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MODULATION OF HUMAN COLONIC T_{84} CELL SECRETION BY HYDROGEN PEROXIDE

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Abstract—Hydrogen peroxide (H_2O_2) is a reactive oxygen species that can be produced in the digestive tract by inflammatory cells or during reperfusion following ischemia. To evaluate a possible direct effect of H_2O_2 on epithelial secretory cells, well-differentiated colonic T_{84} cells were grown to confluence on permeable membranes and studied in Ussing chambers. In this model, where the measured short-circuit current (Isc) reflects electrogenic secretion, we observed that H_2O_2 stimulated a concentration-dependent and transient secretory response: 5.5 mM H_2O_2 produced a peak Isc of 12.4 $\mu A/cm^2$ after 4 min, 2.2 mM H_2O_2 a peak Isc of 7.9 $\mu A/cm^2$ after 4 min, and 1.1 mM H_2O_2 a peak Isc of 5.5 $\mu A/cm^2$ after 16 min ($N = 5$). When 97 experiments using 5.5 mM H_2O_2 were reviewed, the mean peak Isc response was 8.9 ± 0.5 $\mu A/cm^2$. A similar secretory response was elicited whether H_2O_2 was added to the serosal, to the mucosal, or simultaneously to both sides of the T_{84} cell monolayer. This secretory response reflected transcellular chloride secretion because it was inhibited by the depletion of chloride in the medium and by the suppression of the $Na^+,K^+,2Cl^-$ co-transporter activity necessary for the chloride gradient driving chloride secretion. When T_{84} cell monolayer resistance was studied, 5.5 mM H_2O_2 produced a transient decrease in resistance, reflecting transcellular chloride secretion, and a gradual decline in resistance (75% of the initial value after 55 min). The secretory response to H_2O_2 was increased 2-fold in T_{84} cells maximally stimulated with 10 nM vasoactive intestinal peptide (VIP), a neuropeptide which acts via cAMP, demonstrating synergism between the two agents. In contrast, the secretory responses produced by H_2O_2 and carbachol, which acts through the Ca^{2+} pathway, were additive. A late inhibitory effect of H_2O_2 was also observed: in cells previously treated with 5.5 mM H_2O_2, the subsequent secretory responses to either VIP or carbachol were partially inhibited. These secretory effects were specific for the oxidant properties of H_2O_2 because they were inhibited by 450 U/mL catalase and by 5 mM dithiothreitol, but were unaffected by 50 μM deferoxamine B or Fe^{3+}. H_2O_2 may be a potential modulator of intestinal or colonic secretion in certain pathologic conditions such as inflammation or ischemia–reperfusion.

Key words: reactive oxygen species, inflammation, hydrogen peroxide, T_{84} cell, colonic and intestinal secretion, Ussing chamber

In the digestive system, reactive oxygen species (ROS)‡ can be produced naturally by different oxidases in the intestine and liver. They can also be produced pathologically in inflammation, post-ischemic reperfusion, and by ionizing radiation [1–4]. Because these pathologic conditions are often accompanied by diarrhea, the possibility that ROS may directly stimulate colonic secretion was addressed. Compared with the superoxide anion and the hydroxyl radical, hydrogen peroxide (H_2O_2) is an ROS that reacts slowly with organic substrates, has a long half-life, and can diffuse large distances in biological systems. It is therefore the most likely ROS to have a biologic effect and, accordingly, was the compound selected for our studies.

The secretory potential of H_2O_2 was evaluated using the well-differentiated and polarized human colonic adenocarcinoma T_{84} cell line. Because these cells develop intercellular tight junctions, when cultured as confluent monolayers on permeable membranes, they will demonstrate a high electrical resistance between the serosal (or basal) and mucosal (or apical) surfaces of the monolayer [5]. T_{84} cell monolayers also exhibit serosal-to-mucosal chloride transport after exposure to a variety of neurohormonal stimuli acting via cAMP (e.g. vasoactive intestinal peptide (VIP) or prostaglandin E_1 [6, 7]), cGMP (e.g. *Escherichia coli* heat stable enterotoxin [8]), or cytosolic Ca^{2+} (e.g. carbachol, histamine, and calcium ionophores [9, 10]). This chloride transport occurs through activated chloride channels located on the apical membrane of the T_{84} cell and is dependent on the chloride gradient generated between the intracellular and extracellular spaces by the $Na^+,K^+,2Cl^-$ co-transporter located on the basal surface of the cell. When T_{84} monolayers are studied in Ussing chambers, because of the associated high electrical resistance, the electrogenic chloride secretion can be indirectly measured by determining the short-circuit current (Isc) necessary to nullify the difference in potential across the monolayer. Because the secretory response in T_{84} cells is well characterized and reflects the direct action of different secretagogues on a well-defined and uniform

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‡ Abbreviations: Isc, short-circuit current; ROS, reactive oxygen species; VIP, vasoactive intestinal peptide; and LDH, lactate dehydrogenase.

population of cells, we chose this model to characterize the direct effects of H_2O_2 on colonic secretion.

