Uncoupling of Phospholipase C from Receptor Regulation of \([\text{Ca}^{2+}]_c\) in T84 Colonic Cells by Prolonged Exposure to Phorbol Dibutyrate*

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The T84 colonic cell line, a cultured Cl⁻ secretory cell, elevates intracellular free \(\text{Ca}^{2+}\) (\([\text{Ca}^{2+}]_c\)) in a concentration-dependent manner when exposed to carbachol or histamine. As determined with a fluorescence microscope imaging system, exposure of T84 cells to 100 \(\mu\)M carbachol or histamine resulted in an immediate \([\text{Ca}^{2+}]_c\) rise of approximately 50–80 nM in all cells. Preincubation of monolayers for 1 h or longer with 0.4 \(\mu\)M phorbol 12,13-dibutyrate (PDB) reduced the number of cells which responded to histamine or carbachol and reduced the magnitude of the increase in the responding cells. This effect reached its maximum after 2 h and persisted for at least 24 h of PDB incubation.

Binding of quinuclidinyl benzilate, a cholinergic receptor agonist, indicated that down-regulation of external receptors was not an explanation for this effect. Examination of phospholipase C activity in T84 cell membranes showed increased basal activity in PDB-treated compared with control cells. Measurement of inositol phosphates generated by intact cells using \(\text{myo-}[\text{H}]\)inositol incorporation or receptor binding assays showed that 2 h of incubation with PDB elevated basal levels of inositol 1,4,5-trisphosphate and prevented any further carbachol-induced generation of inositol trisphosphate. Probably as a consequence, both total cell calcium and \(\text{Ca}^{2+}\) ionophore-releasable calcium were decreased after 2 h of PDB incubation. Membrane-associated protein kinase C activity was elevated after a 2 h exposure to PDB but was below the level of detection after 24 h with PDB. Protein kinase C antagonists neither duplicated nor blocked the uncoupling of carbachol receptors induced by long term treatment with PDB. The results suggest that prolonged PDB incubation caused uncoupling and elevation of phospholipase C activity from cholinergic and histaminergic receptor regulation resulting in increased basal levels of inositol 1,4,5-trisphosphate.

Protein kinase C apparently is not involved directly in the mechanism that leads to these effects.

The T84 human colonic cancer cell line is a model epithelial Cl⁻ secretory cell. Monolayers respond to physiologic secretagogues (Beuerlein et al., 1987; Cartwright et al., 1985; Dharmasathaphorn et al., 1984, 1985, 1989; Madara and Dharmasathaphorn, 1985; Reinlib et al., 1989), including carbachol and histamine, which act through elevation of intracellular free \([\text{Ca}^{2+}]_c\) (Beuerlein et al., 1989). Histamine also shows a biphasic \([\text{Ca}^{2+}]_c\) response although a larger part of the \([\text{Ca}^{2+}]_c\) response appears to be from an extracellular source (Dharmasathaphorn et al., 1989). A role in T84 cell Cl⁻ secretion for protein kinase C, which can be stimulated by phorbol esters, has recently been suggested (Beuerlein et al., 1987; Vongkovit et al., 1989). Since the simultaneous addition of phorbol 12-myristate 13-acetate and \([\text{Ca}^{2+}]_c\)-dependent secretagogues depressed Cl⁻ secretion in T84 cells without affecting the normal rise of \([\text{Ca}^{2+}]_c\), protein kinase C was postulated to inhibit agonist-induced secretion at a step distal to \([\text{Ca}^{2+}]_c\) elevation (Vongkovit et al., 1989).

Prolonged incubation of fibroblasts with phorbol esters has been shown to down-regulate protein kinase C (Issandou and Rozengurt, 1989; Rodriguez-Pena and Rozengurt, 1984). Prolonged phorbol ester exposure has now been applied to T84 cells to investigate the relationships among \([\text{Ca}^{2+}]_c\), phospholipase C, and protein kinase C. In this study the effects of PDB and the role of protein kinase C on \([\text{Ca}^{2+}]_c\) in T84 cells were investigated at the single cell level using Fura-2 and a fluorescence microscope imaging system. In addition, measurements were made of phospholipase C activity and the levels of cytosolic inositol phosphates under the same conditions that \([\text{Ca}^{2+}]_c\) was monitored. Phospholipase C was activated by prolonged PDB incubation while protein kinase C decreased.

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1The abbreviations used are: \([\text{Ca}^{2+}]_c\), intracellular free \(\text{Ca}^{2+}\) concentration; CCCP, carbonyl cyanide \(m\)-chlorophenylhydrazone; EGTA, \(\alpha\)-[ethyleneglycol](oxyethylene)nitritotetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Fura-2 AM, Fura-2 acetoxymethylester; PDB, phorbol 12,13-dibutyrate; InsP₁, inositol triphosphate; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; InsP₄, inositol monophosphate; InsP₅, inositol bisphosphate; InsP₆, inositol tetraphosphate; QNB, quinuclidinyl benzilate.
over a 24-h period. The results demonstrate an uncoupling of phospholipase C from receptor regulation after PDB incubation.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**Cell culture media were purchased from GIBCO. Ins(1,4,5)P_3 was obtained from Amersham Corp., and [H] Ins(1,4,5)P_3, myo-[H]inositol, [H]polyethylene glycol, and [H]quin-ucilindien benzilate (QNB) were from Du Pont-New England Nuclear. Ionomycin was from Calbiochem. Fura-2 and Fura-2 AM were from Molecular Probes, Inc. (Eugene, OR). EDTA (dissodium salt) was from J. T. Baker, Inc. Liquiscint was from National Diagnostics (Mannville, NJ). All other chemicals were from Sigma.

