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INDUCTION BY THE CALCIUM IONOPHORE A23187 OF A PROTECTIVE EFFECT AGAINST CELL INJURY IN CULTURED GASTRIC MUCOSAL CELLS

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Abstract—An intrinsic protective mechanism against cell injury seems to exist in cultured gastric mucosal cells. Cells, isolated from the stomachs of 10- to 12-day-old rats and subcultured, were examined for damage by the erythrosine B dye exclusion test. Pretreatment with 5 μM A23187 (a calcium ionophore) diminished the cell damage induced by acidified medium (pH 3.5) or 8 mM aspirin (pH 5.0). The effect of A23187 appeared 4 hr after its addition and was reversible. Protection by A23187 against cell injury diminished in the absence of extracellular Ca^{2+} and was dependent on Ca^{2+} concentration. An increase in intracellular Ca^{2+} may induce cell resistance against injury in cultured gastric mucosal cells.

The gastric mucosa is resistant to damage from the acidity of gastric contents. It has several defensive mechanisms, such as bicarbonate and mucus secretion, prostaglandin synthesis and local mucosal blood flow. Also noteworthy is the intrinsic protective mechanism of gastric mucosal cells which has been the subject of several papers [1–3]. Recently, it has been reported that gastric perfusion with $CaCl_2$ enhances the recovery of mucosal integrity in the stomach following ethanol- or hypertonic NaCl-induced erosions [4], and pretreatment with calcium gluconate prevents ethanol-induced mucosal injury in rat stomach [5]. These studies suggest a protective effect of calcium against gastric mucosal injury. In the present work, the role of calcium in the induction of cell resistance to injury was examined in cultured gastric mucosal cells using the calcium ionophore A23187 to increase the intracellular Ca^{2+} concentration. A23187 enhanced cell resistance to injury induced by an acidified medium (pH 3.5), but its protective effect diminished in the absence of Ca^{2+} and was dependent on extracellular Ca^{2+} concentration.

MATERIALS AND METHODS

Cell culture. The stomachs from 10- to 12-day-old rats (Jcl Sprague–Dawley strain, CLEA Japan Inc.) were obtained and placed in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) (pH 7.4) containing 100 units/ml of penicillin and 100 μg/ml of streptomycin. The fundic area was excised from the stomach and incised along the greater curvature. Strips of fundic tissue were rinsed with PBS and the mucosal surface was rubbed thoroughly with wet adsorbent cotton to remove the mucus and lining of the epithelial cells. The strips were shaken in PBS containing 0.1% collagenase and 0.1% $CaCl_2$ for 30 min at 37°. The detached cells were collected by centrifugation (1000 rpm for 5 min) and washed twice with PBS. They were then suspended in an appropriate amount of Eagle's minimal essential medium with Earle's salts (MEM) supplemented with 10% fetal bovine serum (FBS) and 60 μg/ml of kanamycin, inoculated into a 35-mm-diameter plastic dish (2×10^5 cells/ml/dish), and cultured at 37° in 5% CO_2–95% air. When the culture reached confluency, the cells were trypsinized and subcultured at a split ratio of 1:6. Subculture was routinely carried out once a week. Cells from 1-week-old cultures, which were in the confluent state, were used for the experiments.

Assay of cell resistance. The cells were pretreated with various agents for 24 hr, washed once with PBS, and then incubated at room temperature in Eagle's MEM acidified with 1 M HCl to pH 3.5 or in Eagle's MEM containing 8 mM aspirin (pH 5.0). The dye exclusion test was performed according to the method of Ohtsuka *et al.* [6] to assess the cell damage. The incubation media were discarded, and the cell layers were kept for 5 min in 1 ml of 5% erythrosine B in PBS. The dye solution was discarded, and the cell layers were washed four times with PBS. After the cells had been dissolved with 0.5 M NaOH, the concentration of erythrosine B was estimated by measuring the absorbance at 529 nm. The protein concentration of the cell solution was measured by the method of Lowry *et al.* [7]. The dye uptake was expressed as nanomoles per milligram of protein.