MATERIALS AND METHODS

Chemicals

H_2O_2, superoxide dismutase (from bovine erythrocytes), bumetanide, deferoxamine B, barium chloride, carbachol, trypan blue, Fe (III)-EDTA, NADH, and pyruvic acid were purchased from Sigma (St. Louis, MO). Catalase (from beef liver) was obtained from either Sigma or Boehringer Mannheim (Indianapolis, IN). VIP was from Peninsula (Belmont, CA). Culture medium was obtained from the Tissue Culture Facility of the University of North Carolina (Chapel Hill, NC) or from Gibco (Grand Island, NY).

Growth and maintenance of T_{84} cells

T_{84} cells (passes 30–60) were provided by Jonathan A. Cohn (Division of Gastroenterology, Duke University). These cells were cultured at 5% CO_2 and 37° in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 supplemented with 5% (v/v) newborn calf serum. Cells were seeded on collagen-coated Nuclepore filters previously glued onto plastic rings (surface area 2.9 cm^2, approximately 10^6 cells/filter). These filters were then set on glass beads to allow medium (supplemented with 5000 U/L penicillin and 5000 μg/L streptomycin sulfate) to bathe both sides of the cell monolayer. The confluent monolayers used for secretory studies were maintained for at least 7 days after seeding.

Secretory studies [11]

These monolayers were mounted in modified Ussing chambers and both sides of the monolayer were bathed in Ringer's solution (115 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.4 mM KH_2PO_4, 2.5 mM K_2HPO_4, 25 mM $NaHCO_3$, and 10 mM glucose) warmed to 37° with a circulating water jacket and gently mixed and aerated with a constant inflow of 95% air/5% CO_2. During secretory studies, spontaneous tissue potential differences were short-circuited by an automatic voltage clamp (model DVC-1000, WPI, Sarasota, FL) with Ag–$AgCl_2$ electrodes, and the corresponding Isc was recorded at 1-min intervals. Instrument calibration was performed prior to each experiment using a filter/ring unit without cells.

H_2O_2 was diluted with water prior to each experiment from a 30% (w/w) stock solution to produce a 100-fold concentrated solution. This solution was then added to the Ussing chamber compartments at a 1:100 dilution to obtain the desired final concentration. In control experiments, addition of water at a 1:100 dilution did not affect the basal Isc of the T_{84} monolayer.

Studies of cellular injury

Trypan blue exclusion. For each experiment, two confluent T_{84} cell monolayers grown on permeable membranes were each exposed to either Ringer's solution or Ringer's solution containing 5.5 mM H_2O_2 for 90 min at 37° and 5% CO_2. At the end of the incubation period, the solution was removed, and the cell monolayer was overlaid with a 1:4 dilution of trypan blue and inspected using an inverted microscope at 200-fold magnification. The number of cells that were unable to exclude trypan blue was then determined by two independent observers, each sampling three microscopic fields. To calculate the percentage of cells unable to exclude trypan blue, the number of cells examined was estimated using a confluent cell density of ~10,000 cells/mm^2 (obtained by counting trypsinized cells from a matching confluent monolayer) and a microscopic field of 0.785 mm^2 (determined by microscopic examination of known spacings).

Lactate dehydrogenase (LDH) leakage [12, 13]. For each experiment, two confluent T_{84} cell monolayers grown on permeable membranes were each exposed to either Ringer's solution or Ringer's solution containing 5.5 mM H_2O_2 for 90 min at 37° and 5% CO_2. At the end of the incubation period, the overlying medium was removed and the cells were lysed with 4 mL of 1% (w/v) Triton X-100 in Ringer's solution. Enzymatic LDH activity released into the medium and contained in the subsequent cell lysate was assayed using the method of Bergmeyer and Bernt [14]. The percentage of cellular LDH released into the medium was calculated as *LDH in medium/(LDH in medium + LDH in cell lysate)*, and the average obtained for the two control and the two treated monolayers was determined.

T_{84} monolayer resistance. For studies of monolayer resistance, T_{84} cells were grown to confluence on permeable membranes and mounted in Ussing chambers as previously described. At 1-min intervals, a constant current of 100 μA was maintained across the monolayer and the resulting voltage recorded. The corresponding resistance was calculated using Ohm's law.

