The effect of protein kinase C activation on muscarinic-M₃- and K⁺-evoked release of [³H]noradrenaline and increases in intracellular Ca²⁺ in human neuroblastoma SH-SY5Y cells

Nuala P. MURPHY,*† James G. McCORMACK,†§ Stephen G. BALL* and Peter F. T. VAUGHAN*||

*Department of Cardiovascular Studies and †Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K.

Short-term pretreatment (9 min) with the phorbol ester 12-myristate 13-acetate (PMA) alone had no effect on the basal release of [³H]noradrenaline ([³H]NA), but enhanced K⁺ (100 mM)-, acetylcholine (0.1 mM)-, carbachol (1 mM)-, muscarine (1 mM)- and arecoline (1 mM)-evoked release by 2.3-, 6.4-, 3.0-, 2.0- and 2.0-fold respectively in SH-SY5Y cells. Maximum effects of PMA were observed after a 10 min preincubation at a concentration of 0.1 μM. There was a 4-fold decrease in the EC₅₀ values (concentration required for 50 % of maximal stimulation) observed for carbachol- and acetylcholine-evoked release of [³H]NA in the presence of PMA. The inactive phorbol ester 4α-phorbol 12,13-didecanoate did not alter the K⁺- or carbachol-evoked release of [³H]NA. The enhancement of release in the presence of PMA was more potently inhibited by the protein kinase C inhibitors RO 31-7549 [concentration required for 50 % inhibition (IC₅₀) = 0.18 μM] and RO 31-8220 (IC₅₀ = 0.56 μM) than by either polymyxin-B or H-7. Furthermore, in the absence of PMA, both K⁺- and carbachol-evoked release was inhibited by these antagonists. Atropine, hexahydro-sila-difenidol and pirenzepine antagonized the PMA-enhanced carbachol-evoked release of [³H]NA, with K_i values of 2.75 ± 0.25 nM, 2.6 ± 0.64 nM and 294 ± 17 nM respectively. These values were consistent with the coupling of an M₃ muscarinic receptor to the release of [³H]NA in SH-SY5Y cells. Whereas pretreatment with PMA (5 min) enhanced M₃-evoked release of [³H]NA, it decreased the muscarinic-agonist-evoked initial peak (> 85 %) and plateau phase in intracellular Ca²⁺. These results suggest that noradrenaline release evoked by muscarinic agonists was triggered not only by relatively small changes in Ca²⁺ but also by activation of protein kinase C.

INTRODUCTION

In a previous report, we have shown that carbachol-evoked release of [³H]noradrenaline ([³H]NA) in the cell line SH-SY5Y was coupled to an M₃ muscarinic receptor [Murphy *et al.* (1991a); see Bonner (1989) for classification of muscarinic receptors]. Activation of M₃ receptors results in the hydrolysis of phosphoinositides (Bonner, 1989), the primary products of which are *myo*-inositol 1,4,5-trisphosphate (InsP₃), which is linked to the mobilization of intracellular Ca²⁺, and diacylglycerol, which activates protein kinase C (Berridge, 1987). The muscarinic agonists carbachol and acetylcholine induced a biphasic increase in intracellular Ca²⁺ in SH-SY5Y cells, consisting of an initial peak (resulting from InsP₃ mobilization of intracellular Ca²⁺), followed by a decline to a plateau, which was dependent not only on an influx of extracellular Ca²⁺ but also on the continued presence of agonist (Murphy *et al.*, 1991b; Lambert & Nahorski, 1990). The Ca²⁺ signal resulting from muscarinic-receptor activation was similar to that previously reported in SH-SY5Y cells (Lambert & Nahorski, 1990), SK-N-SH cells (Fisher *et al.*, 1989), platelets (Merritt & Hallam, 1988) and human endothelial cells (Hallam *et al.*, 1988). Both the peak and the plateau phases were shown to be mediated via an M₃ muscarinic receptor (Murphy *et al.*, 1991b).

The phorbol ester phorbol 12-myristate 13-acetate (PMA; 'TPA'), which possesses a diacylglycerol-like structure, activates the Ca²⁺-phospholipid-dependent protein kinase C at extremely low concentrations, by increasing its association with the plasma

membrane (Nishizuka, 1984a,b). PMA has been shown to enhance or stimulate exocytotic secretion from several cell types, including rat brain synaptosomes (Nichols *et al.*, 1987), cultured foetal brain neurons (Zurgil & Zisapel, 1985) and adrenal medullary cells (Knight & Baker, 1983). In the present study, we therefore examined the effect of phorbol esters on the K⁺ and cholinergic regulation of [³H]NA release and intracellular Ca²⁺.

MATERIALS AND METHODS

Materials

The SH-SY5Y cell line was kindly provided by Dr June L. Biedler of the Sloan-Kettering Institute for Cancer Research (Rye, NY, U.S.A.). Eagle's minimum essential medium, non-essential amino acids, Ham's F12 nutrient mixture, foetal-calf serum, trypsin and phosphate-buffered saline were supplied by Gibco (U.K.). Carbachol, muscarine, arecoline, ionomycin, fura2/AM, pirenzepine, atropine, EGTA, PMA, 4α-phorbol 12,13-didecanoate (4α-PDD), 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7), polymyxin B sulphate and d-tubocurarine were obtained from Sigma Chemical Co. (U.K.). Hexahydro-sila-difenidol (HHSD) hydrochloride was kindly given by Dr. Gunter Lambrecht and Dr. Reinhold Tacke, University of Karlsruhe, Germany.

