Contrasting Duration of Inhibition of Cell-Cell Communication in Primary Mouse Epidermal Cells by Phorbol 12,13-Dibutyrate and by Bryostatin 1

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ABSTRACT

The bryostatins, macrocyclic lactones isolated from the phorbol esters, activate protein kinase C in vitro and inhibit phorbol ester binding to the enzyme. In intact cells, the bryostatins induce some phorbol ester responses, such as neurontin phosphorylation, but paradoxically they not only fail to induce other responses, e.g., differentiation in HL-60 promyelocytic leukemia cells, but actually block response to the phorbol esters. We compare here bryostatin 1 and phorbol 12,13-dibutyrate as inhibitors of cell-cell communication in cultured primary mouse epidermal cells. Like phorbol 12,13-dibutyrate, bryostatin 1 at nanomolar concentrations markedly inhibited cell coupling. It differed from the phorbol esters, however, in that its action was more transient. For 4 h of incubation bryostatin 1 caused little inhibition of coupling. Moreover, coincubation of bryostatin 1 and phorbol 12,13-dibutyrate gave no greater response at this time than that found for bryostatin 1 alone. Time-dependent inhibition of the protein kinase C pathway could account for many of the observed differences between the actions of the phorbol esters and bryostatin 1.

INTRODUCTION

Protein kinase C, the major receptor for the phorbol ester tumor promoters, is thought to mediate one arm of the signal transduction pathway for that large class of hormones and cellular effectors which act to induce enhanced phosphoinositide turnover (1). The phorbol esters at nanomolar concentrations activate the enzyme, apparently by functioning as analogues of sn-1,2-diacylglycerol, a product of phosphoinositide breakdown (2). Several other classes of structurally distinct products, including the indole alkaloid lyngbyatoxin and the polycatecyl aplysiatoxin, have been identified which inhibit phorbol ester binding and induce similar biological responses (3). These compounds have proven of great value for modeling studies of the phorbol ester pharmacophore (4). In addition, the parallelism of their actions strongly supports the role of protein kinase C as the major effector of phorbol ester responses.

The bryostatins, macrocyclic lactones isolated by Pettit and coworkers from Bugula neritina (5), likewise activate protein kinase C (6, 7), bind directly to the enzyme, and inhibit phorbol ester binding (6, 7). In contrast to the indole alkaloids and polycatecyls, however, the bryostatins induce only some of the same responses as do the phorbol esters. In HL-60 cells, depending on the subline, bryostatin 1 partially (8) or completely (7) failed to induce terminal differentiation and correspondingly blocked the differentiation response to the phorbol esters. In Friend erythroleukemia cells, where phorbol esters block differentiation induced by hexamethylene bisacetamide, treatment with bryostatin 1 overcame the phorbol ester block and restored differentiation (9). Provocatively, the action of bryostatin 1 in this system was noncompetitive with respect to the phorbol ester. In Sencar mice, bryostatin 1 was inactive as a complete tumor promoter and inhibited tumor promotion by phorbol 12-myristate 13-acetate (10). In contrast to the findings with the above systems, bryostatin 1 closely resembled the phorbol esters in its mitogenic activity on Swiss 3T3 cells (6) and in its activation of polymorphonuclear leukocytes (11). Understanding the mechanisms which account for the divergent responses to the phorbol esters and the bryostatins may shed new light on control of the protein kinase C pathway.

In primary mouse epidermal cells, bryostatin 1 causes some effects similar to those of the phorbol esters, such as inhibition of epidermal growth factor binding or induction of ornithine decarboxylase, but fails to induce differentiative responses such as induction of epidermal transtamatinase or cornified envelope formation (12). In this report, we compare the activity of bryostatin 1 and PDBu on cell-cell communication in the primary mouse epidermal cells.

Inhibition of cell-cell communication in response to the phorbol esters has been characterized in a number of cell systems (13-16). It has attracted considerable attention because it represents one postulated mechanism whereby initiated cells could escape suppression by surrounding normal cells. The inhibition of cell-cell communication is of further interest because it has a rapid onset, permitting analysis of the time course of response over a broad time range.