Statistical analysis

Unless mentioned otherwise, the values given are means ± SEM of the indicated number of observations. All comparative studies used matched monolayers seeded at the same time and studied either concurrently or on the same day. Unless otherwise noted, statistical significance was determined using both the two-tailed paired Student's *t*-test and the unpaired nonparametric Mann–Whitney U test.

RESULTS

Stimulation of T_{84} electrogenic secretion by H_2O_2

In these comparative experiments, matched T_{84} monolayers were exposed to different concentrations of hydrogen peroxide and the resulting electrogenic secretion studies in Ussing chambers. After 5 min of baseline recording, H_2O_2 was added to both sides of the monolayer to a final concentration of 1.1, 2.2, and 5.5 mM and the Isc recorded every minute for another 55 min. As illustrated in Fig. 1A, H_2O_2 stimulated a transient increase in the Isc that was concentration dependent: 5.5 mM produced the largest secretion with a peak Isc change of 12.4 ± 2.9 μA/cm^2 after 4 min, 1.1 mM produced the smallest secretion with a broad and delayed Isc

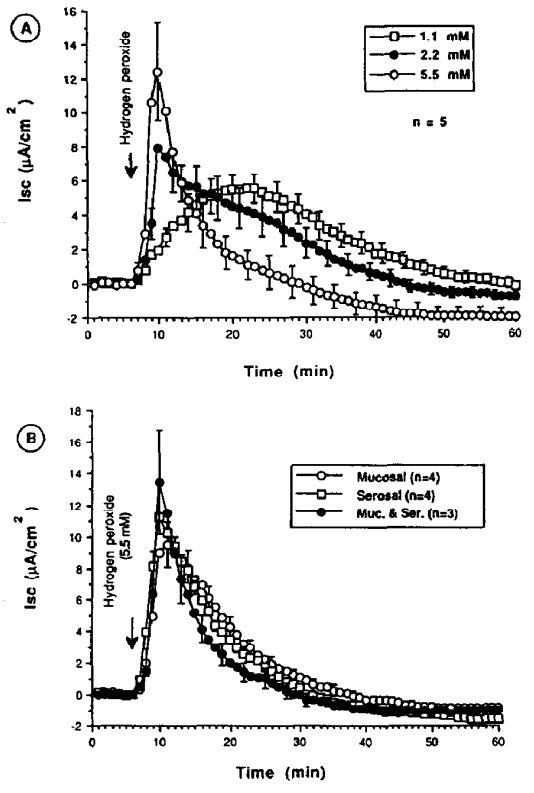

Fig. 1. Characteristics of H_2O_2-stimulated T_{84} secretion. T_{84} monolayers were prepared and studied in Ussing chambers as described in Materials and Methods. In panel A, after 5 min of baseline recording, H_2O_2 was added to both the mucosal and serosal sides of the monolayer to produce final concentrations of 1.1 mM (□), 2.2 mM (●), and 5.5 mM (○). The Isc change was recorded every minute, and the means and SEM derived from five experiments, each studying three monolayers seeded at the same time and studied on the same day, are shown. In panel B, after 5 min of baseline recording, H_2O_2 was added to either the mucosal (○), the serosal (□), or simultaneously to both sides (●) of the monolayer at a final concentration of 5.5 mM. The Isc was recorded every minute, and the means and SEM derived from four experiments are shown (three experiments for simultaneous mucosal and serosal addition).

peak of 5.5 ± 0.8 μA/cm² after 16 min, and 2.2 mM produced an intermediate response with a peak Isc of 7.9 ± 2.6 μA/cm² (N = 5). The difference in the amplitude of the secretory responses to 5.5 and 1.1 mM H_2O_2 was significant by both the paired *t*-test (P = 0.038) and the Mann–Whitney U test (P = 0.048), whereas the difference between the secretory responses to 5.5 and 2.2 mM H_2O_2 was only statistically significant with the paired *t*-test (P = 0.0052).

To estimate more accurately the amplitude of these responses, all experiments where 5.5 or 1.1 mM H_2O_2 was added to both sides of the monolayer were reviewed: the mean peak Isc change obtained with 97 experiments using 5.5 mM was 8.9 ± 0.5 μA/cm², while the mean peak response obtained with 11 experiments using 1.1 mM was 4.0 ± 0.8 μA/cm². The difference in the secretory responses to 5.5 and 1.1 mM H_2O_2 was again significant by both the unpaired *t*-test (P = 0.0033) and the Mann–Whitney U test (P < 0.003).

The secretory responses to H_2O_2, added to either the serosal, the mucosal, or to both sides of the T84 monolayer, were next compared. As shown in Fig. 1B, these secretory responses were equivalent.