**Cell Culture—**T_{60} cells, used to passage number 69, were cultured on glutaraldehyde-cross-linked rat tail collagen-coated glass cover slips as described (Dharmsathaphorn and Pandol, 1984; Reinlib et al., 1989). Monolayers were maintained in 1:1 Dulbecco-Vogt modified Eagle's and Ham's F-12 media with 15 mM HEPES, pH 7.5, 1.2 g/liter NaHCO_3, 40 mg/liter penicillin, 8 mg/liter streptomycin and 5% newborn calf serum and studied between 4 and 14 days after confluence. The cells were a complete monolayer, as examined by thin histologic sections. In rare areas, cells appeared stratified (Reinlib et al., 1989).

**Fura-2 Loading and Single Cell Analysis—**T_{60} cells were loaded with Fura-2, and [Ca^{2+}] was studied in single cells as described previously (Gryciewicz et al., 1985; Reinlib et al., 1989). Coverslips of cells were incubated at 37 °C for 20 min (for determination of a fluorescence medium containing 10 μM Fura-2 AM followed by a 30-min incubation at 37 °C. Before study, the monolayer was washed three times with 1 ml of Ringer's-HCO_3, supplemented with 10 mM HEPES, pH 7.40, and 10 mM glucose (Buffer A). The coverslip was mounted in a water-tight Dvorak perfusion chamber, with a total volume of 0.25 ml, aimed at 30 °C. The coverslip was visualized through a 63X Zeiss Planapo lens (numerical aperture 1.3), and emitted fluorescence (480–520 nm) was collected via a DAGE 66 SIT camera (DAGE, Indianapolis, IN). The excitation light was controlled by a MicroVax II computer (Perkin-Elmer Co., Marlborough, MA). A computer-generated bandpass filters of 350 and 380 nm and a motorized wheel in front of the 75-watt xenon source. The time required for exposure to a pair of excitation filters and data acquisition was approximately 1 s. Data were determined for 8-frame averaged images at 10-s intervals although some experiments were performed using 1-s, nonaveraged intervals. Data were acquired and stored as the average fluorescence intensity within 7.8-μm² areas within each cell, and calculations were made by a MicroVax II computer with 100 megabytes of memory and IP-512 imaging boards (Imaging Technology, Inc., Woburn, MA). Approximately eight cells/field were measured simultaneously by these experiments with previously defined (Reinlib et al. et al., 1989). Calculations of [Ca^{2+}] were made following Gryciewicz et al. (1985). The K_{0} for Fura-2 was confirmed as 224 nm at 30 °C in the fluorescence microscope. The average R_{o} (FI 350/FI 385) values for 1 μM Fura-2 dye standards in the absence (R_{a}) or presence of [Ca^{2+}] of 10 μM, 0.01 and 7.50 ± 0.47, respectively (n = 10). [Ca^{2+}] changes of less than 10 nM were considered indistinguishable from system noise and calculated as zero change. All values were corrected for background and cellular autofluorescence, measured separately. Cells containing punctate fluorescence were rare and were not studied when found. Thus, as shown previously (Reinlib et al., 1989), the dye did not appear to accumulate subcellularly. In some single cells were studied and the peak [Ca^{2+}] occurred at variable times after secretagogue exposure, the mean peak [Ca^{2+}] in the data tables differed slightly from the peak responses illustrated in the figures.

For fluorometry of whole monolayers, T_{60} cells were seeded onto glass coverslips coated with rat tail collagen. The coverslips were glued to plastic supports with silicone rubber adhesive (General Electric Co., Waterford, NY) and studied 7–15 days after seeding. Cells were loaded with Fura-2 by incubation of the cells at 23 °C for 60 min in “Na medium” containing (in mM) 130 NaCl, 5 KCl, 2 CaCl_2, 10 NaHCO_3, 25 glucose, 20 HEPES, pH 7.4, and radioactivity was determined in a liquid scintillation counter. Nonspecific binding was defined as binding in the absence of excess atropine (1 μM), and specific binding was determined by subtracting nonspecific from total binding. The K_{0} and the maximum density of binding sites (B_{max}) for [H]QNB binding were calculated by nonlinear least-squares regression to the equation of Scatchard HEPES, NaCl, and EDTA (as described above).

**Determination of Total Cell Calcium—**To evaluate the effect of PDB on total cell calcium, plasma emission spectroscopy was carried out on T_{60} monolayers. Monolayers were incubated for 2 h at 37 °C in the presence or absence of 0.4 μM PDB and washed three times in Buffer A and then once in buffer A that contained no added Ca^{2+}.
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(Muscholl, 1979). The cells were then scraped into 2 ml of ice-cold 0.3 mM LaCl₃, 5 mM HCl and drill homogenized 10 times. The suspension was centrifuged at 100,000 × g for 5 min, and the supernatant was frozen in liquid N₂ until the Ca²⁺ determination. In parallel, cells were treated identically except that 0.5 mg/ml [³¹P]polyethylene glycol (10 µM cold isotope weight 95%) was included in the incubation solution for 1 h to assess the volume of cell and for correct Ca²⁺ in the extracellular space. Data are expressed as mg of Ca²⁺/mg of cell protein.