MATERIALS AND METHODS

Cell Culture. Epidermal cells from newborn BALB/c mouse epidermis were prepared by a trypsin floation procedure (17). Cells (4 x 10^6) were plated in 60-mm plastic dishes in Eagle's minimum essential medium containing 0.05 M Ca^2+ and 8% chelexed fetal bovine serum. Medium containing 0.05 M Ca^2+ and 8% chelexed fetal bovine serum was prepared by a trypsin flotation procedure (17). Cells (4 x 10^6) were plated in 60-mm plastic dishes in Eagle's minimum essential medium containing 0.05 M Ca^2+ and 8% chelexed fetal bovine serum and incubated at 37°C in 95% air:5% CO2. The next day the cells were washed with Ca^2+, Mg^2+-free phosphate-buffered saline to remove unattached cells; they were fed with the appropriate medium at this time and after an additional 2 days. Subconfluent cultures 3-5 days after plating were used for the experiments.

Measurement of Cell-Cell Communication. Fluorescent Lucifer Yellow CH and PDBu were purchased from Sigma Chemical Company, St. Louis, MO.

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2 To whom requests for reprints should be addressed.
3 The abbreviations used are: PDBu, phorbol 12,13-dibutyrate; ED_{50}, 50% effective dose.

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bryostatin 1 on dye transfer was studied, the cells received Eagle's minimum essential medium containing 1.2 mM Ca²⁺ and 8% chelated fetal bovine serum [high (Ca²⁺) medium] together with or without (controls) the chemicals. The number of neighboring cells containing dye was determined 10 min after injection.

RESULTS

Primary mouse epidermal cells can be maintained in a proliferating state when cultured in low-calcium medium (0.05 mM Ca²⁺) (18). Under such conditions, the cells show efficient cell-cell communication, as measured by intercellular transfer of microinjected Lucifer Yellow CH dye. Addition of PDBu at 200 nM did not affect dye transfer by the cells grown in low-calcium media (data not shown).

Upon medium change to high-calcium medium (1.2 mM Ca²⁺), the extent of cell coupling was maintained for 4–6 h (Fig. 1A) and then gradually declined to 25–40% of the initial value by 14 h (Figs. 2 and 5). Addition of 200 nM PDBu following the shift to high-calcium medium dramatically inhibited the extent of cell coupling, reducing it to 10% of the control value within 30 min (Figs. 1B and 2). Suppression of dye transfer was maintained for at least 16 h, although the decrease in the extent of cell coupling in the controls at later times renders interpretation problematic.

The ED⁵₀ for inhibition of cell coupling by PDBu, measured at 4 h in cells shifted to high-calcium medium, was 18 nM (Fig. 3), a value between the dissociation constants of PDBu for the high- and low-affinity receptors in these cells (19). It should be noted that the dose-response curve for inhibition of cell coupling by PDBu was more abrupt than typical, with the majority of the inhibition occurring as PDBu was increased from 10 to 30 nM.

After incubation for 4 h, the effect of bryostatin 1 on cell coupling in the absence or presence of PDBu was determined (Fig. 4). By itself, bryostatin 1 caused measurable inhibition of cell coupling at this time in many but not all experiments. The maximal extent of inhibition, 0–30%, was much less than that observed with PDBu. In the presence of 200 nM PDBu, the effect of bryostatin 1 was the opposite. Here, increasing concentrations restored the level of cell coupling (Fig. 1C), until at 100 nM bryostatin 1 a similar extent of cell coupling was seen in either the absence or presence of PDBu. The ED⁵₀ of bryostatin 1 as an inhibitor of the PDBu responses was 14 nM.