Identification of H_2O_2-stimulated secretion as Cl^- transport

In T_{84} cells, the change in the Isc stimulated by different known secretory agents has usually reflected the transepithelial transport of Cl^-. To evaluate whether H_2O_2 also activated the same process, we studied the effect of the depletion of Cl^- in the medium on H_2O_2-stimulated secretion. This was achieved by substituting 57.5 mM Na_2SO_4 for the 115 mM NaCl in the Ringer's medium. As shown in Fig. 2A, compared with control studies using unsubstituted medium, this manipulation inhibited the Isc change stimulated by H_2O_2 by 95%, from a peak Isc of 16.5 ± 3.1 μA/cm² to 0.7 ± 0.6 μA/cm² (N = 4, P = 0.007 by the *t*-test with 3 df, P = 0.021 by the Mann–Whitney U test). Similarly, when sodium gluconate or sodium isethionate was substituted for NaCl in the medium, the peak control secretion stimulated by 5.5 mM H_2O_2 was inhibited, respectively by 73% (N = 4, P = 0.006 by the *t*-test with 3 df, P = 0.021 by the Mann–Whitney U test) and by 94% (N = 3, P = 0.002 by the *t*-test with 2 df, P = 0.049 by the Mann–Whitney U test).

Because the transepithelial transport of Cl^- is dependent on the cellular chloride gradient produced by the Na^+,K^+,$2Cl^-$ co-transporter, we also examined the effect of bumetanide, an inhibitor of this co-transporter. As shown in Fig. 2B, a 15-min preincubation with 0.3 mM bumetanide inhibited the secretory response to H_2O_2 by 55%, from a mean peak Isc in control monolayers of 7.2 ± 0.2 μA/cm² to a mean peak Isc of 3.3 ± 0.9 μA/cm² in treated monolayers (N = 4, P = 0.034 by the *t*-test with 3 df, P = 0.021 by the Mann–Whitney U test). Of note, when bumetanide was used to inhibit the sustained secretory response elicited by agents acting via cAMP, such as pituitary adenylate cyclase activating polypeptide, the maximal inhibitory effect was only reached 20 min after the stimulation of secretion [15]. Therefore, the effect of bumetanide on the peak secretory response to hydrogen peroxide, which occurs 4 min after the addition of the secretagogue, may not reflect the full inhibitory potential of this agent.

Studies of possible cellular toxicity produced by H_2O_2

Trypan blue exclusion. To evaluate for the potential toxicity of hydrogen peroxide on T_{84} cells, both sides of confluent monolayers grown on permeable membranes were exposed to 5.5 mM H_2O_2 in Ringer's solution for 90 min at 37° and 5% CO_2. At the end of the incubation period, the solution was removed, the cell monolayer was overlaid with a 1:4 dilution of trypan blue and then inspected using an inverted microscope at 200-fold magnification. For both untreated and hydrogen

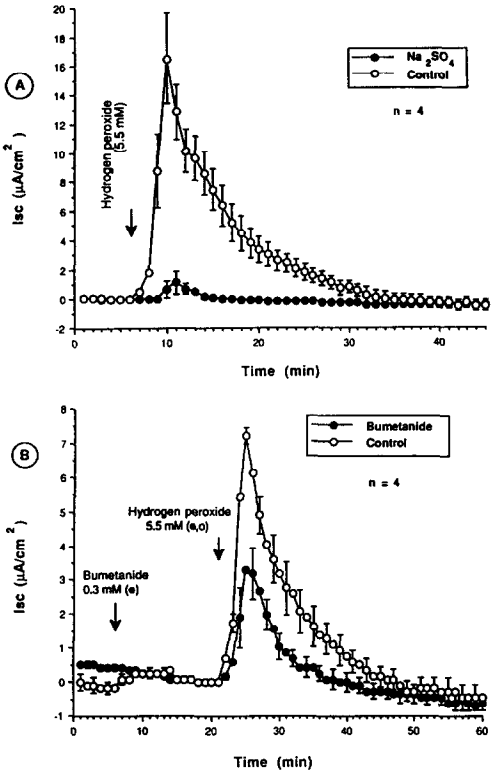

Fig. 2. Identification of H_2O_2-stimulated T_{84} cell secretion as transcellular Cl^- transport. T_{84} monolayers were prepared and studied in Ussing chambers as described in Materials and Methods. For each experiment, two monolayers seeded at the same time were studied simultaneously. In panel A, one monolayer was bathed in normal Ringer's solution (○), while the other monolayer was bathed in Ringer's solution in which Na_2SO_4 was substituted for NaCl (●). After 5 min of baseline recording, H_2O_2 was added to both sides of the T_{84} cell monolayer at a final concentration of 5.5 mM, and the Isc was recorded every minute. The means and SEM from four experiments are shown. In panel B, for each experiment, two monolayers seeded at the same time were studied simultaneously. After 5 min of baseline recording, bumetanide was added to the serosal side of one monolayer at a final concentration of 0.3 mM (●). After an additional 15 min, H_2O_2 was added to both sides of both monolayers at a final concentration of 5.5 mM. The means and SEM from four experiments are shown. Because the bumetanide was dissolved in 0.1 M NaOH, the serosal sides of the bumetanide-treated and control monolayers had a final concentration of 1 mM NaOH.

