Protein kinase C is involved in desensitization of muscarinic receptors induced by phorbol esters but not by receptor agonists

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Preincubation with receptor agonists or phorbol esters desensitized muscarinic-receptor-mediated [³H]cyclic GMP responses in mouse neuroblastoma N1E-115 cells. However, desensitization mediated by phorbol esters was heterologous, whereas that effected by receptor agonist was specific towards the muscarinic receptors. In addition, there was no loss of cell surface muscarinic receptors, as measured by the binding of the hydrophilic ligand [³H]N-methylscopolamine, when cells were treated with phorbol esters, but receptor-agonist-induced desensitization was accompanied by a decrease in cell surface receptor density. We examined the role of protein kinase C (PKC) in the desensitization of muscarinic receptors by employing a kinase inhibitor and by down-regulation of PKC by long-term incubation of cells with phorbol esters. Whereas these manoeuvres had marked effects on phorbol-ester-induced desensitization of muscarinic responses, they did not block agonist-induced down-regulation and desensitization of muscarinic receptors. In addition, when phosphoinositide hydrolysis was suppressed, the muscarinic agonist was still capable of mediating receptor sequestration and desensitization. These results suggest that the mechanisms for regulating muscarinic receptor sensitivity could be both PKC-dependent and PKC-independent, being mediated by phorbol esters and receptor agonists respectively.

INTRODUCTION

Muscarinic receptors, like many other receptor systems, are susceptible to agonist-induced desensitization. Thus prolonged interaction between agonists and muscarinic receptors results in desensitization of the receptor-mediated increases in cyclic GMP formation [1–3], phosphoinositide (PI) hydrolysis [4–6] and intracellular Ca²⁺ [4,7], and inhibition of adenylate cyclase [8]. Although the precise molecular and biochemical events underlying muscarinic receptor desensitization remain unclear, it has been shown that a good correlation exists between the relative efficacy of a series of muscarinic agonists for stimulation of PI breakdown and their effectiveness in inducing receptor sequestration [9] and desensitization of receptor-mediated electrophysiological responses [10]. The breakdown of phosphatidylinositol biphosphate upon stimulation of a variety of neurotransmitter receptors generates diacylglycerol (DAG) [11], an endogenous activator of protein kinase C (PKC) [12]. The tumour-promoting phorbol esters activate PKC by substituting for the endogenous DAG in a variety of tissues [13], and they have been useful in studying the role of this kinase in cell regulation. There are numerous examples where phorbol esters induce a decrease in the accumulation of inositol phosphates [14–17] and cyclic GMP [18] in response to muscarinic agonists. Therefore involvement of PKC has been implicated in desensitization of muscarinic receptors [19,20], representing a negative feedback control in response to increased PI breakdown (for discussion, see [21]).

In the light of this hypothesis, the goal of the present study was to rigorously assess the role of PKC in desensitization of muscarinic receptors induced by either receptor agonists or phorbol esters using the widely studied murine neuroblastoma N1E-115 cell line [22]. The muscarinic-receptor-mediated re-

sponses we have chosen to monitor are cyclic GMP formation and PI hydrolysis. These two responses are mediated by the same muscarinic receptor subtype (M₁) in N1E-115 cells, as defined pharmacologically by the use of the novel antagonist pirenzepine [23,24]. In addition, both are susceptible to desensitization by phorbol esters and receptor agonists [18,25,26]. The major finding is that there appear to be two distinct and independent pathways underlying muscarinic receptor desensitization in N1E-115 cells. Once pathway, evoked by phorbol esters, is mediated by PKC, whereas the other mechanism, evoked by muscarinic agonists, is independent of PKC.

MATERIALS AND METHODS

Culture of N1E-115 cells

Mouse neuroblastoma cells (clone N1E-115) were grown in 75 cm² tissue culture flasks in 20 ml of Dulbecco's modified Eagle's medium (DMEM) without antibiotics and supplemented with 10% newborn calf serum (Gibco, Grand Island, NY, U.S.A.). Cells were kept at 37 °C in a humidified atmosphere consisting of CO₂/air (1:9). Cells of passages 11 to 17 were used for the experiments. Phorbol esters (Behring Diagnostics, La Jolla, CA, U.S.A.) were added to cultures in monolayer in 20 ml of DMEM containing 2% serum. Phorbol esters were first dissolved in dimethyl sulphoxide (Me₂SO) and then diluted with DMEM. The final concentrations of Me₂SO in culture flasks were 0.002–0.02% (v/v), and the same concentrations of Me₂SO were also present in corresponding control flasks. For prolonged treatment of the cells with phorbol 12,13-dibutyrate (PDBu), the phorbol ester was dissolved in Me₂SO and diluted with DMEM to a concentration of 100 μM, and sterilized as described previously [25].