In the experiments on the Ca^{2+} concentration–effect relationships, the Ca^{2+} concentration in the culture medium was varied using calcium–EGTA* buffers. The medium with a low Ca^{2+} concentration was composed of 9 vol. of calcium-free Eagle's MEM, 1 vol. of dialyzed FBS, and 250 μM EGTA. Ca^{2+} concentration in the medium was controlled by addition of a calculated amount of $CaCl_2$. Ca^{2+} concentrations were calculated according to the following equation, using $3 \times 10^6\ M^{-1}$ as K which was calculated from the data of Harrison and Bers [8].

* EGTA, ethyleneglycolbis(amino-ethylether)tetra-acetate.

$$[EGTA]_{total} = \frac{([Ca]_{total} - [Ca]_{free})(1 + K \cdot [Ca]_{free})}{K \cdot [Ca]_{free}}$$

The cell layers were washed twice with low Ca^{2+}-medium (8 nM) and cultured for 24 hr in the medium containing various concentrations of Ca^{2+} in the presence or absence of A23187.

Materials. Both Eagle's MEM and calcium-free Eagle's MEM were purchased from Nissui Seiyaku (Tokyo, Japan); FBS was from Flow Laboratories (Staumore, Australia), collagenase from Boehringer (Mannheim, F.R.G.), and trypsin (1:250) from Difco Laboratories (Detroit, MI, U.S.A.). A23187, W-7 and trifluoperazine were obtained from the Sigma Chemicals Co. (St Louis, MO, U.S.A.). Erythrosine B, 1-oleoyl-2-acetylglycerol and EGTA were obtained from Nakarai Chemicals (Kyoto, Japan).

RESULTS

Cell culture. Recent studies have shown that prostaglandin treatment does not prevent cell damage of superficial mucus cells by ethanol but can reduce the depth of the mucosal damage [9, 10]. If the gastric mucosal cells possess intrinsic protective properties against cell injury, they may be in the cells underlying the surface mucus cells, namely the mucus neck cells. The superficial cells were removed by rubbing with a wet adsorbent cotton, and the intactness of the underlying mucus neck portion was confirmed histologically. The rubbed whole fundic tissues were digested with collagenase for about 30 min. Since there was only a small amount of cells, they were allowed to proliferate by serial subcultures. After five to six passages, the cells were used for the experiments.

The cell layer was macroscopically covered with a mucus-like gelatinous layer. Histochemical study showed that over 90% of the cells contained PAS-positive granules, and clots of PAS-positive substances were widely distributed over the cell layer. Not only were most of the surface mucus cells removed by rubbing the mucosal surface, but such cells isolated from the stomach of a suckling rat lost their proliferative ability after a few subcultures [11]. On the other hand, mucus neck cells are proliferative stem cells which also secrete mucus.

Effect of A23187. Figure 1 shows the effect of A23187 concentration on the dye uptake of cells injured by acidified medium (pH 3.5). Pretreatment with A23187 dose-dependently suppressed the dye uptake caused by the acidified medium. Before the injury caused by the acidified medium, there was little difference of dye uptake between control cells (9.1 ± 0.3 nmol/mg protein, mean ± SE for three dishes) and A23187 (5 μM)-treated cells (10.7 ± 0.3 nmol/mg protein, mean ± SE for three dishes). The time course of cell damage caused by treatment with the acidified medium is shown in Fig. 2. The progressive increase in dye uptake caused by prolonged treatment with the acidified medium was retarded in the cells pretreated with A23187. Figure 3 shows that the effect of A23187 against cell injury appeared 4 hr after its addition; it was not found at 2 hr. Once the protective effect of A23187 was

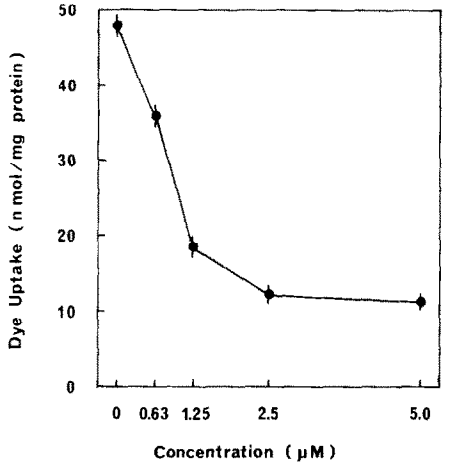

Fig. 1. Effect of A23187 concentration on dye (erythrosine B) uptake of cells injured by acidified medium in cultured gastric mucosal cells. Cells were cultured with A23187 for 24 hr. After removal of medium and washing with PBS, the cell layers were treated for 25 min in acidified medium (pH 3.5) and then the dye exclusion test was performed. Values are means ± SE (N = 4).