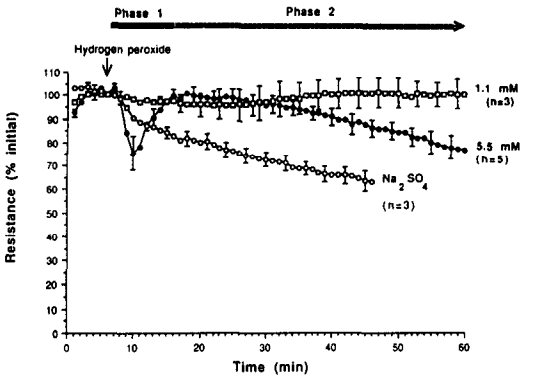

Fig. 3. Effect of H_2O_2 on T_{84} monolayer electrical resistance. T_{84} monolayers were prepared and mounted in Ussing chambers as described in Materials and Methods. After 5 min of baseline recording, hydrogen peroxide was added to both sides of the monolayer at a final concentration of 1.1 mM (□) or 5.5 mM (●, ○). In some monolayers (○), Na_2SO_4 was substituted for NaCl in the Ringer's medium (chloride-depleted medium). At 1-min intervals, a constant current of 100 μA was maintained across the monolayer and the corresponding voltage was recorded. The resistance was calculated using Ohm's law and expressed as a percentage of the initial resistance. The initial resistance values for the experiments involving 5.5 mM H_2O_2, 5.5 mM H_2O_2 in chloride-depleted medium, and 1.1 mM H_2O_2 were, respectively, 1730 ± 120, 2500 ± 340 and $1450 \pm 230\ \Omega \cdot cm^2$. The means and SEM from three (□, ○) and five (●) experiments are shown.

peroxide-treated monolayers, in each microscopic field, 1–3 cells, or <0.05% of the cells examined, were unable to exclude trypan blue (three experiments done in duplicate). Therefore, no cellular toxicity to H_2O_2 was detected using this method.

LDH leakage. LDH leakage by damaged cells was also used to evaluate the potential cellular toxicity produced by H_2O_2 [12, 13]. Confluent T_{84} monolayers, grown to confluence on permeable membranes, were exposed to 5.5 mM H_2O_2 in Ringer's solution for 90 min at 37° and 5% CO_2. At the end of the incubation period, the overlying medium was removed and the cells were lysed with 4 mL of 1% (w/v) Triton X-100 in Ringer's solution. Enzymatic LDH activity released into the overlying medium and contained in the cell lysate was assayed. The percentage of LDH leaked into the medium [LDH in medium/(LDH in medium + LDH in cell lysate)] was $1.6 \pm 0.5\%$ for control cells and $1.4 \pm 0.1\%$ for hydrogen peroxide-treated cells (N = 3, in duplicate). Again no significant cellular toxicity to H_2O_2 was detected using this method.

T_{84} monolayer resistance. More subtle effects of hydrogen peroxide on T_{84} cellular biology, such as those related to monolayer resistance, were also evaluated. Confluent T_{84} cell monolayers on permeable membranes were mounted in Ussing chambers and exposed to 5.5 mM H_2O_2. At 1-min intervals, a constant current of 100 μA was maintained across the monolayer, the corresponding voltage recorded, and the resistance calculated using Ohm's law.

As shown in Fig. 3, hydrogen peroxide produced two different types of changes in monolayer resistance. In phase 1, a transient decrease in cellular resistance occurred from 2 to 10 min after the addition of 5.5 mM H_2O_2. The transient decrease in resistance was reversible, paralleled Cl^- secretion, and was blocked when Cl^- in the medium was depleted by substituting Na_2SO_4 for NaCl in the medium. These properties suggest that, as previously observed with other secretagogues, this transient decrease in resistance merely reflected increased

Table 1. Effects of different modulators of H_2O_2 metabolism

	Isc (% of control)	N (df)	P (paired two-tailed *t*-test)	P (Mann–Whitney U)
Catalase (450 U/mL)	4.2 ± 4.2	3 (2)	0.002	0.049
Dithiothreitol (5 mM)	25 ± 6.4	5 (4)	0.003	0.009
Deferoxamine B (50 μM)	97 ± 8.4	3 (2)	NS	NS
Iron (50 μM)	101 ± 15	7 (6)	NS	NS