Examination of the time course for the inhibition by bryostatin 1 of cell coupling in the absence of PDBu indicated that the limited response was a function of incubation time rather than intrinsic to bryostatin 1 itself (Fig. 5). At incubation times of 30–90 min, bryostatin 1 inhibited cell coupling to a similar extent as did PDBu, 80–90%. However, inhibition was rapidly lost thereafter. Recovery of cell coupling upon incubation in the presence of bryostatin 1 together with PDBu was similar to recovery of coupling in the presence of bryostatin 1 alone. Because the effect of bryostatin 1 at these later times could be detected by its inhibition of phorbol ester action, it is unlikely that bryostatin 1 was being degraded. In support of this conclusion, no degradation was observed upon direct analysis using [³H]bryostatin 4, a related derivative. The dose-response curve for bryostatin 1 as an inhibitor of cell coupling, measured with a 60-min incubation time, yielded an ED⁵₀ of 3 nM (Fig. 6). This value was similar, but not identical, to the ED⁵₀ of 15 nM observed for the blockade of the PDBu response by bryostatin 1 at 4 h.

In order to determine whether the inhibitory effect of bryostatin 1 on phorbol ester action required macromolecular synthesis, cells were treated in the presence of actinomycin D or cycloheximide at concentrations shown previously to inhibit their RNA and protein synthesis by >95% (20). In agreement with previous findings in other cell lines (16), neither agent affected the level of cell coupling of either the control or the PDBu-treated cells (Table 1). When added to cells treated with PDBu and bryostatin 1 in combination, once again these agents had no effect, arguing that the inhibition of the PDBu response by bryostatin 1 was independent of macromolecular synthesis.

DISCUSSION

Studies using the phorbol esters have implicated protein kinase C in tumor promotion and in many other cellular responses (21–24). The possible utility of inhibiting protein kinase C in order to block any specific response is clouded, however, by the variety of other protein kinase C-mediated responses that would also be affected. Intense interest has therefore been directed at evidence for dissociation of responses mediated by protein kinase C.

Some dissociation of responses has been observed for diacylglycerols. Although they mimic many phorbol ester effects, including inhibition of cell-cell communication (25, 26), they have been reported not to induce differentiation of HL-60 cells (27) or to inhibit maturation of granulosa cells (28). Likewise, in HL-60 cells they fail to cause the full range of substrate phosphorylations seen in response to PDBu (27). Analysis of these differences has been complicated, however, by the low potency of the diglycerides, by their rapid metabolism, and by the accumulation in membranes of unusual phospholipids as a consequence of this metabolism.

In contrast, bryostatin 1 is a metabolically stable, highly potent (nM) activator of protein kinase C (6, 7, 11). We report here that bryostatin 1 differs from the phorbol esters in having a markedly more transient effect on cell-cell communication in mouse primary epidermal cells. The response of the cells to PDBu for this endpoint is lost concomitant with the loss of response to bryostatin 1. Conceptually, this transient activity of bryostatin 1 may reflect a quantititative rather than a qualitative difference. In BALB/c 3T3 cells (25) and rat liver epithelial cells (29) the inhibition of dye transfer in response to phorbol 12-myristate 13-acetate (100 ng/ml) was also temporary, with restoration of communication to control levels by 10–12 h.

The model that bryostatin 1 first activates and then blocks the protein kinase C pathway in a time- and cell-type-dependent fashion could rationalize much of our current knowledge about the effects of these compounds (Table 2). Motivated by our observations on cell-cell communication, we have confirmed that the inhibition of epidermal growth factor binding by bryostatin 1 in the keratinocytes was also time dependent, ranging from a response comparable to that for the phorbol esters at 30 min to little effect at 4 h (12).

Studies are currently underway to characterize the mechanism of this time-dependent inhibition. On the one hand, the inhibitory effects of bryostatin 1 in Friend erythroleukemia cells were not competitive with phorbol ester concentration, implying that the inhibition in this system was mediated by interaction at a site distinct from the high-affinity phorbol ester binding site of protein kinase C. On the other hand, using dye transfer in keratinocytes, we could separately measure the potencies of bryostatin 1 for both its phorbol ester-like activity at early times and its phorbol ester inhibitory activity at later times. These were the same order of magnitude although not
identical; the nontheoretical shape of the dose-response curves and the tendency of bryostatin to absorb to plasticware make exact comparison difficult. These latter results suggest that the binding site for bryostatin 1 on protein kinase C which mediates enzymatic activation and the target in the cell which mediates the inhibitory effects of the bryostatin may be the same or related. For example, bryostatin binding to protein kinase C might directly cause accelerated down-regulation. Alternatively, bryostatin binding to a distinct target evolutionarily related to the regulatory domain on protein kinase C might have a negative regulatory effect on the direct protein kinase C activation by bryostatin.