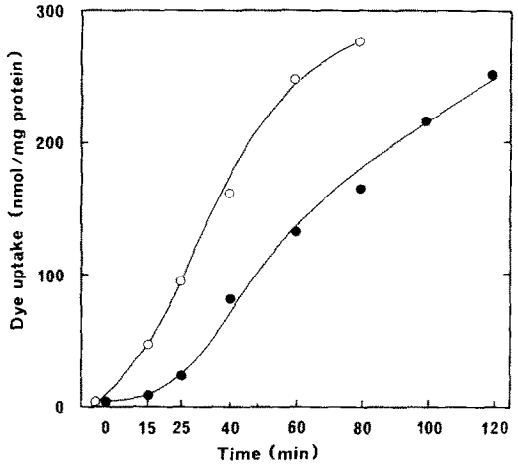

Fig. 2. Protective effect of A23187 in cells exposed over various times to acidified medium. Cells were cultured with 5 μM A23187 for 24 hr. After removal of medium and washing with PBS, the cell layers were treated for the indicated periods in acidified medium (pH 3.5) and then the dye exclusion test was performed. Key: (○) control; and (●) A23187. Values are averages for two dishes.

attained, it was sustained for at least 6 hr after removal of A23187 from the medium.

The effect of pretreatment with A23187 on cell injury caused by 8 mM aspirin (pH 5.0) is shown in Fig. 4. Aspirin increased the dye uptake by the control cells, but this increase was suppressed markedly by the A23187 pretreatment.

Figure 5 shows the effect of Ca^{2+} concentration on A23187-induced protection against cell injury. The cells were cultured for 24 hr, in the presence or absence of A23187, in calcium–EGTA buffered Eagle's MEM with Ca^{2+} concentration from 8 nM through 1 μM. The cells markedly shrank and became partly detached from the dish during culture with 8 nM Ca^{2+}; their resistance to injury was not

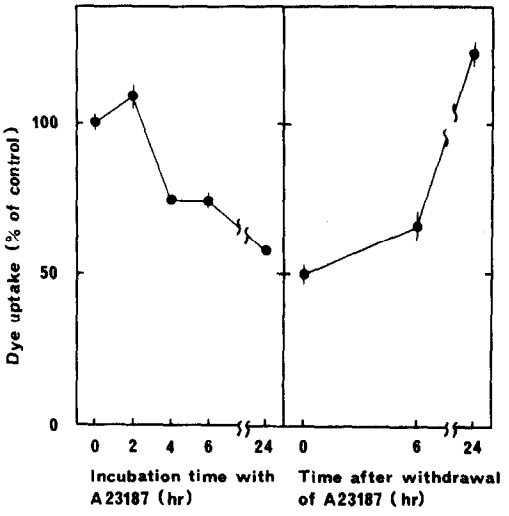

Fig. 3. Time course of effect of A23187 on dye uptake of cells injured by acidified medium in cultured gastric mucosal cells. Cells were cultured for indicate periods after addition or withdrawal of 5 μM A23187. After removal of medium and washing with PBS, the cell layers were treated with acidified medium for 25 min and then the dye exclusion test was performed. The dye uptake of control cells was 83.8 ± 3.7 nmol/mg protein. Values are means ± SE (N = 3 or 4).