T_{84} monolayers were prepared and studied in Ussing chambers as described in Materials and Methods. For each experiment, two monolayers seeded at the same time were studied simultaneously. After 5 min of baseline recording, the agent studied (catalase, dithiothreitol, deferoxamine B, and iron) was added to both sides of one monolayer. After additional incubation (5 min for catalase, 10 min for dithiothreitol, 15 min for deferoxamine and iron), H_2O_2 was added to both sides of both monolayers at a final concentration of 5.5 mM. The peak Isc changes in the treated cells are expressed as a percentage of the peak Isc change in the control untreated cells. The mean control Isc changes for the experiments corresponding to catalase, dithiothreitol, deferoxamine, and iron were, respectively, 8.7 ± 2.5, 5.1 ± 1.38, 4.0 ± 0.8 and 8.0 ± 2.0 μA/cm². The means ± SEM, number of experiments, degrees of freedom, and P values are shown.

cellular plasma membrane ion conductances resulting from the activation by H_2O_2 of the apical Cl^- channel, the basal Na^+,K^+,$2Cl^-$ co-transporter, and the basal K^+ efflux channels [8, 16]. In addition, as displayed in phase 2, H_2O_2 also produced a late gradual decrease in monolayer resistance: 55 min after addition of 5.5 mM H_2O_2, the monolayer resistance was 74 ± 6% (N = 5) of the initial baseline value. Because this change was also apparent with monolayers studied in chloride-depleted medium, it probably reflected an additional effect of hydrogen peroxide on T_{84} cells which was distinct from chloride secretion. When the concentration of H_2O_2 was decreased to 1.1 mM, the effect of monolayer resistance was reduced markedly.

Specificity of H_2O_2-stimulated secretion

Inhibition by catalase and dithiothreitol. To establish that the demonstrated chloride secretion was dependent on the presence of H_2O_2, the effect of catalase, an enzyme that converts H_2O_2 to water, was studied. As shown in Table 1, when the cells were preincubated with 450 U/mL catalase for 5 min, the Isc change produced by H_2O_2 in control monolayers was decreased by 96% (N = 3, P = 0.002 by the *t*-test with 2 df, P = 0.049 by the Mann–Whitney U test), confirming the specificity of the secretory effect for hydrogen peroxide.

The dependence of the secretory effects of H_2O_2 on the oxidative properties of this compound was next evaluated using the reducing agent dithiothreitol. As shown in Table 1, preincubation of the cells with 5 mM dithiothreitol for 10 min also inhibited the peak control secretion stimulated by 5.5 mM H_2O_2 by 75% (N = 5, P = 0.003 by the *t*-test with 4 df, P = 0.009 by the Mann–Whitney U test). This inhibition is unlikely to result from non-specific toxicity of dithiothreitol on T_{84} cells because preincubation with 5 mM dithiothreitol in the same manner did not inhibit the secretory effect of VIP (98 ± 20% control response, N = 3).

Lack of effect of iron and deferoxamine B. Hydrogen peroxide can be converted into the hydroxyl radical through the Haber–Weiss reaction. Because this reaction is dependent on iron, we evaluated the possibility that the hydroxyl radical may mediate the secretory action of H_2O_2 by studying the effect of either 50 μM iron or of the iron chelator, deferoxamine B. These agents should have opposite effects on the conversion of H_2O_2 to the hydroxyl radical. As shown in Table 1, preincubation with deferoxamine B or iron had no significant effect on H_2O_2-stimulated secretion, producing, respectively 97% (N = 3) and 101% (N = 7) of the maximal response obtained in matched controls. The secretion stimulated by H_2O_2 is, therefore, independent of the conversion of this oxidant to the hydroxyl radical.

Interaction between H_2O_2, VIP, and carbachol

Synergism between H_2O_2 and VIP. To evaluate the interaction between H_2O_2 and VIP, in each matched pair of T_{84} monolayers (seeded at the same time and studied simultaneously), 10 nM VIP was initially added to the serosal side of one monolayer and 5.5 mM H_2O_2 was initially added to both sides of the other monolayer. After an additional 15 min, 5.5 mM H_2O_2 was added to the monolayer that previously received 10 nM VIP and vice versa. As shown in Fig. 4A, 5.5 mM H_2O_2 by itself stimulated a secretion that was 29 ± 4% (N = 3) of the secretion stimulated by 10 nM VIP. In contrast, when the same concentration of H_2O_2 was added to cells that have been stimulated previously with 10 nM VIP, the additional increase was equivalent to 66% of the maximal VIP response (from 99 ± 1 to 165 ± 11%). Synergism between VIP and H_2O_2 was, therefore, demonstrated because H_2O_2 produced a larger secretory response in cells stimulated with VIP (N = 3, P = 0.034 by the *t*-test with 2 df, or P = 0.049 by the Mann–Whitney U test).