Determination of Insositol Phosphate Levels and Phospholipase C Activity—Insitol phosphates were assayed in cultures incubated in 1.5 ml of carbachol-free medium (Raben et al., 1987) supplemented with 5 µCi/ml myo-[³¹P]inositol for 50-60 h at 37°C. In half of the cultures, 0.4 µM PDB was included in the medium for the final 2 h of incubation. LiCl was also present in the wash and carbachol-containing solutions. The monolayers were washed once in Ringer's-HCO₃ containing 10 mM glucose, 10 mM LiCl and 30 mM HEPES, pH 7.4.

The solution was drawn off, and monolayers were incubated at 30°C in the same buffer in either the absence or presence of 100 µM carbachol.

After 30 min the solution was withdrawn and the reaction stopped by the addition of 2 ml of acidified methanol (MeOH/ concentrated HCl, 500:3, v/v), InsP₁, InsP₂, and InsP₃ were separated and assayed as described (Downes and Mitchell, 1981; Raben et al., 1987) by anion-exchange column chromatography on Bio-Rad AG 1-X₈ resin (formate form). The 0.1 M-0.2 M (formate/ammonium form) eluate was defined as InsP₁; 0.1 M-0.4 M as InsP₂; 0.1-0.1 M as InsP₃ and 0.1 M-0.5 M as InsP₄. Two-mI fractions were collected, 10 µl of scintillation mixture added, and radioactivity determined in a liquid scintillation counter.

Total free Ins(1,4,5)P₃ at specified times after carbachol addition in nonradioabeled T₄₄ monolayers was also measured with a receptor binding assay. This assay was also used to measure phospholipase C activity in crude membranes prepared from T₄₄ monolayers. T₄₄ crude membranes were prepared by washing two T₇5 flasks of cells with 1 mM EDTA, 1 mM β-mercaptoethanol, 20 mM HEPES, pH 7.50, 1 µg/ml phosphoramidone, 40 µg/ml phenylmethylsulfonyl fluoride. The scraped cells or membranes were homogenized three times and centrifuged at 45 min at 100,000 × g.

The pellets were then washed two T₇5 flasks of cells with 1.2 mg of membranes were prepared by washing two T₇5 flasks of cells with 1 mM EDTA, 1 mM β-mercaptoethanol, 20 mM HEPES, pH 7.50, 1 µg/ml phosphoramidone, 40 µg/ml phenylmethylsulfonyl fluoride. The scraped cells or membranes were homogenized three times and centrifuged at 45 min at 100,000 × g. The pellets were resuspended in a minimal volume of the homogenizing buffer and used for phospholipase C generation assays. For the assay, rat cerebellar microsomal membranes, which contain the specific Ins(1,4,5)P₃ receptor (S), were prepared from adult, male Sprague-Dawley rats killed by decapitation. The cerebellum was homogenized with an Ultra-turrax tissue disrupter (Tekmar Co., Cincinnati, OH) at top setting, 10 s, in 30 volumes of ice-cold 50 mM Tris, pH 7.7, 1 mM EDTA, 1 mM β-mercaptoethanol, pelleted by centrifugation at 20,000 × g, and resuspended in 30 volumes of the same buffer. The latter step was repeated three times, and the final pellet was resuspended in the same buffer at 1.2 mg of protein/ml. T₄₄ monolayers were preincubated in the absence or presence of PDB, washed with 1 ml of Buffer A, and exposed to Buffer A plus 100 µM carbachol at 30°C for specified time intervals. Then the solution was drawn off and the reaction stopped by addition of 0.5 ml of ice-cold 1 M trichloroacetate. In the case of T₄₄ crude membranes, the generation of InsP₃ was stopped by the addition of an equal volume of 2 N trichloroacetate. The scraped cells or membrane pellets were homogenized three times and centrifuged at 100,000 × g for 10 min, and the supernatants were centrifuged four times with water-saturated ether. The aqueous layers were used undiluted to quantify the amount of Ins(1,4,5)P₃ present as follows. 100 µl of sample was incubated in a total volume of 500 µl of 50 mM Tris, 1 mM EDTA, 1 mM β-mercaptoethanol with 1 nM [³¹P]Ins(1,4,5)P₃ (5 µCi) and 60 µg of rat cerebellar membranes. Displacement of [³¹P]Ins(1,4,5)P₃ from the rat brain membrane was determined after 10 min by centrifuging the assay mixture at top speed in a Beckman Microfuge and determining the radioactivity in the pellet. Computation of cytosolic Ins(1,4,5)P₃ was made by comparison with a standard curve after correcting for nonspecific binding not removed by 2 µM cold Ins(1,4,5)P₃.

