluted in buffer B at 880 r.p.m. (234 g_{max}). Two cell populations were obtained at flow rates of 20 ml/min (F1, 600 ml total volume collected) and 40 ml/min (F2, 1000 ml) and the remainder of the cells was discarded. For this investigation we used F2 cells, consisting of about 75% mucous cells, 15% chief cells and less than 2% parietal cells, as determined by cell staining (13). Cell viability, as checked by trypan blue exclusion, was $>90\%$.

Measurement of adenylate cyclase activity

F2 cells were suspended in 25 mM Tris-HCl buffer, pH 7.8, containing 100 µM ethylenedinitrilotetraacetic acid (EDTA) and 100 µM dithiothreitol and homogenized by sonication (3 × 5 sec, 30 watt, Branson sonifier). Adenylate cyclase activity in the homogenate was measured by determining the rate of cAMP formation from ATP at 30°C in 0.2 ml incubation medium containing (mM) Tris-HCl buffer pH 7.8 25, $MgCl_2$ 10, 3-isobutyl-1-methylxanthine (IBMX) 0.1, ATP 1, creatine phosphate 15.6, GTP 0.01, creatine kinase 10 U/ml, cell protein 40 µg and the agents to be tested. In the case of the ranitidine experiments the cell protein was preincubated for 5 min with the H_2-receptor antagonist and then histamine was added. After 15 min of incubation the reaction was stopped by heat (3 min, 90°C). The samples were cooled to 4°C and subsequently 0.2 ml water and 30 mg aluminium oxide were added. After centrifugation, the cAMP concentration was determined in 50 µl aliquots of the supernatant according to Gilman (14).

Measurement of intracellular calcium

F2 cells (5×10^6 cells/ml) were incubated at 37°C for 30 min with 5 μM FURA2-pentaacetoxymethyl ester (FURA2/AM) in buffer C, pH 7.4, containing (in mM) HEPES 20, Tris 20, NaCl 118, KH_2PO_4 3, K_2HPO_4 2, $MgCl_2$ 1.2, $CaCl_2$ 1, glucose 11 and BSA 1 mg/ml. Incubation was continued for 30 min at 37°C after dilution ($\times 8$) of the incubation mixture with buffer C. The cells were pelleted by centrifugation, washed twice in BSA-free buffer C, resuspended and stored in 1 ml of this buffer at 4°C until use. The fluorescence of continuously stirred FURA2-loaded cells (3×10^5 cells/ml) was measured in a thermostatically controlled cuvette in a computer-equipped Shimadzu RF-5001 PC spectrofluorimeter with excitation at 340 and 380 nm and emission at 500 nm. At the end of each experiment the maximum fluorescence was recorded by lysing the cells with 0.1% Triton X-100 and the minimum fluorescence was subsequently determined by adding ethyleneglycol-bis (β-aminoethylether) tetraaceticacid (EGTA, final concentration 7.5 mM) to the cell suspension. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio (340/380 nm) according to Grynkiewicz et al. (15) for dual wavelength measurements assuming a K_D of 224 nM. It should be mentioned that these values may represent only approximations of the absolute $[Ca^{2+}]_i$, since compartmentalization of fura-2 by gastric mucous cells may occur (6).

Protein determination

Protein was determined according to Lowry et al. (16) using BSA as standard.

Statistical analysis

Data from adenylate cyclase activity measurements were evaluated by analysis of variance in combination with Dunnett's t-test (17). Data from calcium measurements were analysed by Dunnett's t-test for paired comparisons. Values are given as mean ± SEM. *n* indicates the number of different cell preparations used.

Substances

Collagenase type V, PGE_2, $PGF_{2\alpha}$, PGD_2, histamine dihydrochloride, carbachol chloride, EDTA, EGTA, IBMX, Triton X-100 (Sigma, München, FRG); FURA2/AM (Calbiochem, LaJolla, USA); ranitidine hydrochloride (Glaxo, Bad Oldesloe, FRG); iloprost (Schering, Berlin, FRG); U46619 (Upjohn, Kalamazoo, USA); BSA, HEPES (Serva, Heidelberg, FRG); dB-cAMP, sodium salt (Boehringer, Mannheim, FRG). All other substances were of analytical grade and obtained from Merck (Darmstadt, FRG).