This potentiated Isc increase was followed, however, by a progressive Isc decline so that baseline values were obtained after 25 min, suggesting a late inhibitory effect of H_2O_2. This late inhibitory effect was also demonstrated when 10 nM VIP was added to cells previously treated with H_2O_2. In this case,

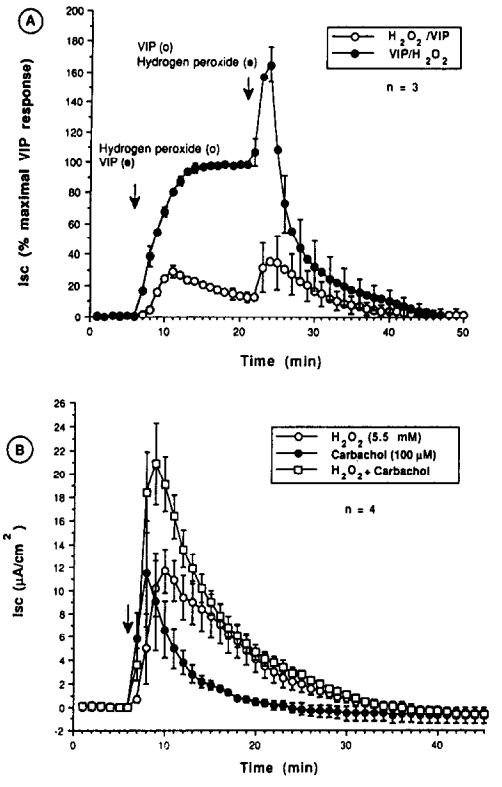

Fig. 4. Interaction between H_2O_2 and VIP (panel A) and carbachol (panel B). T_{84} monolayers were prepared and mounted in Ussing chambers as described in Materials and Methods. In panel A, for each experiment, two monolayers seeded at the same time were studied simultaneously. After 5 min of baseline recording, 5.5 mM H_2O_2 was added to both sides of one monolayer (○) while 10 nM VIP was added to the serosal side of the other monolayer (●). After another 15 min, H_2O_2 was added to the monolayer that received VIP and vice versa. The Isc was recorded every minute and expressed as a percentage of the maximal Isc change obtained with 10 nM VIP (the mean maximal Isc change obtained with VIP was $19.5 \pm 3.9\ \mu A/cm^2$). The means and SEM from three experiments are shown. In panel B, for each experiment, three monolayers seeded at the same time were studied on the same day. After 5 min of baseline recording, either 5.5 mM H_2O_2 (○), 100 μM carbachol (●), or 5.5 mM H_2O_2 plus 100 μM carbachol (□) were added. H_2O_2 was added to both sides of the monolayer, while carbachol was added to the serosal side of the monolayer. The Isc was recorded every minute, and the means and SEM from four experiments are shown.

the additional Isc increase was only 23% of the maximal response obtained with VIP alone (from 12 ± 4 to $35 \pm 18\%$).

In separate experiments, when forskolin, another agent that acts via increased production of cAMP, was substituted for VIP, similar responses were obtained (data not shown).

Additive effects of H_2O_2 *and carbachol.* Because the secretory response stimulated by carbachol was transient, lasting only ~8 min, We studied the interaction between H_2O_2 and carbachol by comparing the secretory responses produced by H_2O_2 and carbachol, added either alone or in combination. As shown in Fig. 4B, in these experiments, 100 μM carbachol added to the serosal side of the monolayer produced a secretory response with the peak Isc of $11.5 \pm 4.5\ \mu A/cm^2$ (N = 4) after 2 min, whereas 5.5 mM H_2O_2 added to both sides of the cell monolayer stimulated a secretory response with a peak Isc of $11.7 \pm 1.9\ \mu A/cm^2$ after 4 min. By comparison, the peak Isc produced by the combined addition of H_2O_2 and carbachol was $20.9 \pm 3.5\ \mu A/cm^2$, approximately equivalent to the sum of the individual responses.

DISCUSSION

In summary, we have shown that H_2O_2 at 1–5 mM concentrations stimulated electrogenic secretion by T_{84} cell monolayers. This secretion was inhibited by the depletion of chloride in the medium (obtained by substituting Na_2SO_4, sodium isethionate, or sodium gluconate for NaCl) and by the disruption of the cellular chloride gradient (obtained by inhibiting the $Na^+,K^+,2Cl^-$ co-transporter with bumetanide). These finding suggested that, similar to other known secretagogues, H_2O_2 produced an electrogenic secretion by stimulating active transcellular chloride transport.