Abbreviations used: PI, phosphoinositide; PKC, protein kinase C; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; [³H]NMS, [³H]N-methylscopolamine; PDBu, phorbol 12,13-dibutyrate; PDAC, phorbol 12,13-diacetate; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride; CBC, carbamylcholine; DMEM, Dulbecco's modified Eagle's medium; Me₂SO, dimethyl sulphoxide; IC₅₀, concn. causing 50% inhibition.

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[³H]N-Methylscopolamine ([³H]NMS) binding assay in intact cells

Cells were harvested by gentle shaking and centrifuged at 250 *g* for 1 min at 4 °C, followed by washing twice with Hepes buffer containing (mM): NaCl, 110; KCl, 5.3; CaCl₂, 1.8; MgSO₄, 1.0; glucose, 25; Hepes, 20. The pH was titrated to 7.4 with NaOH and osmolality was adjusted to 333 ± 5 mosmol/l by adding sucrose. The cells (approx. 1 mg of protein) were then incubated for 90 min at 15 °C in 1 ml of Hepes buffer containing 0.2–0.5 nM final concentration of [³H]NMS (73–84 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.). Non-specific binding was defined in the presence of 2 μM-atropine. Assays were terminated by filtration as described [18,25].

[³H]Cyclic GMP measurements

Relative changes in cyclic GMP formation in intact cells were measured as described [26]. Briefly, cells were collected by incubation with 10 ml of Puck's D₁ solution [27] followed by centrifugation at 250 *g* for 1 min, and the pellet was washed twice with Hepes buffer. The cells were then suspended at approx. 4 × 10⁶ cells/ml of Hepes buffer containing 7.5 μCi of [³H]guanosine/ml (5 Ci/mmol; ICN Biochemicals, Irvine, CA, U.S.A.) and incubated in a shaking water bath at 37 °C for 60 min at 40 rev./min and then washed once with Hepes buffer. The cells were resuspended in Hepes buffer and distributed into the wells of a multiwell plate (approx. 0.2 × 10⁶ cells/well; 0.3 ml final reaction volume) and equilibrated at 37 °C for 15 min in shaking water bath. Phorbol 12-myristate 13-acetate (PMA) (in Me₂SO; final concentrations of solvent of 0.0001–0.1 % in the wells) was added in a 30 μl volume. Carbamylcholine (CBC) or histamine (30 μl) was added to each well to give a final concentration of 1 mM. Basal [³H]cyclic GMP levels were determined by adding 30 μl of Hepes buffer only. The reaction was terminated after 30 s and [³H]cyclic GMP was isolated by ion-exchange chromatography as previously described [18]. All samples were corrected for recovery of added [¹⁴C]cyclic GMP, which was usually 65–80 %.