RESULTS

Adenylate cyclase activity

Basal enzyme activity in isolated porcine gastric mucous cells averaged 11 – 12 pmol of cAMP per mg of protein per min. *Table 1* shows the effects of different prostanoids on adenylate cyclase activity. PGE_2 in a concentration of 1 μM stimulated enzyme activity to 224% of the control value ($p < 0.01$),

Table 1. Effects of different prostanoids on adenylate cyclase activity in enriched porcine gastric mucous cells

Treatments	Adenylate cyclase activity [pmol cAMP/(mg protein × min)]
Control	11.8 ± 0.7
PGE_2	26.4 ± 2.5*
$PGF_{2\alpha}$	10.1 ± 0.4
Iloprost	10.8 ± 0.6
U46619	10.4 ± 1.8

Cell homogenates were incubated for 15 min without (control) or with the prostanoids in a concentration of 1 μM and adenylate cyclase activity was determined as described in "Methods". Each value represents the mean ± SEM of triplicate determinations from 3 different cell preparations. * $p<0.01$ versus control.

Table 2. Effects of histamine and the H_2-receptor antagonist ranitidine on adenylate cyclase activity in enriched porcine gastric mucous cells

Treatments	Adenylate cyclase activity [pmol cAMP/(mg protein × min)]
Control	12.1 ± 0.8
Ranitidine 100 μM	14.9 ± 1.4
Histamine 10 μM	28.4 ± 1.5*
Histamine 10 μM + Ranitidine 100 μM	15.5 ± 1.1**

Cell homogeates were incubated for 15 min without or with histamine after a 5 min preincubation in the absence or presence of ranitidine. Adenylate cyclase activity was determined as described in "Methods". Each value represents the mean ± SEM of triplicate determinations from 3 different cell preparations. * $p<0.05$ when compared with control. ** $p<0.05$ when compared with histamine alone.

whereas $PGF_{2\alpha}$, PGD_2, iloprost (stable anlogue of prostacyclin) and the thromboxane-mimetic U46619 were ineffective in this concentration. Histamine in a concentration of 10 μM stimulated adenylate cyclase activity to 235% of the control value ($p<0.05$), an effect which was inhibited by the H_2-receptor antagonist ranitidine (*Table 2*). Forskolin 100 μM stimulated adenylate cyclase activity to 2190±470% of the control value (triplicate determinations, $n=4$, $p<0.01$, not shown).

Intracellular calcium

The $[Ca^{2+}]_i$ of unstimulated porcine gastric mucous cells was 107±3 nM ($n=12$). The m-cholinoceptor agonist carbachol, previously shown by us to increase $[Ca^{2+}]_i$ in guinea-pig enriched gastric mucous cells (5, 18), induced