RESULTS

Effect of PDB Preincubation on the [Ca²⁺]i Response to Carbachol and Histamine—Carbachol, a muscarinic secretagogue that operates through calcium-dependent pathways, was studied for its effects on [Ca²⁺]i in single T₄₄ cells. Single T₄₄ cells were monitored using a fluorescence microscope video camera system. After the addition of 100 µM carbachol to control cells, [Ca²⁺]i rose quickly to a peak and then declined slowly over the next few minutes (Fig. 1A), as reported previously (Reimbl et al., 1989). All the T₄₄ cells studied under control conditions in Ringer's-HCO₃, 10 mM glucose, 10 mM HEPES, pH 7.4, responded in this manner (Table I). The effects of incubation with PDB on basal [Ca²⁺]i in T₄₄ cells and on the elevation in [Ca²⁺]i, caused by carbachol were studied at intervals up to 24 h (Fig. 1B). One min of phorbol ester exposure had no effect on basal [Ca²⁺]i (data not shown) or on the changes caused by carbachol in terms of peak [Ca²⁺]i, rises, plateau level, and duration of [Ca²⁺]i elevation (Fig. 1B), as earlier suggested (Beuerlein et al., 1987). In the absence or presence of PDB (0.4 µM) for 1 min, 100% of the monitored cells responded to carbachol with a rise in [Ca²⁺]i. Prolonged PDB exposure decreased the percentage of cells which responded to carbachol and also decreased the magnitude of the response in those cells which did increase [Ca²⁺]i. (Table I and Fig. 1B). After 1 h of PDB treatment, a rise in [Ca²⁺]i, occurred in 80% of cells, and the magnitude of the increase in responding cells was only 60% of the response in control cells (Table I). Two h of PDB exposure decreased to 45% the cells that elevated [Ca²⁺]i, in response to carbachol with responding cells elevating [Ca²⁺]i, only to 37% of the control response (Table I). The effect of PDB on the carbachol-induced [Ca²⁺]i elevation continued for 24 h, at which time only 49% of cells elevated [Ca²⁺]i, in response to carbachol, with the magnitude of the increase in responding cells being only 44% of the control response. In Fig. 1B, time courses are shown for the carbachol-induced increase in [Ca²⁺]i, averaged for all cells and for responding cells alone. Basal [Ca²⁺]i levels did not vary significantly from the control value of 86 ± 5 nM when cells were pretreated with PDB for 2 h and for up to 24 h. With 2 h of PDB treatment, basal [Ca²⁺]i was 81 ± 6 nM (Table I), and basal...

![Fig. 1. Time course of 10⁻⁵ M carbachol-induced A[Ca²⁺]i in T₄₄ cells and effects of PDB preincubation. Cells grown on glass coverslips were loaded with Fura-2 and studied individually in the fluorescence microscope as under "Experimental Procedures." Up to eight cells/cover slip were analyzed simultaneously by measuring fluorescence at fixed locations in individual cells at 1-s intervals. Data in panel A are means ± S.E., expressed as A[Ca²⁺]i, where the [Ca²⁺]i immediately before carbachol addition was set to 0. In panel B only the mean values are plotted. Panel A, untreated cells; all responding to carbachol (n = 75 cells), Panel B, cells pretreated with 0.4 mM PDB for 24 h. with (n = 12 cells, all responding); and 2, h (n = 30; 45%; responding); 1 and 3, 24 h (n = 47, 36%; responding). Open symbols (O, □) represent the average of all cells; solid symbols (●, ■) are the average of responding cells only.](image-url)
Phospholipase C Uncoupling in T₄₄ Cells

TABLE 1

| Addition | No treatment | 1 h of PDB | p | 2 h of PDB | p |
|----------|--------------|------------|---|------------|---|
| Basal [Ca²⁺], (nM) | 86 ± 5 (96) | 81 ± 6 (46) | NS* | 5.0 ± 0.7 (16) | NS |
| Carbachol (100 μM) | | | | | |
| Maximal Δ[Ca²⁺], (nM) | 81 ± 5 (75) | 49 ± 5 (5) | <0.05 | 17 ± 2 (3) | <0.05 |
| % Cells responding | 100% | 80% | | 19% |
| R₁: ionomycin, CCCP, 4 mM Ca²⁺ | 6.0 ± 0.7 (75) | 38 ± 2 (8) | <0.01 | 17 ± 2 (3) | <0.05 |
| Histamine (100 μM) | | | | | |
| Maximal Δ[Ca²⁺], (nM) | 47 ± 4 (20) | 36 ± 2 (8) | <0.01 | 17 ± 2 (3) | <0.05 |
| % Cells responding | 95% | 100% | | 19% |
| R₁: ionomycin, CCCP, 4 mM Ca²⁺ | 4.5 ± 0.5 (21) | 5.0 ± 0.7 (16) | NS |

*NS, not significant.

FIG. 2. Inhibition of 10⁻⁴ M histamine-induced [Ca²⁺] elevation by PDB preincubation. [Ca²⁺], was monitored at 10-s intervals in control cells ( ●, ○), in which 20 of 21 cells responded, or after pretreatment for 2 h with 0.4 μM PDB ( ■, □), in which 3 of 16 cells elevated [Ca²⁺]. Mean change in [Ca²⁺] with 100 μM histamine is shown for responding cells (●, ■) and for all cells (○, □).

[Ca²⁺], after 24-h exposure to PDB was 90 ± 20 nM.