Cell fractionation and PKC assay

N1E-115 cells detached from culture flasks by incubation with D₁ buffer were washed twice with ice-cold HEE buffer (20 mM-Hepes/2 mM-EDTA/2 mM-EGTA, pH 7.5) containing 250 mM-sucrose. The cells were then resuspended in HEE buffer containing 25 μg of leupeptin/ml and sonicated with 3 × 10 s bursts. The homogenates were centrifuged at 100 000 *g* for 60 min at 4 °C to separate the cytosolic and particulate fractions. The cytosolic fraction was kept on ice, with Nonidet P-40 (Sigma Chemical Co., St. Louis, MO, U.S.A.) added to a final concentration of 1 %. The pellet was resuspended in HEE/leupeptin buffer containing 1 % Nonidet P-40 and stirred on ice for 1 h, and then centrifuged at 100 000 *g* for 30 min. The supernatant of this solubilized particulate fraction and the cytosolic fraction were passed on to DE-52 cellulose columns (Whatman Bio-systems, Clifton, NJ, U.S.A.) pre-equilibrated with HEE buffer (pH 7.5) at 4 °C. After washing with 6 bed vol. of HEE buffer, PKC was eluted with HEE buffer containing 100 mM-NaCl in 8 × 0.7 ml fractions. Fractions with highest PKC activity were pooled and used for the enzymic assay. PKC activity was quantified by measuring incorporation of ³²P from [γ-³²P]ATP into histone type III-S (Sigma), as modified from [28]. The reaction mixture in a final volume of 0.25 ml contained 30 mM-Hepes (pH 7.5), DE-52 column eluate containing 3–5 μg of protein, 60 μM-[γ-³²P]ATP [(0.5–1) × 10⁶ c.p.m., 3000 Ci/mmol, New England Nuclear], 10 mM-magnesium acetate and 25 μg of type III-S histone, with or without 70 μM free Ca²⁺, 10 μg of

phosphatidylserine and 4 μg of dioleoylglycerol (all from Sigma). After 3 min at 37 °C, the reaction was stopped by adding 1 ml of cold 1 M-NaH₂PO₄ in 20 % trichloroacetic acid, and the reaction mixture was filtered through a GF/C filter, washed with 2 × 3 ml of 1 M-NaH₂PO₄ in 10 % trichloroacetic acid and counted for radioactivity by liquid scintillation spectrometry. The Ca²⁺- and phospholipid-dependent phosphorylation was determined by subtracting the amount of ³²P incorporated under the basal conditions from that in the presence of phospholipid and Ca²⁺. In the absence of phospholipid, the amount of ³²P incorporated with the addition or omission of Ca²⁺ were the same (results not shown); therefore it is unlikely that other Ca²⁺-dependent protein kinases contributed to ³²P incorporation into the histone when both phospholipid and Ca²⁺ were present.

The effect of H-7 [1-(5-isoquinolinesulphonyl)-2-methyl-piperazine dihydrochloride] was tested as follows. Enzyme preparations were incubated with increasing concentrations of H-7 at room temperature for 10 min before addition of histone, phospholipids and Ca²⁺, and the reaction was initiated by adding 1 μM-ATP. The enzymic activity was inhibited by H-7 in a concentration-dependent fashion with an IC₅₀ (concn. causing 50 % inhibition) of 15 μM. Protein determination was according to the method of Bradford [29], using γ-globulin as standard.

RESULTS

Characteristics of desensitization induced by muscarinic agonists and phorbol esters

After N1E-115 cells were treated with the muscarinic receptor agonist CBC (1 mM) for 30 min and then washed, the cyclic GMP formation stimulated by a second addition of CBC was reduced to 12.5 ± 0.8 % of that in control cells. A similar refractoriness of the cyclic GMP response to CBC (18.2 ± 3.9 % of control) was observed after the cells were exposed to 100 nM-PMA for 60 min (results not shown). CBC-induced desensitization was specific toward the muscarinic receptors as CBC pretreatment caused only a slight desensitization of the histamine-mediated cyclic GMP response (results not shown). However, desensitization induced by PMA was heterologous, since preincubation of the cells with PMA also desensitized histamine-H₁-receptor-mediated [³H]cyclic GMP formation.

CBC, but not phorbol esters, down-regulates muscarinic receptors

Preincubation of N1E-115 cells with CBC for 60 min significantly down-regulated subsequent [³H]NMS binding to cell surface receptors (Fig. 1), as in previous reports [6,30]. On the other hand, pre-exposure of the cells to 100 nM-PMA for up to 2 h had no significant effect on [³H]NMS binding (Fig. 1). Although statistically significant decreases in [³H]NMS binding occurred after treatment with PMA for longer time periods, down-regulation brought about by such treatment was rather small in magnitude. Thus specific [³H]NMS binding only decreased to 72.5 ± 3.7 % of control values after 4 h of PMA preincubation and to 71.9 ± 3.0 % after 12 h, in comparison with a value of 41.7 ± 0.5 % of control when cells were treated with 1 mM-CBC for 60 min. The absence of phorbol-ester-induced receptor down-regulation was in spite of the fact that treatment of cells with phorbol esters caused redistribution of PKC, an index of the kinase activation. Thus incubation of the cells with 1 μM-PDBu caused a significant increase in specific PKC activity in the particulate fraction as early as 5 min after the addition of the phorbol ester to the medium. This increase was sustained for up to 2 h of incubation with PDBu and was accompanied by a parallel decrease in cytosolic activity (Fig. 2). PDBu was used in these experiments since it is much less lipophilic, and therefore