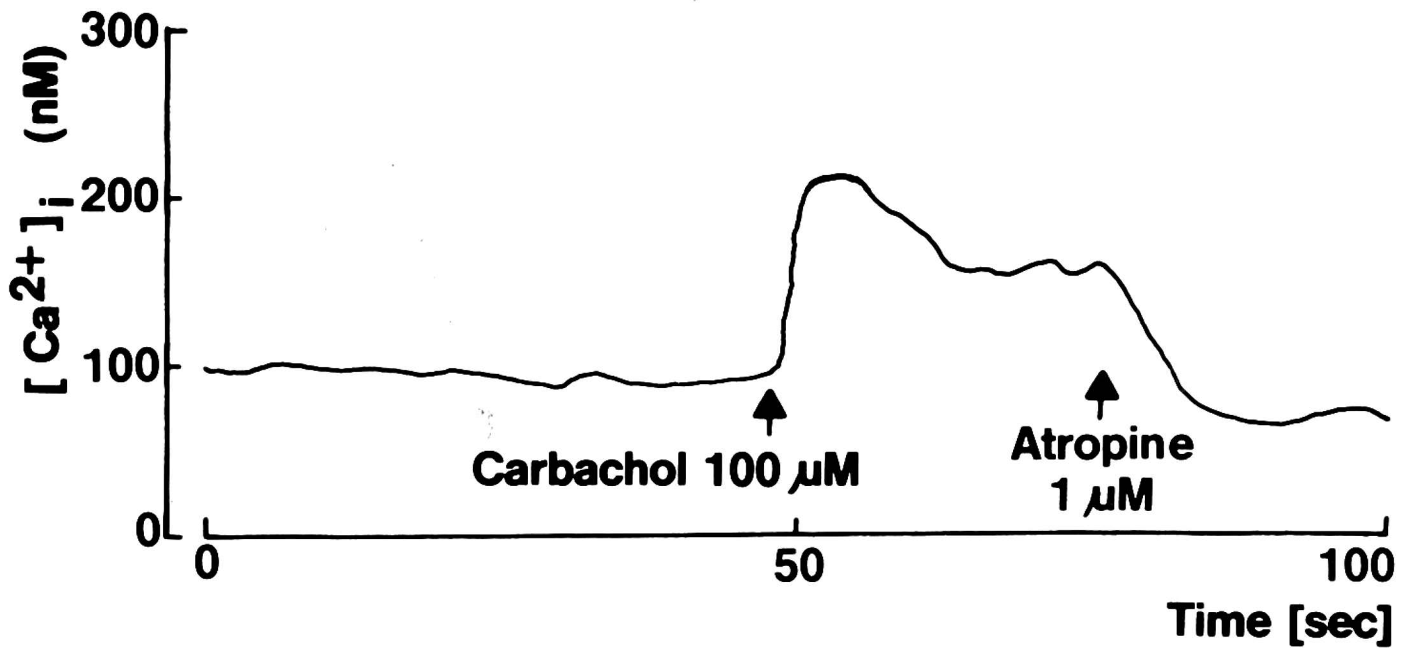

Fig. 1. Effects of carbachol and atropine on $[Ca^{2+}]_i$ in isolated porcine gastric mucous cells.

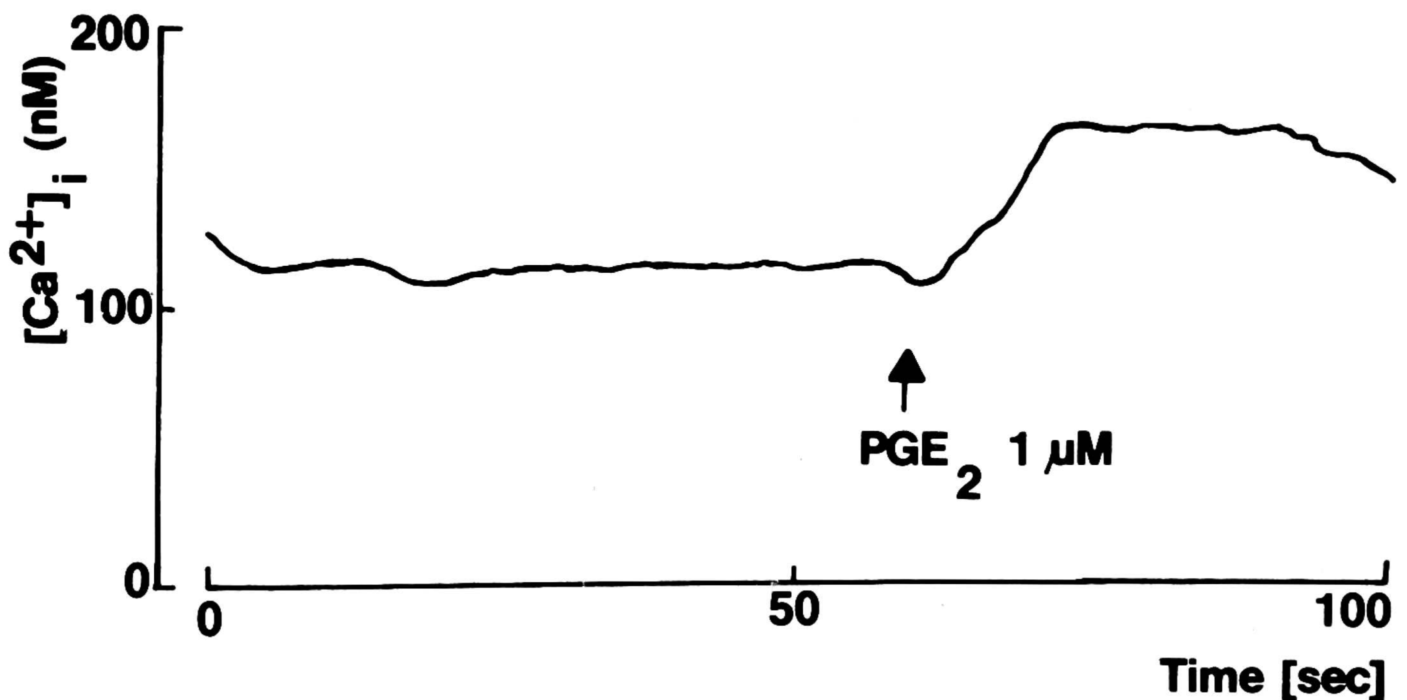

Fig. 2. Effect of PGE_2 on $[Ca^{2+}]_i$ in isolated porcine gastric mucous cells.