Differences in responsiveness to the bryostatins between cell types may reflect in part different durations for the transient activity of the bryostatins in the different cells. Thus, partial response in the rat pituitary cells was observed at 48 h (30) and complete response in the Swiss 3T3 cells at 40 h (6), in contrast to the much shorter effective duration in the mouse primary epidermal cells. Comparison of an identical endpoint in these different systems would help to better evaluate this hypothesis.

The inhibition of cell coupling by the phorbol esters in the primary mouse epidermal cells was dependent upon the change of medium from low to high calcium. The basis for this dependence is currently being investigated. The medium change is associated with elevated intracellular calcium. Since high levels of calcium alone can block cell coupling in other systems...


Fig. 2. Time course of inhibition by PDBu of cell coupling. Cells were prepared and used as described in "Materials and Methods." The medium was changed to high-calcium medium and PDBu (200 nM) was added to cultures at 0 h. Lucifer Yellow CH solution was injected at the times indicated, and the number of coupled cells per injection was measured 10 min later. Points, mean of 5 to 11 separate injections in a single experiment; bars, SE; O, control; •, PDBu.

Fig. 3. Inhibition of cell coupling as a function of PDBu concentration. At 0 h, the medium was changed to high-calcium medium and different doses of PDBu were added to cultures. Lucifer Yellow CH solution was injected at 4 h. Data are expressed as the mean of 6 to 9 separate injections; bars, SE. A second experiment gave similar results.

Fig. 4. Effect of bryostatin 1 on PDBu-mediated inhibition of cell coupling at 4 h of incubation. At 0 h the medium was changed to high-calcium medium and cells were treated with various doses of bryostatin 1 in the absence of PDBu (O) or in the presence of 200 nM PDBu (•). Lucifer Yellow CH solution was injected at 4 h. Values are the mean of 6 to 11 separate injections; bars, SE. A second experiment gave similar results.

Fig. 5. Time course of inhibition of cell coupling by bryostatin 1. At 0 h the medium was changed to high-calcium medium and the cells were treated with 120 nM bryostatin 1. The fluorescent dye was injected at the times indicated. Points, mean of 11 to 18 separate injections from 2 experiments; bars, SE; O, control; •, bryostatin 1.

Fig. 6. Inhibition of cell coupling as a function of bryostatin 1 concentration at 1 h of incubation. At 0 h the medium was changed to high-calcium medium and bryostatin 1 was added. Lucifer Yellow CH was injected 1 h later. Values are mean of 14 to 17 separate injections from 2 experiments; bars, SE.

Table 1 Lack of effect of inhibitors of macromolecular synthesis on the block of PDBu action by bryostatin 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Cell coupling</th>
<th>% of control</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>100 ± 11</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Cycloheximide</td>
<td>84 ± 17</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PDBu + cycloheximide</td>
<td>13 ± 3</td>
<td>10</td>
<td></td>
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<tr>
<td></td>
<td>PDBu + bryostatin 1</td>
<td>98 ± 4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PDBu + bryostatin 1 + cycloheximide</td>
<td>90 ± 19</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>100 ± 14</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Actinomycin D</td>
<td>96 ± 12</td>
<td>11</td>
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</tr>
<tr>
<td></td>
<td>PDBu + actinomycin D</td>
<td>10 ± 2</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PDBu + bryostatin 1</td>
<td>114 ± 6</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PDBu + bryostatin 1 + actinomycin D</td>
<td>114 ± 14</td>
<td>11</td>
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</tbody>
</table>

(31), it may be that the observed effect represents a synergistic response to elevated but lower intracellular calcium levels together with protein kinase C activation. Alternatively, the elevated intracellular calcium may be modulating the protein kinase C pathway directly.

Natural products chemistry has played a critical role in the identification and elucidation of the function of protein kinase C. Because of their differential activity on responses mediated by protein kinase C, the bryostatins promise to afford exciting insights into selective control of this system.
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REFERENCES


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