Fig. 1. Effect of pretreatment with PMA or CBC on [3 H]NMS binding to N1E-115 cells

Cells were incubated in DMEM for the time periods indicated in the absence (□) or presence (▨) of 100 nM-PMA or with 1 mM-CBC (■) for 60 min at 37 °C, then washed twice with Hepes buffer and incubated with 0.2 nM-[3 H]NMS for 90 min at 15 °C. The incubation was terminated by rapid filtration through GF/B filters. Data presented are averaged results from between three and eleven independent experiments, each performed in quadruplicate, and the bars represent the S.E.M. *Significantly different from control, $P < 0.05$. Numbers in parentheses indicate numbers of experiments performed.

more readily washable, than PMA. Upon incubation of cells with [3 H]PDBu followed by washing twice with cold Hepes buffer, there was no detectable specific [3 H]PDBu binding [25]. Therefore the changes in PKC activity assessed in these assays was not caused by residual phorbol ester. The same extent of PKC translocation was induced by treatment of the cells with 100 nM-PMA (results not shown). Increasing Ca^{2+} availability to the intracellular environment by the use of Ca^{2+} ionophores is thought to facilitate phorbol ester activation of PKC [31]. Supplementation of the incubation medium containing PMA with 0.3 μM of the Ca^{2+} ionophore A23187, however, did not modify [3 H]NMS binding as compared with cells treated with PMA alone (results not shown). Therefore activation of PKC by

Fig. 2. Translocation of PKC induced by PDBu

Cells were incubated without (control) or with 1 μM -PDBu and prepared for PKC assays as described in the Materials and methods section. Data shown are means $\pm$ S.D. ($n = 2-5$) of percentage distributions of PKC activity induced by PDBu pretreatment as compared with the corresponding fraction of control cells in a parallel preparation.

phorbol esters seems to correlate with desensitization of the receptor-mediated cyclic GMP response but has no effect on muscarinic receptor density.

CBC induces sequestration and desensitization of muscarinic receptors under conditions where agonist-mediated PI hydrolysis is significantly suppressed

To test the role of PKC in agonist-induced down-regulation of muscarinic receptors, cells were incubated with 100 nM-PMA for 1 h, a condition in which the CBC-induced PI response was inhibited to $21.2 \pm 1.3\%$ of control values [25]. The addition of submaximal desensitization-inducing concentrations of CBC for another 1 h down-regulated [3 H]NMS binding in a concentration-dependent fashion that was not different from cells treated with CBC alone (Fig. 3). Moreover, incubation of cells for 45 min with 20 μM -phorbol diacetate (PDAC), a readily washable phorbol ester, suppressed agonist-mediated inositol phosphate accumulation to approx. 10% of that in the control (Fig. 4, inset, assayed as described previously [25]), and agonist-induced [3 H]cyclic GMP formation was inhibited to $30.4 \pm 2.3\%$ of control values. The reasons for this discrepancy are not clear at present. Under these conditions, an additional 15 min of incubation with 1 mM-CBC desensitized further the subsequent CBC-mediated cyclic GMP response (Fig. 4) resulting in $48 \pm 11.8\%$ of the response compared with cells treated with PDAC alone. This extent of desensitization of the cyclic GMP response was comparable with agonist-induced desensitization of cyclic GMP formation ($43.1 \pm 3.2\%$ of control) in cells which were not pretreated with PDAC (Fig. 4).