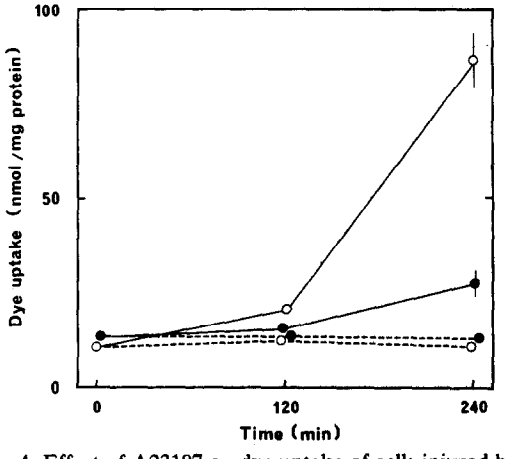

Fig. 4. Effect of A23187 on dye uptake of cells injured by aspirin in cultured gastric mucosal cells. Cells were cultured with 5 μM A23187 for 24 hr. After removal of medium and washing with PBS, the cell layers were treated with 8 mM aspirin for the indicated period and then the dye exclusion test was performed. Key: (○) control; (●) A23187; (- - -) treated with control medium (pH 5.0); and (——) treated with 8 mM aspirin (pH 5.0). Values are means ± SE (N = 3).

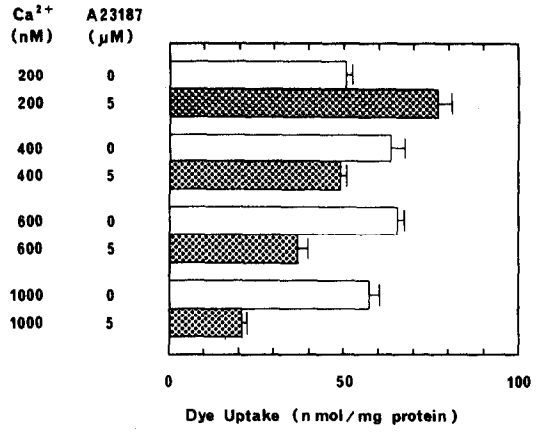

Fig. 5. Effect of Ca^{2+} concentration on A23187-induced protection against cell injury. Cells were cultured for 24 hr, in the presence or absence of A23187, in calcium–EGTA buffered medium with Ca^{2+} concentration from 200 through 1000 nM. After removal of medium and washing with PBS, the cell layers were treated with acidified medium (pH 3.5) for 25 min and the dye exclusion test was performed. Values are means ± SE (N = 3).

Table 1. Effects of pretreatment with A23187, W-7 and trifluoperazine (TFP) on cell injury caused by acidified medium

Addition to medium	Dye uptake (% of control)
None	100.0 ± 3.2
A23187 5 μM	33.5 ± 5.8
W-7 10 μM	125.0 ± 5.4
W-7 10 μM + A23187 5 μM	35.5 ± 4.4
W-7 25 μM	153.1 ± 6.8
W-7 25 μM + A23187 5 μM	41.9 ± 2.4
W-7 50 μM	189.9 ± 1.1
W-7 50 μM + A23187 5 μM	50.0 ± 6.0
None	99.9 ± 4.8
A23187 5 μM	50.7 ± 3.8
TFP 20 μM	167.1 ± 4.7
TFP 20 μM + A23187 5 μM	77.7 ± 2.7
TFP 30 μM	214.0 ± 13.3
TFP 30 μM + A23187 5 μM	118.1 ± 8.9

Cells were pretreated with calmodulin inhibitors for 30 min. Next, 5 μM A23187 was added to the medium and the cells were cultured for an additional 24 hr. After removal of the medium and washing with PBS, the cell layers were treated with acidified medium (pH 3.5) for 25 min and then the dye exclusion test was performed. Absolute values for the controls were, respectively, 92.7 ± 3.0 and 65.4 ± 2.1 nmol/mg protein. Values are means ± SE (N = 4).

assayed. The protective effect of A23187 against cell injury was not observed at a Ca^{2+} concentration of 200 nM but was at 400 nM and above with increasing Ca^{2+} concentration.

Effects of calmodulin inhibitors. A23187 seems to exert its protective effect on cell injury via an increase in intracellular Ca^{2+}. The effects of calmodulin inhibitors on the A23187-induced protection against cell injury were examined so as to obtain a clue to the function of Ca^{2+}. Pretreatment with calmodulin inhibitors dose-dependently facilitated the dye uptake caused by the acidified medium (Table 1). A23187 weakened their effects. When high concentrations of calmodulin inhibitors (W-7, 100 μM; trifluoperazine, 50 μM) were used, the cells shrank and became partly detached from the surface of the dishes during incubation with inhibitors for 24 hr. Addition of A23187 did not prevent these severe effects of calmodulin inhibitors. An increase in intracellular Ca^{2+} also shows its effect via activation of protein kinase C [12]. Because a specific inhibitor of

protein kinase C was not available, the effect of 1-oleoyl-2-acetylglycerol, an activator of protein kinase C [13], was examined to test the involvement of activation of the kinase in the resistance to cell injury, but no protective effect was found (data not shown).