Results similar to the carbachol experiments were found using 100 μM histamine, an agonist that also stimulates Cl⁻ secretion through calcium-dependent mechanisms. In control cells, histamine elevated [Ca²⁺], immediately, with a peak increase of 47 nM within 30 s, with 95% of the cells responding (Fig. 2 and Table I). As with carbachol, the [Ca²⁺], response after histamine was inhibited by preincubating with 0.4 μM PDB (Fig. 2). One h of PDB exposure significantly inhibited the peak increase in [Ca²⁺], caused by histamine by 23%, with the inhibitory effect becoming maximum at 2 h of PDB exposure, at which time the peak Δ [Ca²⁺], was inhibited by 64% (Fig. 2 and Table I). When pretreated with PDB for 2 h, only 19% of T₄₄ cells elevated [Ca²⁺], in response to histamine (Table I), and with 24-h PDB exposure only 16% of cells responded (data not shown).

Effect of PDB on Cholinergic Receptors—As long term incubation with phorbol esters has been reported to downregulate cholinergic receptors, membrane fractions prepared from T₄₄ monolayers incubated in the absence or presence of PDB for 2 h were assayed for specific binding of the cholinergic receptor antagonist QNB. As shown in Fig. 3, the QNB binding characteristics in untreated and PDB-treated cells were similar. Equilibrium binding of [³H]QNB was found to be saturable and of high affinity. In one experiment it was calculated that control membranes had a Kₐ of 1.2 nM and Bₘₐₓ of 18 fmol/mg, and PDB-treated membranes had a Kₐ of 0.8 nM and Bₘₐₓ of 17 fmol/mg. Results in a second preparation were similar, with control and experimental membranes both having a Kₐ of 0.4 nM, and Bₘₐₓ values were 12.5 and 13.0 fmol/mg, respectively.

PDB Incubation Stimulates Membrane-associated Phospholipase C Activity in T₄₄ Cells—As carbachol receptors are functionally linked to phospholipase C in multiple cell types (Berridge, 1987; Berridge et al., 1987), the effect of prolonged PDB incubation on the activity of this enzyme was examined. Phospholipase C activity was first measured in membranes prepared from control and 2-h PDB-preincubated T₄₄ cells. The generation of Ins(1,4,5)P₃ by the membranes was monitored at room temperature and was saturable and of high affinity (Kₐ, 1.2 and 0.8 nM for control and PDB-treated membranes, respectively. The results shown are representative of two experiments.

FIG. 3. Binding of [³H]QNB to T₄₄ membranes was unchanged by pretreatment with PDB. T₄₄ cells, control ( ▲) or pretreated for 2 h with 0.4 μM PDB ( ○), were washed three times in Buffer A (see “Experimental Procedures”), and the 100,000 x g pellet was assayed for equilibrium binding of [³H]QNB. Binding was determined at room temperature and was saturable and of high affinity (Kₐ, 1.2 and 0.8 nM for control and PDB-treated membranes, respectively. The results shown are representative of two experiments. .
Phospholipase C activity in T₄₅₅ cell membranes is elevated by PDB incubation

Phospholipase C activity was assayed by monitoring Ins(1,4,5)P₃ generation in a crude membrane preparation. Immediately after preparation, the membranes (15–30 μg of protein) were incubated 30 min at 37 °C in 200 μl (final volume) containing 1 mM EGTA; 1 mM β-mercaptoethanol, 1 mM NaATP, 20 mM HEPES, pH 7.5, 20 mM β-glycerophosphate or in an identical solution except with the free Ca²⁺ raised to 2 mM. Ins(1,4,5)P₃ was assayed by a competitive binding assay (Bredt et al., 1989). Phospholipase C activity was defined as the Ca²⁺-dependent amount of Ins(1,4,5)P₃. The zero-time levels of Ins(1,4,5)P₃ (generated during the 2-h preparation of membranes) were subtracted to obtain the values shown below and were as follows: control, 853 ± 494 pmol/mg protein; 2 h + 0.4 μM PDB, 12,980 ± 1,280 pmol/mg protein (p < 0.005). The experiment is representative of two preparations. Each condition was studied in triplicate; results are mean ± S.E. Student’s unpaired t test compared PDB-treated with control membranes.

| Condition          | Control | 2 h + 0.4 μM PDB | p       |
|--------------------|---------|-----------------|---------|
| No additions       | 3,257 ± 1,403 | 2,861 ± 1,110 | NS¹     |
| 2 mM Ca²⁺          | 8,095 ± 903   | 14,795 ± 3,967 | <0.025  |
| Phospholipase C activity | 4,838 | 11,934 |         |

¹ NS, not significant.