The kinase inhibitor H-7 attenuates PMA-induced desensitization of the CBC-mediated response but fails to prevent muscarinic-agonist-induced desensitization

The enzymic activity of PKC was inhibited by H-7 in a concentration-dependent manner in N1E-115 cells, with an IC_{50} of 15 μM (results not shown). The inhibitory effect of PMA on CBC-induced cyclic GMP formation in these cells was also reduced significantly by H-7 (Fig. 5). However, preincubation of the cells with 200 μM -H-7 for 30 min followed by an additional

Fig. 3. Effect of preincubation with PMA on CBC-induced decrease in [3 H]NMS binding to N1E-115 cells

After the cells were incubated for 1 h in DMEM at 37 °C in the absence or presence of 100 nM-PMA, CBC was added at the concentrations indicated and incubated with the cells for an additional 1 h. The cells were then washed twice with cold Hepes buffer and incubated with 0.2 nM-[3 H]NMS for 90 min at 15 °C. Data shown are averages of four or five independent experiments, each performed in quadruplicate, and the bars represent S.E.M.

Fig. 4. Preincubation with CBC induces desensitization of [^3H]cyclic GMP under conditions of suppressed PI hydrolysis

Cells were prepared for [^3H]cyclic GMP experiments as described in the Materials and methods section, and then diluted to approx. 0.25×10^6 cells/ml and incubated with or without $20 \mu\text{M}$ -PDAc for 45 min at 37°C . CBC (1 mM) was then added for an additional 15 min. Drug treatments were terminated by rapid dilution and washing with HEPES buffer. [^3H]cyclic GMP assays were done as described. Results shown are from a representative of three independent experiments each with similar outcomes. The inset shows a representative on three experiments of CBC-induced [^3H]inositol phosphates (IP) formation in control cells (C) and in cells pretreated with $20 \mu\text{M}$ -PDAc for 45 min.

Fig. 5. H-7 attenuates the PMA inhibitory effect on [^3H]cyclic GMP formation but fails to prevent CBC-induced desensitization

Cells were prepared for [^3H]cyclic GMP assays as described in the Materials and methods section, and then incubated with (□) or without (▨) $200 \mu\text{M}$ -H-7 for 30 min at 37°C . Cells were treated with or without 50 nM -PMA (60 min) or 1 mM-CBC (30 min), and the subsequent measurements of [^3H]cyclic GMP mediated by CBC were done as described in the Materials and methods section. Data shown are averages of three independent experiments, each performed in triplicate. *Significantly different from control; $P < 0.05$.

15 min of CBC treatment did not block the desensitization of the subsequent CBC-mediated cyclic GMP response (Fig. 5).

PKC down-regulation attenuates the PMA inhibitory effect while having no influence on agonist-induced desensitization and sequestration of muscarinic receptors

Incubation of cells with $1 \mu\text{M}$ -PDBu for 24 h down-regulated [^3H]PDBu binding to $12.8 \pm 0.6\%$ of that in control cells (results not shown). Upon this treatment, PKC activity, as shown in Fig. 6, was also decreased from 7.66 ± 1.5 to 0.75 ± 0.1 nmol/min in the cytosolic fraction, and from 1.0 ± 0.1 to 0.26 ± 0.04 nmol/min in the particulate fraction. The overall activity of PKC was

Fig. 6. Down-regulation of PKC by long-term incubation with PDBu

Cell monolayers were incubated with (▨) or without (□) $1 \mu\text{M}$ -PDBu for 24 h, then washed and prepared for PKC assays as described in the Materials and methods section. The inset shows percentages of total (cytosolic plus particulate) PKC activity in untreated (UnT) and PDBu-treated (T) cells. Data are averages of three independent experiments performed in quadruplicate, and bars show S.E.M.

Fig. 7. Desensitization of CBC-mediated cyclic GMP response induced by PMA is dependent on PKC activity whereas that induced by CBC is not

Cell monolayers were incubated with (▨, ●) or without (□, ○) $1 \mu\text{M}$ -PDBu for 24 h and prepared for [^3H]cyclic GMP measurements as described in the Materials and methods section. (a) After shaking in a multiwell plate for 15 min at 37°C , various concentrations of PMA were added for another 60 min before CBC (1 mM) was added to induce cyclic GMP formation. (b) Cells were treated with various concentrations of CBC for 30 min, washed, then stimulated with 1 mM-CBC. Data are averages of four independent experiments each done in triplicate; bars are S.E.M.