a rapid rise of $[Ca^{2+}]_i$ also in porcine gastric mucous cells and was used as a control of cell responsiveness in the following experiments. *Fig. 1* shows a typical change in $[Ca^{2+}]_i$ evoked by carbachol (100 µM). Atropine (1 µM) rapidly reduced carbachol-stimulated $[Ca^{+}]_i$ to the prestimulation level.

PGE_2 (1 µM, *Fig. 2*) and 16,16-dimethyl PGE_2 (1 µM, data not shown) increased $[Ca^{2+}]_i$ in the porcine gastric mucous cells, however, the response was lower (PGE_2 $182 \pm 13\%$, $n=6$, $p<0.01$ versus control; carbachol $225 \pm 11\%$ of control value, $n=6$, $p<0.01$ versus control) and slower than that to carbachol, reaching a peak value within 10 sec after the addition of PGE_2 to the cell suspension.

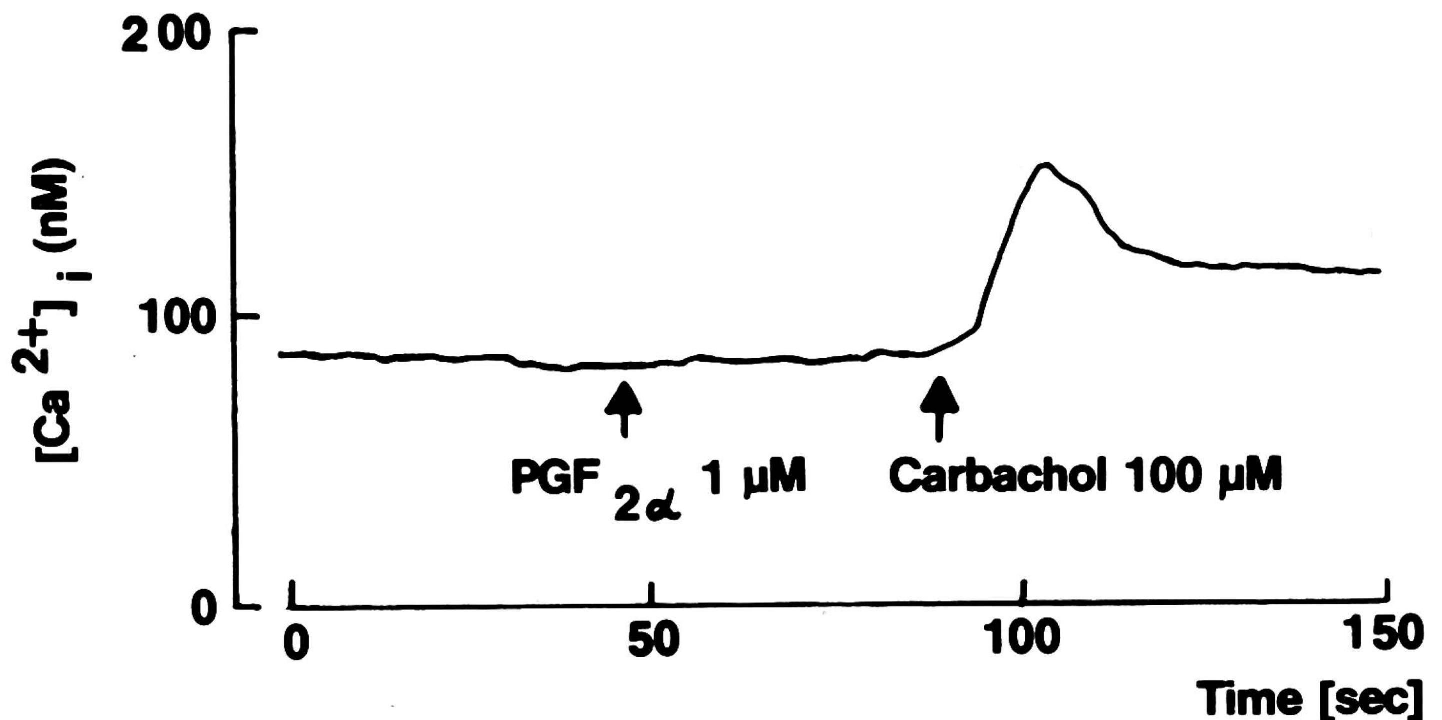

Fig. 3. Effects of $PGF_{2\alpha}$ and carbachol on $[Ca^{2+}]_i$ in isolated porcine gastric mucous cells.