DISCUSSION

The calcium ionophore A23187 has been used to demonstrate Ca^{2+}-dependent processes in various cells. On the other hand, A23187 induces cell injury in cultured hepatocytes [14, 15]. Increased intracellular Ca^{2+} has been proposed to mediate the toxic injury induced by A23187, based upon the fact that the toxic effect of A23187 depends on extracellular Ca^{2+}. However, a study has shown that cell injury is not observed in hepatocytes incubated with intracellular Ca^{2+} and a low concentration of A23187 (2 μM), though this treatment markedly increases the total intracellular calcium content [16]. In cultured gastric mucosal cells, 5 μM A23187 did not influence cell viability, as described in Results.

Coexistence of A23187 potentiates toxic injury induced by CCl_4 in cultured hepatocytes [17]. In this experiment, A23187 was used in order to increase intracellular Ca^{2+} and to find out whether increased intracellular Ca^{2+} induces protective mechanisms against cell injury. The culture medium containing A23187 was thus discarded, and the cell layers were washed before treatment with cell-injuring agents. Pretreatment of gastric mucosal cells with A23187 diminished cell injury caused by the acidified medium (pH 3.5) (Figs. 1 and 2) and also markedly suppressed cell injury induced by aspirin, an agent erosive to gastric mucosa (Fig. 4). As shown in Fig. 3, induction of cell resistance against injury was observed 4 hr after addition of the ionophore, and the decay of cell resistance after its removal took time. Recently, a protective effect of A23187 on cell damage has been reported in the induction of resistance against hyperthermia (45°) in Chinese hamster ovary HA-1 fibroblasts [18]. These fibroblasts are also exposed to A23187 for 4 hr to induce the thermal resistance and longer survival decays of up to 24 hr after removal of the ionophore. Thus, A23187 induces similar protective mechanisms against cell injury both in rat gastric mucosal cells and in Chinese hamster ovary fibroblasts. In the gastric mucosal cells, the protective effect of A23187 on cellular injury was abolished at a low concentration of Ca^{2+} (200 nM) and was dose-dependently shown at above 400 nM (Fig. 5). A23187-induced thermotolerance in HA-1 cells also diminishes markedly in calcium-free medium [18]. The ionophore-induced resistance against cell injury depends on the extracellular calcium and seems to be brought about by increased intracellular Ca^{2+}.

Pretreatment with calmodulin inhibitors W-7 and trifluoperazine reduced the resistance against cell injury (Table 1). The effect of trifluoperazine on cellular resistance was more potent than that of W-7, in agreement with the fact that the affinity of trifluoperazine for calmodulin is higher than that of W-7 [19]. Calmodulin may be important for the resistance against cell injury.

When A23187 was added with the calmodulin inhibitors, the ionophore diminished the reduction of cellular resistance induced by calmodulin inhibitors. These inhibitors bind to calmodulin in a calcium-dependent fashion [20, 21]. Therefore, increased intracellular Ca^{2+} may not reduce the binding of calmodulin to its inhibitors. It seems to intensify the resistance against cell injury by acting on sites other than calmodulin.

Recent work in cultured Chinese hamster fibroblasts has shown that the induction of glucose-regulated proteins by A23187 requires its continuous presence for over 2 hr [22]. This induction is reduced by calmodulin inhibitor W-7 and is not related to activation of protein kinase C [22]. The appearance of A23187-induced resistance against cell injury in cultured gastric mucosal cells required a relatively long period (over 2 hr), and the resistance to cell injury was diminished by calmodulin inhibitors and was not intensified by activation of protein kinase C. Though the physiological roles of glucose-regulated proteins are still unknown, these proteins may be involved in cellular resistance to injury.

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