decreased to $12.4 \pm 1.5\%$ of control after 24 h of PDBu treatment (Fig. 6, inset). The basal PKC activity present in the same preparations was not significantly changed upon long-term phorbol ester incubation. It is noteworthy that after incubation of the cells with PDBu for 24 h, there was no detectable increase in Ca^{2+} - and phospholipid-independent kinase activity as assayed using the 400 mM-NaCl eluate from DE-52 columns (results not shown). Under these conditions, CBC-mediated cyclic GMP formation was $76 \pm 6.9\%$ of that in cells not treated with PDBu (results not shown). After the cells had been incubated with $1\ \mu\text{M}$ -PDBu for 24 h, then washed free of PDBu and reincubated with increasing concentrations of PMA for 1 h, it was found that the inhibitory effect of PMA on CBC-mediated cyclic GMP formation was almost lost, except with very high concentrations of the phorbol ester (Fig. 7a). In comparison with this, when PKC-down-regulated cells were incubated with increasing concentrations of CBC for 30 min, an agonist-concentration-dependent desensitization of subsequent CBC-induced cyclic GMP form-

ation was observed, in the same fashion and to the same extent as in untreated cells (Fig. 7b). Furthermore, incubation of PKC-down-regulated cells with 1 mM-CBC induced desensitization of the subsequent CBC-mediated cyclic GMP response with a time course that was essentially identical with that in cells which were not treated with PDBu (Fig. 8a). In cells whose PKC was down-regulated by long-term phorbol ester treatment, [^3H]NMS binding was equal to that in control cells ($99.3 \pm 1.9\%$ of control; results not shown). Treatment of the cells with CBC for an additional 1 h induced a concentration-dependent down-regulation of [^3H]NMS binding in the same fashion and to the same extent as seen in control cells (Fig. 8b). Thus it appears that CBC-induced down-regulation of muscarinic receptors is independent of PKC.

DISCUSSION

It is well documented that exposure of N1E-115 cells to PKC-activating phorbol esters [18,25,32] or receptor agonists [1-3,33,34] results in desensitization of muscarinic-agonist-mediated responses. The results reported here provide strong evidence that, although the effects of phorbol esters on muscarinic receptor sensitivity are mediated by PKC, this kinase is not involved in the desensitizing effects of receptor agonists.

The diminished cellular cyclic GMP response resulting from prolonged incubation of N1E-115 cells with CBC, but not with phorbol esters, is accompanied by an apparent internalization of muscarinic receptors as demonstrated by the diminished binding of the hydrophilic ligand [^3H]NMS. This lack of effect of phorbol esters was despite the observation that, during these treatment periods, PKC activity translocated to the particulate fraction remained elevated, a phenomenon which is considered to be related to PKC activation [35]. Additionally, the presence of the Ca^{2+} ionophore A23187 did not reveal any effect of phorbol esters on [^3H]NMS binding, although an increase in intracellular Ca^{2+} concentration is believed to potentiate the effects of phorbol esters in activating PKC [36]. Thus, from our and others' findings [15], cell surface muscarinic receptor number does not seem to be affected by PKC activation. However, these results are in disagreement with those reported by other investigators [19]. Furthermore, whereas agonist-induced desensitization was specific towards the muscarinic receptors, that caused by phorbol esters was heterologous, suggestive of different mechanisms of action. This is also supported by the finding that desensitization by both groups of agents was additive.

Under conditions of phorbol ester treatment where CBC-mediated accumulation of inositol phosphates was suppressed ([25,32]; this study), CBC still induced a concentration-dependent decrease in [^3H]NMS binding, as well as desensitization of subsequent cyclic GMP responses which were not different from those obtained in control cells. Under these conditions, the amount of DAG produced by CBC during the desensitization step is presumably negligible. In addition, although NaF mediates a much stronger signal in inositol phosphate accumulation than muscarinic agonists in N1E-115 cells (W. S. Lai, unpublished work), it did not down-regulate [^3H]NMS binding (results not shown). These data, in addition to those discussed above, suggest that PI hydrolysis products, and subsequent PKC activation, are not involved in agonist-induced desensitization or down-regulation of muscarinic receptors.