Fig. 4. Effect of PDB incubation on carbachol-induced Ins(1,4,5)P₃ generation in T₄₅₅ cells. Total aqueous Ins(1,4,5)P₃ was determined in control (●) and 2-h PDB-pretreated (○) cultures by a competitive binding assay for the Ins(1,4,5)P₃-specific receptor freshly prepared from rat cerebellum. Stairs denote significant differences between PDB-treated monolayers and untreated controls; the plus signs show significant changes caused by carbachol. Data are mean ± S.E. of three experiments.

lipase C, it was of interest to determine whether the enzyme was still responsive to receptor activation. Ins(1,4,5)P₃ levels were measured in T₄₅₅ monolayers after incubation in the absence or presence of PDB (Fig. 4). Control cells had a significantly increased amount of Ins(1,4,5)P₃, 10 s after carbachol addition. The Ins(1,4,5)P₃ level peaked at 20 s, at which point it had doubled, and returned to base line by 30 s. When cells were first exposed for 2 h to PDB the resting level of Ins(1,4,5)P₃ was significantly higher than in the untreated cells, and in fact the basal value in this group was not significantly different from the carbachol-induced peak of the controls (83 ± 8 versus 104 ± 14 pmol/monolayer, respectively; Fig. 4). Carbachol, however, had no further effect on Ins(1,4,5)P₃ in PDB-treated cells. 30 s after carbachol exposure, Ins(1,4,5)P₃ was again significantly greater in PDB-treated cells than in the corresponding control cells. A similar pattern of Ins(1,4,5)P₃ elevation was found when T₄₅₅ monolayers were preincubated for 24 h with PDB before carbachol exposure, with PDB-treated cells having a basal Ins(1,4,5)P₃ level that was increased compared with untreated cells and no further elevation of Ins(1,4,5)P₃ in response to carbachol (data not shown).

The effect of PDB treatment on carbachol-evoked changes in Ins(1,4,5)P₃ was studied further using cells labeled with myo-[³H]inositol. T₄₅₅ monolayers were radiolabeled with myo-[³H]inositol, the inositol phosphates were extracted, and the fractions were separated by anion-exchange chromatography into InP₁, InP₂, InP₃, and InP₄ (Table III). Total incorporation of the radiolabel was not changed significantly by PDB incubation. The inclusion of 10 mM LiCl in all the aqueous solutions, which inhibits the further metabolism of inositol phosphates, was necessary to achieve radioactivity. As shown in Table III, 100 μM carbachol caused significant increases in untreated T₄₅₅ cells in the levels of InP₁, InP₂, and InP₄, and InP₃, of 76, 86, and 55%, respectively. After preincubation for 2 h with 0.4 μM PDB, generation of InP₁, InP₂, and InP₄ was not elevated significantly by carbachol. Although increased 5'-phosphomonoesterase activity by PDB could explain the lack of increase in InP₁, InP₃, and InP₄, the level of InP₁ would be expected to be comparatively high, which was not the case. These data indicate uncoupling of carbachol receptors from phospholipase C.

TABLE II

Phospholipase C Uncoupling in T₄₅₅ Cells

| Condition          | 2 h + 0.4 μM PDB | p       |
|--------------------|-----------------|---------|
| No additions       | 3,257 ± 1,403 | 2,861 ± 1,110 | NS¹     |
| 2 mM Ca²⁺          | 8,095 ± 903   | 14,795 ± 3,967 | <0.025  |
| Phospholipase C activity | 4,838 | 11,934 |         |

¹ NS, not significant.

The effect of PDB treatment on carbachol-evoked changes in Ins(1,4,5)P₃ was studied further using cells labeled with myo-[³H]inositol. T₄₅₅ monolayers were radiolabeled with myo-[³H]inositol, the inositol phosphates were extracted, and the fractions were separated by anion-exchange chromatography into InP₁, InP₂, InP₃, and InP₄ (Fig. 4) or generation of [³H]InsP₁ derived from incorporated myo-[³H]inositol. In these T₄₅₅ cells, abnormally activated phospholipase C could potentially be compensated by raised activities of 5'-phosphomonoesterase or InP₄ kinase. The actions of these enzymes would tend to keep basal Ins(1,4,5)P₃ at close to normal levels. However, if the basal level of Ins(1,4,5)P₃ was indeed elevated after PDB preincubation, an effect on cellular calcium stores would be expected. Therefore, the amount of stored calcium after PDB treatment was estimated. Two techniques were used: plasma emission spectroscopy of whole monolayers, to measure total cell calcium; and Ca²⁺ ionophore-releasable calcium, measured in single cells using Fura-2, which reflects intracellular stored calcium.

After incubation for 2 h in the absence or presence of PDB, monolayers were prepared for plasma emission spectroscopy. Parallel sets of cells were used to correct for Ca²⁺ present in the extracellular spaces by use of [³H]polyethylene glycol. PDB exposure did not significantly alter the extracellular space (32 and 35 μl/mg protein for untreated and PDB-incubated monolayers, respectively). In two experiments, after PDB treatment total cell Ca²⁺ decreased 28% (control values were 4.0 and 3.8 mg of Ca²⁺/mg of protein, and PDB-treated cells contained 2.9 and 2.7 mg of Ca²⁺/mg of protein).

Intracellular calcium stores were also evaluated by studies of [Ca²⁺], in T₄₅₅ cells at the single cell level using ionomycin to permeabilize the endoplasmic reticulum and CCCP to release mitochondrial calcium. After control or PDB incubation for 2 h, cells were perfused with Ca²⁺-free Ringer's-HCO₃ solution (see “Experimental Procedures”) for 5 min and then treated with 10 μM ionomycin plus 10 μM CCCP. Peak changes in [Ca²⁺] were taken as an indication of the amount of calcium available in the internal stores. As shown in Fig. 5, the total stored Ca²⁺ was significantly reduced by preexposure to PDB, with controls able to elevate [Ca²⁺], 548 ± 84 nM above the basal level whereas PDB-treated cells raised [Ca²⁺], 301 ± 54 nM above basal (p < 0.05). The cells were perfused at the end of the study with Ringer's-HCO₃ solution containing 10 μM ionomycin, 10 μM CCCP, 4 mM Ca²⁺ to ensure that under all conditions the intracellular dye responded in a manner similar to free dye. The latter treatment caused elevation of [Ca²⁺], in both control and PDB-treated cells to similar values. In summary, prolonged PDB treatment decreased total calcium storage.
TABLE III

Generation of inositol phosphate with carbachol is inhibited by 0.4 μM PDB preincubation

TABLE IV

α-PDB, did not significantly reduce the response of [Ca++], to 100 μM carbachol (Table IVA).