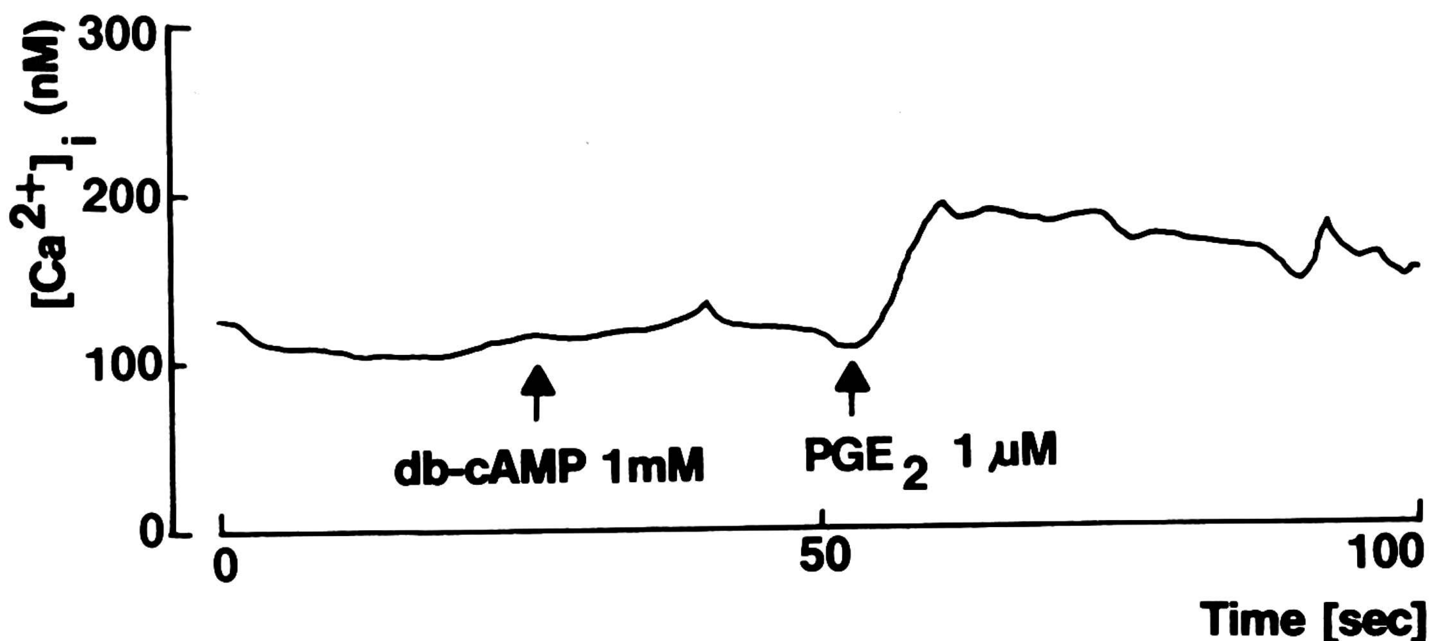

Fig. 4. Effects of dB-cAMP and PGE_2 on $[Ca^{2+}]_i$ in isolated porcine gastric mucous cells.

$PGF_{2\alpha}$ (*Fig. 3*), PGD_2, iloprost and U46619 (data not shown), each in a concentration of 1 μM, had no significant effect on $[Ca^{2+}]_i$ in porcine gastric mucous cells. Histamine (100 μM), forskolin (100 μM, data not shown) and dibutryryl cAMP (1 mM, *Fig. 4*) also failed to increase $[Ca^{2+}]_i$.