Although the kinase inhibitor H-7 [37] suppressed the enzymic activity of PKC as well as PMA-induced desensitization of muscarinic-receptor-mediated responses in N1E-115 cells, it did not influence muscarinic-agonist-induced receptor desensitization. Furthermore, treatment of N1E-115 cells with a PKC-activating phorbol ester for 24 h caused a loss of 90% of the

Fig. 8. CBC-induced receptor desensitization and down-regulation in the presence or absence of PKC activity

(a) Cell monolayers were incubated with (◆) or without (◇) $1\ \mu\text{M}$ -PDBu for 24 h, then prepared for [^3H]cyclic GMP assays as described in the Materials and methods section, and preincubated with 1 mM-CBC for the indicated time periods. Cells were washed and stimulated with 1 mM-CBC in triplicate. Data presented are averaged results of six independent experiments. Bars show S.E.M. (b) Cell monolayers were incubated with (▲) or without (△) $1\ \mu\text{M}$ -PDBu for 24 h, CBC was then added into flasks at the indicated concentrations and incubated for an additional 1 h at 37°C . Cells were then washed twice with Hepes buffer and incubated with $0.5\ \text{nM}$ -[^3H]NMS for 90 min at 15°C and the assay was terminated by rapid filtration through GF/B filters. Data presented are averaged results of five independent experiments each performed in quadruplicate, and the bars show S.E.M.

detectable PKC activity as well as 90 % of [^3H]PDBu binding, (this work; [25]), similar to findings in other cell types [38], and resulted in significant attenuation of the inhibition of muscarinic receptor function by phorbol esters. In contrast, PDBu-treated cells were able to undergo muscarinic-agonist-induced desensitization of the cyclic GMP response and receptor down-regulation with a time course and concentration-dependence similar to those observed in untreated cells. Thus these findings support the notion that PKC is involved in the observed actions of phorbol esters, whereas it does not play an important role in receptor-agonist-induced desensitization. A dichotomy of mechanisms of desensitization induced by these two classes of agents has been suggested for the β -adrenergic-receptor-adenylate cyclase system in rat glioma C6 cells using a similar approach [39]. However, we cannot exclude the remote possibility that residual PKC activity (10 % of control) might play a role in agonist-induced desensitization. It is noteworthy that this residual enzyme did not represent the Ca^{2+} - and phospholipid-independent form of PKC which is formed by its proteolytic cleavage [40].

Taken together, several experimental designs presented herein indicate that possible activation of PKC by muscarinic agonists does not play an important role in their ability to desensitize receptor responses. Similarly, recent studies suggest that agonist-induced desensitization of other receptors coupled to PI hydrolysis may be independent of PKC activation [41,42]. In fact, phorbol esters desensitize the function of other neurotransmitter receptors which are not coupled to PI hydrolysis, e.g. β -adrenergic receptors in certain types of cells [39]. Therefore, although the effects of pretreatment of a certain tissue with either a phorbol ester or a receptor agonist on receptor sensitivity might appear similar, these effects might be mediated by different cellular mechanisms.

In summary, we have found that exposure of mouse neuroblastoma N1E-115 cells to phorbol esters resulted in desensitization of the CBC-mediated cyclic GMP response, and this resembled the desensitization induced by preincubation of the cells with a muscarinic receptor agonist, except in being heterologous. It is evident, however, that phorbol-ester-mediated suppression of receptor function is PKC-dependent, whereas agonist-induced desensitization is PKC-independent. Evidence for such divergent mechanisms of muscarinic receptor regulation is severalfold. First, down-regulation of cell surface receptor density occurred upon agonist pretreatment but not phorbol ester incubation. Secondly, receptor agonist was fully capable of inducing down-regulation and desensitization of the muscarinic response under conditions where the PI response was suppressed. Thirdly, a protein kinase inhibitor diminished the effects of phorbol esters without influencing agonist-induced desensitization. Fourthly, although long-term phorbol ester treatment rendered a major loss of the ability of phorbol esters to desensitize the CBC-mediated biochemical response, PKC-depleted cells showed unaltered muscarinic-agonist-mediated receptor desensitization and internalization. Finally, whereas it has been shown that muscarinic-agonist-induced desensitization in cardiac tissue is accompanied by receptor phosphorylation, phorbol ester treatment was ineffective in this respect [43]. In conclusion, alterations in muscarinic receptor sensitivity could be mediated by both PKC-dependent and PKC-independent mechanisms which are triggered by phorbol esters and receptor agonists respectively.

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