The current studies are in agreement with the earlier observation that H_2O_2 stimulated the secretion by rat and rabbit colons mounted in Ussing chambers. However, this secretory effect, which was studied using whole mucosal layers, was postulated to be indirect because it was reduced by cyclooxygenase inhibition (e.g. with piroxicam or indomethacin) and by enteric nervous system blockade (e.g. with tetrodotoxin, hexamethonium, or atropine). It was likely to be mediated by the stimulation of colonic immune or mesenchymal cells by H_2O_2 to release prostaglandins. Prostaglandins would, in turn, trigger the release from the enteric nervous system of the different neurotransmitters, which would interact directly with the epithelial cells to stimulate secretion [17, 18]. In contrast, the use of a uniform population of T_{84} cells in this report establishes that H_2O_2 can also interact directly with colonic epithelial cells to cause secretion.

Because prolonged exposure of intestinal cells to ROS has been shown to be toxic to intestinal cells (e.g. IEC-18 cells [19]) the possibility that this secretion may reflect gross and non-specific cellular toxicity remains to be excluded. When chemical and histologic methods were used to assess for possible H_2O_2-induced cellular injury (extracellular release of LDH and ability of cells to exclude trypan blue), no toxicity was demonstrated. On the other hand, more subtle effects of hydrogen peroxide on cellular metabolism may occur. For example, when T_{84} monolayer resistance was assessed, 5.5 mM H_2O_2 caused two types of changes in resistance. The initial transient decrease in resistance coincided with the electrogenic Cl^- secretion, was absent when medium was depleted of Cl^-, and most likely reflected increased cellular plasma membrane ion conductances associated with the activation of the apical Cl^- channel, the basal $Na^+,K^+,2Cl^-$ co-transporter, and the basal K^+ efflux channels by H_2O_2 [8, 16].

This transient decrease in resistance is, therefore, a manifestation of Cl^- secretion and not the mechanism responsible for it. When epithelial MDCK cells were exposed to H_2O_2, a gradual decrease in monolayer resistance, reflecting an increased paracellular conductance resulting from a separation of the intercellular tight junctions, was demonstrated [20]. A similar phenomenon might produce the late and gradual decrease in T_{84} monolayer resistance observed after the addition of 5.5 mM hydrogen peroxide. This decreased resistance might be partially responsible for the late inhibitory effect produced by H_2O_2 on the electrogenic secretion detected when T_{84} cells were stimulated by VIP (Fig. 4A).

H_2O_2 is a potent oxidant, and, relative to the other ROS (the superoxide anion and the hydroxyl radical), has a longer half-life, reacts slowly with organic substrates, and can diffuse large distances in biological systems. The inhibitions of the secretory effect of H_2O_2 by catalase and by the reducing agent dithiothreitol support the hypothesis that the oxidative properties of H_2O_2 were responsible for its secretory effects. The inability of the modulators of the Haber–Weiss reaction, iron and its chelator, deferoxamine B, to affect significantly the secretory response of H_2O_2 also suggests that it is H_2O_2 rather than the derived hydroxyl radical which stimulated secretion. These findings are consistent with earlier observations made in whole colonic mucosa that H_2O_2 was the most effective ROS in stimulating secretion [18].

Because H_2O_2 can act from either the mucosal or the serosal side of the T_{84} monolayer, it is likely that, due to its small size and lack of charge, it can move easily across plasma membranes to produce its effect intracellularly. This property is different from the recent partially characterized neutrophil-derived secretagogue, 5′-AMP, which becomes metabolized to adenosine on the apical surface of the cell and then interacts with specific adenosine receptors on the serosal surface of the cell [21, 22].

These findings may also be of clinical relevance. Up to 15 μM H_2O_2 can be produced by a suspension of 3×10^5 neutrophils/mL [4], and this production can be further potentiated when neutrophils are adherent to biological surfaces [23]. The inflamed colonic crypts found in ulcerative and granulomatous colitis can contain 2.6×10^7 neutrophils/cm^3 [24], and these activated cells may conceivably generate locally the millimolar concentrations of H_2O_2 used in our studies. Furthermore, the secretory response elicited by hydrogen peroxide may also be potentiated by different endogenous factors, such as VIP, as shown in Fig. 4A. In addition, it has been demonstrated that the small secretory response to 1 mM hydrogen peroxide can also be enhanced markedly by the presence of fibroblasts (either cocultured or acutely juxtaposed) [25]. Along with other agents derived from inflammatory cells, such as monochloramine [13] or the neutrophil-derived secretagogue, 5′-AMP [21, 22], hydrogen peroxide may be a factor in the pathogenesis of the diarrhea associated with inflammatory bowel diseases or reperfusion injury following ischemia.

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