In order to investigate the involvement of extracellular and/or intracellular Ca^{2+} in the cell response we measured changes in $[Ca^{2+}]_i$ in response to the same agonists after exposure of the cells to Ca^{2+}-free incubation medium, containing EGTA 1 mM, for 1 min. As shown in *Table 3* $[Ca^{2+}]_i$ at basal conditions in Ca^{2+}-free medium was 66 ± 5 nM (n=9). PGE_2 increased

Table 3. Effects of PGE_2, $PGF_{2\alpha}$ and carbachol on $[Ca^{2+}]_i$ in enriched porcine gastric mucous cells in Ca^{2+}-free incubation medium

Treatments	n	$[Ca^{2+}]_i$ [nM]
Control	9	66.6 ± 5.2
Carbachol 100 μM	8	144.1 ± 10.2*
PGE_2 1 μM	5	109.6 ± 8.0*
$PGF_{2\alpha}$ 1 μM	5	64.2 ± 7.9

Cells were incubated with the indicated compounds in Ca^{2+}-free medium, containing 1 mM EGTA and $[Ca^{2+}]_i$ was determined as described in "Methods". Each value represents the mean ± SEM of n different cell preparations. * $p < 0.01$ versus control.

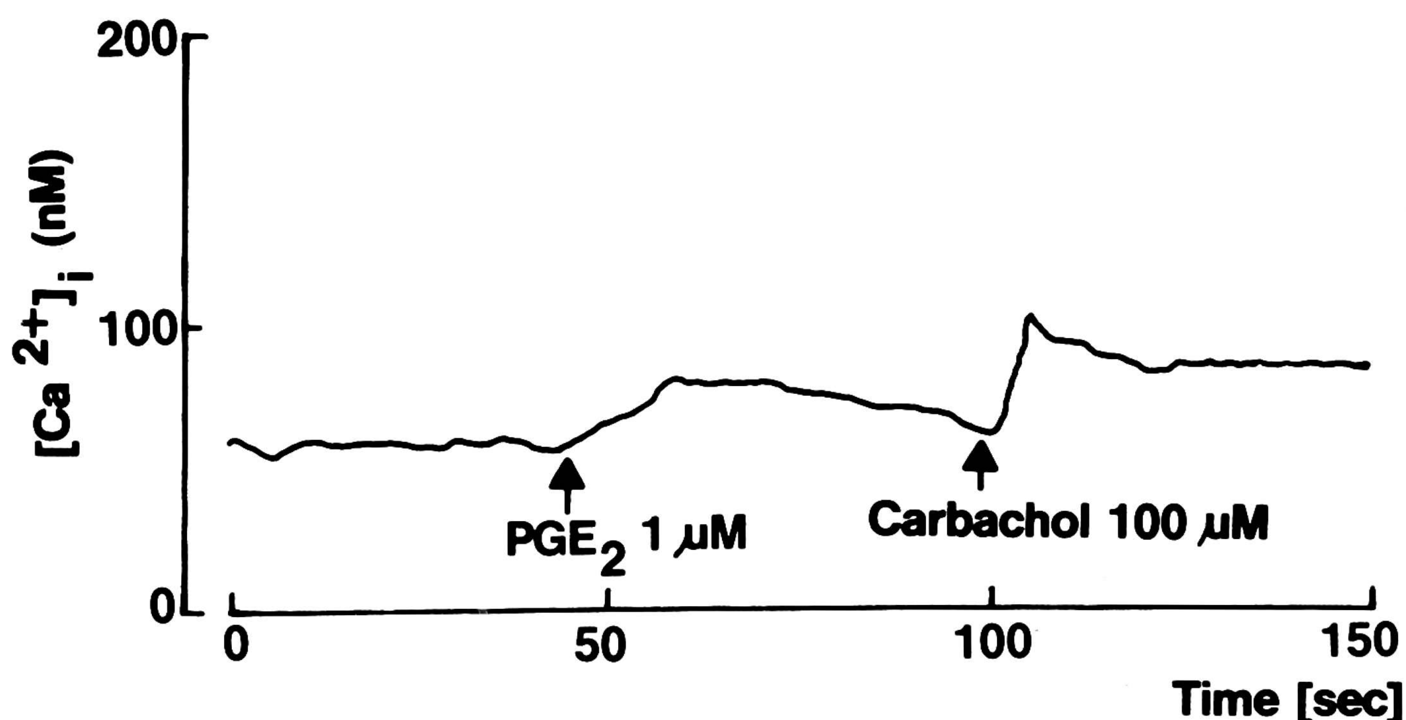

Fig. 5. Effects of PGE_2 and carbachol on $[Ca^{2+}]_i$ in isolated porcine gastric mucous cells in Ca^{2+}-free medium.