That the inhibitory effect of 2-h PDB exposure on the carbachol-induced elevation of [Ca++], was not exerted directly by protein kinase C was suggested by studies with the protein kinase C inhibitors H-7 and staurosporine. Neither H-7 nor staurosporine altered basal [Ca++], (data not shown), nor did they inhibit the rise in [Ca++], following exposure to 100 μM carbachol (Table IV, A and B). The carbachol-induced rise in [Ca++], after incubation with H-7 (60 or 100 μM) for either 15 min or 2 h before carbachol addition was not significantly

stores, consistent with the effective level of Ins(1,4,5)P3 in the intact cells being elevated.

Effect of Prolonged PDB Incubation on T84 Cell Protein Kinase C—PDB is known to activate protein kinase C. To determine whether the demonstrated uncoupling of phospholipase C from its receptor(s) was through protein kinase C, the kinase inhibitors staurosporine and H-7 were used, as well as the "nonactivating" phorbol ester, α-PDB, as a control. These agents were first applied to control and PDB-incubated T84 monolayers, and [Ca++], was measured in a fluorometer after carbachol addition. In a population of control T84 cells grown on glass coverslips, carbachol elevated [Ca++], by 171 ± 10 nM (Table IVA). The response was abolished completely by incubation of the cells for 2 h with 0.4 μM PDB, similar to the observations made on single cells. Incubation of the cells under similar conditions with the inactive phorbol analogue, α-PDB, did not significantly reduce the response of [Ca++], to 100 μM carbachol (Table IVA).
different from the control carbachol response (Table IV, A and B). Similar results were obtained when the cells were incubated for 15 min or 2 h with staurosporine (1 μM); again the carbachol stimulation of [Ca\(^{++}\)] was no different from the control response (Table IVA). Not only did H-7 and staurosporine not duplicate the effect of 2-h treatment with PDB, but a 2-h exposure to H-7 did not significantly reverse the effect of 2-h PDB exposure to inhibit the carbachol-induced elevation of [Ca\(^{++}\)]. (Table IVB). As shown in Table IVB, PDB continued to inhibit the carbachol-induced [Ca\(^{++}\)], increase in the presence of the protein kinase C blocker H-7. Although a marginal increase in [Ca\(^{++}\)], was observed under these conditions, only 31% of the cells responded. Thus the [Ca\(^{++}\)], elevation that occurred with carbachol in cells preincubated with PDB plus H-7 was significantly less than in control cells.

The effects of prolonged PDB exposure were studied to determine whether any correlation could be found between protein kinase C activity and [Ca\(^{++}\)] response to carbachol. Specific activity of protein kinase C in Ts4 cell homogenate, cytosol, and a crude membrane preparation was determined after 2- and 24-h of PDB exposure and compared with untreated control cells. Protein kinase C activity in cell homogenates decreased in a time-dependent manner after PDB addition to the medium (Fig. 6 and Table V). Homogenate activity of Ts4 monolayers fell measurably after 30 min of exposure to PDB and continued to a low of approximately 18% of control after 24 h. Membrane and cytosolic fractions, prepared from Ts4 monolayers after incubation with or without PDB, were assayed for protein kinase C activity. Table V shows that after 2 h with PDB, membrane-associated protein kinase C rose by 175%, constituting 44% of the total cellular activity, as compared with 9% of the total activity found in the membrane of control cells. Cytosolic protein kinase C activity was decreased by 2 h of PDB treatment and was nearly absent after 24 h with PDB (5% of control). After 24 h of PDB incubation the membrane-associated level was undetectable (Table V). Therefore, total protein kinase C activity decreased over a 24-h incubation period while membrane-associated activity increased at 2 h and was below detection after 24-h exposure to PDB. No correlation was found between membrane-associated protein kinase C activity and the inhibition of receptor-stimulated increases in [Ca\(^{++}\)].

![Fig. 6. Total cellular protein kinase C activity decreases in a time-dependent manner upon PDB exposure.](image)

**DISCUSSION**

Carbachol and histamine, which have been shown to stimulate Cl\(^-\) secretion in colonic epithelia and Ts4 cells, act at least partially through mobilization of intracellular Ca\(^{++}\). As shown previously for Ts4 cells, the peak increase in [Ca\(^{++}\)], in response to carbachol was highly dependent upon stored calcium and could be eliminated by the addition of dantrolene, which immobilizes stored calcium, or by prolonged preincubation of cells with 100 μM EGTA to deplete the cellular calcium stores (Renli et al., 1989). The latter phase of [Ca\(^{++}\)], elevation required the presence of extracellular Ca\(^{++}\) and could be inhibited by the Ca\(^{++}\) channel blockers, verapamil or nifedipine (Renli et al., 1989). These studies further probe the mechanism of the carbachol-induced rise in [Ca\(^{++}\)].