$[Ca^{2+}]_i$ to 165 ± 7% ($p < 0.01$) and carbachol stimulated $[Ca^{2+}]_i$ to 216 ± 6% of control value ($p < 0.01$), whereas $PGF_{2\alpha}$ again had no significant effect on $[Ca^{2+}]_i$. In Ca^{2+}-containing (data not shown) as well as in Ca^{2+}-free medium (*Fig. 5*) stimulation of $[Ca^{2+}]_i$ by PGE_2 did not prevent a subsequent stimulation of $[Ca^{2+}]_i$ by carbachol (*Fig. 5*) or vice versa (data not shown).

DISCUSSION

The purpose of this study was to examine effects of prostaglandins on adenylate cyclase activity and $[Ca^{2+}]_i$ in isolated porcine gastric mucous cells. Our results indicate that in these cells PGE_2 exerts stimulatory effects on both parameters.

Data for stimulation of adenylate cyclase activity (*Table 1*) correspond well with those previously obtained for PGE_2 in guinea-pig gastric mucous cells (5). They are also in accordance with data from isolated rabbit gastric cells, where PGE_2 stimulated cAMP formation most potently in nonparietal cell fractions (19). $PGF_{2\alpha}$, PGD_2, iloprost and U46619, activating preferentially FP-, DP-, IP- and TP-receptors, respectively (20), failed to stimulate adenylate cyclase activity suggesting that prostanoid effects on adenylate cyclase activity in porcine gastric mucous cells are mediated via EP-receptors.

In contrary to the results documented for guinea-pig (5) and rabbit (6) gastric mucous cells, PGE_2 increased also $[Ca^{2+}]_i$ in porcine gastric mucous cells. The response to PGE_2 was lower and slower than that to carbachol and detectable in the presence (*Fig. 2*) or absence (*Fig. 5* and *Table 3*) of extracellular Ca^{2+}, indicating that liberation of calcium from intracellular stores is involved in this process. PGD_2, $PGF_{2\alpha}$, iloprost and U46619 failed to stimulate $[Ca^{2+}]_i$, suggesting that prostanoid effects on $[Ca^{2+}]_i$ in porcine gastric mucous cells are also mediated via EP-receptors.

Stimulatory effects of PGE_2 on more than one second messenger system at a time have been reported for other cell types and it has been suggested that an increase in $[Ca^{2+}]_i$ in response to PGE_2 may occur either as a consequence, e.g in T lymphocytes (21), or independent of an activation of the cAMP system, e.g. in cortical collecting duct cells (22) or osteoblastic cells (23). To evaluate this issue in the gastric mucous cells we determined effects of known activators of the cAMP system on both parameters.

Histamine (10 µmol/l) stimulated adenylate cyclase activity (*Table 2*), however, failed to increase $[Ca^{2+}]_i$. Stimulation of adenylate cyclase activity by histamine is obviously mediated via H_2-receptors, since it could be prevented by the H_2-receptor antagonist ranitidine. It may be mentioned that histamine stimulates adenylate cyclase activity and/or cAMP levels in porcine (this paper), guinea-pig (5) and dog (24), but not in rat gastric mucous cells (25), suggesting species differences for this histamine effect. Forskolin (12), a receptor-independent activator of the adenylate cyclase, and dB-cAMP, an activator of cAMP-dependent protein kinases failed to stimulate $[Ca^{2+}]_i$ in gastric mucous cells, although the cells from the same preparations responded to PGE_2 and the m-cholinoceptor agonist carbachol (*Fig. 3 and 4*). In conjunction with the histamine data this indicates that an activation of the cAMP system per se does not result in an increase in $[Ca^{2+}]_i$. We therefore suggest that the simultaneous stimulation of adenylate cyclase activity and of $[Ca^{2+}]_i$ by PGE_2 is mediated *via* separate effects on both second messenger systems. Dual effects of other secretagogues on second mesenger systems in gastric mucosal cells have been reported. In parietal cells, for example, histamine stimulates both adenylate cyclase acitivity and $[Ca^{2+}]_i$ (26).

In conclusion, our data indicate that in enriched porcine gastric mucous cells PGE_2 increases adenylate cyclase activity and, different from that documented for other species, also intracellular free Ca^{2+}, whereas histamine activates only the adenylate cyclase. We suggest that the effects of PGE_2 are mediated via activation of EP-receptors linked to both the cAMP- and the Ca^{2+}-related second messenger system. A further pharmacological and biochemical characteristion and the physiological relevance of the prostanoid receptors involved remain to be established.

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Received: November 15, 1993
Accepted: December 13, 1993

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