Recent investigations suggest a role for protein kinase C (Beuerlein et al., 1987; Dhamarsathphorn et al., 1989; Weymer et al., 1985) in Cl\(^-\) secretion in Ts4 cells. Specifically, exposure to phorbol 12-myristate 13-acetate for 1-2 min before stimulating Ts4 monolayers with carbachol blocked Cl\(^-\) secretion without inhibiting the rise in [Ca\(^{++}\)]. However, incubations of greater than 15 min with phorbol 12-myristate 13-acetate also inhibited [Ca\(^{++}\)], rises stimulated by carbachol addition. Previous work did not go beyond measurements of Cl\(^-\) fluxes and [Ca\(^{++}\)], elevation of whole monolayers. The present work was initially intended to investigate receptor-mediated [Ca\(^{++}\)], increases under conditions that either activated (2-h exposure to PDB; see Table V) or decreased membrane levels of protein kinase C (24-h exposure to PDB). However, after both 2- and 24-h exposure to PDB there was increased basal phospholipase C activity compared with controls, based either on measurement of membrane bound phospholipase C activity or of total Ins(1,4,5)P\(_3\) (Tables II and III and Fig. 4).

Overall, the results demonstrate that prolonged PDB incubation induces an uncoupling of phospholipase C from receptor regulation and results in prolonged activation of phospholipase C. The mechanism by which uncoupling from the receptor activates the phospholipase C is unknown, although possibilities include an effect on a guanine nucleotide binding protein. Nishizuka (1988) suggested a negative feedback role for protein kinase C in signal transduction in which the C kinase lowers InsP\(_3\) levels, either by blocking receptor-mediated hydrolysis of inositol phosphates or increasing hydrolysis of InsP\(_3\). An example of the latter has been demonstrated in human platelets in which protein kinase C action regulates inositol trisphosphate 5'-phosphomonoesterase ac-
tivity (Connolly et al., 1986). These negative feedback aspects of protein kinase C action occur rapidly and are distinct from the more prolonged effect of phorbol ester exposure demonstrated here. The uncoupling of phospholipase C resulting in prolonged phospholipase C activation and inhibition of further stimulation of phospholipase C via plasma membrane receptors occurs distal to the plasma membrane receptor and initially does not produce a negative feedback effect on the entire system. In fact, the consequence of such a mechanism would be predicted to be an initial, short lived activation of InsP3 generation, which would persist until the intracellular calcium stores were emptied, followed by inhibition of signal transduction. Thus, this would ultimately complement the above described negative feedback on the system by protein kinase C. However, in interpreting the physiologic significance of these results it must be remembered that a phorbol ester, not a physiologic stimulus, was being studied.

The results of studying generation of inositol phosphates using myo-[3H]inositol incorporation were consistent with results of the competitive binding assay, showing that carbachol could activate inositol phosphate turnover in T44 cells but not in PDB-incubated cells (Fig. 4 and Table III). Basal levels of inositol phosphates are not easily determined using myo-[3H] inositol, and high pressure liquid chromatography was not performed. The fact that basal levels of [3H]InsP2 were not changed by PDB pretreatment whereas basal total Ins(1,4,5)P3, measured by a competitive binding assay, was increased suggests that separate inositol pools were involved in generation of carbachol-stimulated [3H]InsP2 (Table III) and total Ins(1,4,5)P3. Table III also shows no significant difference in PDB-treated versus control cells in the amounts of InsP2 and InsP3 produced following carbachol, which suggests that the 5'-phosphomonoesterase was similarly active under both conditions. Additionally, in the studies, PDB did not increase basal or carbachol-induced InsP2 levels, indicating that InsP2 kinase(s) was not activated.

Finally, it was of interest to determine whether the effects of prolonged PDB incubation described here involved protein kinase C. Long term PDB exposure lowers protein kinase C in many systems (Rodriguez-Pena and Rozengurt, 1984). Assays of protein kinase C indicated that the enzyme was gradually decreased in both cytosol and whole cell homogenate with prolonged PDB exposure, but the membrane fraction had elevated protein kinase C at 2 and undetectable levels at 24 h after PDB. In contrast, the membrane fraction at both 2 and 24 h had highly activated phospholipase C (Table I and Fig. 4). Thus, phospholipase C activation after PDB incubation did not correlate with membrane-bound activity of protein kinase C. In addition, as further indication that the results described were not a result of a direct effect of protein kinase C is that antagonists of protein kinase C, H-7 and staurosporine, neither prevented nor duplicated the uncoupling of carbachol receptors induced by long term treatment with PDB (Table IV). The possibility cannot be eliminated, however, that the effects demonstrated here could be caused by a nuclear effect of protein kinase C or a protein kinase C isoform that is not detected by the histone II S assay and/or is insensitive to the protein kinase C antagonists used in these studies. Thus our results suggest that the effects of prolonged PDB exposure are ultimately carried out by some mediator, which is initially altered by PDB, but that protein kinase C is not the direct cause of the prolonged phospholipase C activation shown here.

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