Tissue culture

Cells of the human adrenergic neuroblastoma cell line SH-SY5Y were cultured in a 1:1 mixture of Ham's F12 nutrient

Abbreviations used: HBS, Hepes-buffered saline; HHSD, hexahydro-sila-difenidol; [³H]NA, [³H]noradrenaline; 4α-PDD, 4α-phorbol 12,13-didecanoate; PMA, phorbol 12-myristate 13-acetate.

†Present address: Laboratoire de Neuropharmacologie, Collège de France, 11 place M. Berthelot, 75231 Paris Cedex 05, France.

§ Present address: Department of Pharmacology, Syntex Research Centre, Research Avenue South, Heriot-Watt University Research Park, Riccarton, Edinburgh EH14 4AP, U.K.

|| To whom correspondence and reprint requests should be addressed.

mixture and Eagle's minimum essential medium containing 1% non-essential amino acids supplemented with 10% (v/v) foetal-calf serum in air/CO₂ (39:1) in a humidified incubator at 37 °C. The cell monolayers were harvested by treatment with phosphate-buffered saline containing 5% (w/v) trypsin and then cultured in either 6-well plates (to measure the release of [³H]NA) or 25 cm² flasks (to monitor intracellular Ca²⁺) at a seeding density of 10⁵ cells/ml.

Measurement of [³H]NA release

This was done by the method described by Murphy *et al.* (1991a). Briefly, culture medium was removed from SH-SY5Y cells in 6-well plates and replaced by 1.0 ml of Hepes-buffered saline (HBS; 135 mM-NaCl, 5 mM-KCl, 0.6 mM-MgSO₄·7H₂O, 2.5 mM-CaCl₂·2H₂O, 10 mM-Hepes, 6 mM-glucose, 0.2 mM-pargyline and 0.2 mM-ascorbic acid) containing 40 nM-[³H]NA. After incubation for 1 h at 37 °C, the ³H-supplemented medium was removed. The unaccumulated ³H was removed by washing the monolayer consecutively with 6 × 2.0 ml of HBS containing desmethylinipramine (1 µM) at 37 °C. The release of radioactivity was then monitored by exposing the monolayer to 1.0 ml of HBS for 3 min at 37 °C. Release was induced by incubating the cells with elevated concentrations of K⁺, carbachol, acetylcholine, muscarine or arecoline. [³H]NA released into the medium was expressed as a percentage of the total amount of radioactivity in the cell monolayer at the beginning of the incubation period.

Measurement of intracellular Ca²⁺

Intracellular Ca²⁺ was monitored by the method previously described by Murphy *et al.* (1991b). Briefly, SH-SY5Y cells were cultured in 25 cm² flasks for 7 days until confluent. The medium was removed and replaced by 1 ml of phosphate-buffered saline containing 1 mM-EDTA and 10 µM fura2/AM. Preliminary experiments showed that loading under these conditions was convenient and reproducible. After a 15 min incubation period, the cells were centrifuged at 1000 *g* for 3 min and resuspended in 2 ml of HBS* (137 mM-NaCl, 5 mM-KCl, 1 mM-CaCl₂, 5 mM-glucose, 1.2 mM-MgCl₂, 0.44 mM-KH₂PO₄, 4.2 mM-NaHCO₃ and 20 mM-Hepes) containing 5% (w/v) Ficoll to prevent sedimentation. Intracellular Ca²⁺ was then measured in 2 ml suspensions of constantly stirred fura2-loaded SH-SY5Y cells. Fura2 fluorescence was monitored for 5 min before addition of agonists. The response did not vary provided that the agonist was added between 1 and 8 min after resuspending the cell pellet. Fura2 fluorescence, after addition of carbachol or acetylcholine, was generally monitored in a Perkin-Elmer LS-5 spectrofluorimeter at 37 °C with an excitation wavelength of 340 nm and an emission wavelength of 500 nm. Control experiments using the isosbestic point for fura2 (362.5 nm excitation) established that no non-specific changes occurred during the experiments. After measurement of the fluorescence values during the experiments (*F*), the cells were then lysed with 0.1% (v/v) Triton X-100 to obtain *F*_{max}, the fluorescence of Ca²⁺-saturated dye (Cobbold & Rink, 1987). *F*_{Mn}, the fluorescence signal after the dye is quenched, was monitored by addition of 4 mM-MnCl₂ and is used to calculate *F*_{min}. (fluorescence of free dye) with the equation: *F*_{min} = *F*_{Mn} + [(*F*_{max} - *F*_{Mn})/6]. Hence the concentration of intracellular Ca²⁺ for any value of *F* may be calculated by using the equation: [Ca²⁺]_i = *K*_d[(*F* - *F*_{min})/(*F*_{max} - *F*)] (Grynkiewicz *et al.*, 1985), where the *K*_d of fura2 is taken as 224 nM (Tsien *et al.*, 1982).

RESULTS

Effect of PMA on release of [³H]NA

The effect of preincubation time with PMA on carbachol-

evoked release of [³H]NA is illustrated in Fig. 1. Maximum enhancement was observed after a 10 min preincubation period. On the basis of these results, it was decided to preincubate SH-SY5Y cells for 9 min in experiments to determine the role of

Fig. 1. Effect of preincubating SH-SY5Y cells with PMA for various times on carbachol-evoked release of [³H]NA in SH-SY5Y cells

Cells were preincubated with 0.1 µM-PMA for the times indicated, before the addition of 1 mM-carbachol for 3 min. The zero value represents release in the presence of PMA added simultaneously with carbachol. Results are expressed as percentage release from which the basal release (2.0 ± 0.2%; *n* = 23) has been subtracted, and are means ± S.E.M. (*n* = 4).

Fig. 2. Dose-response profile of PMA on carbachol-evoked release of [³H]NA in SH-SY5Y cells

SH-SY5Y cells preloaded with [³H]NA were preincubated with the indicated concentration of PMA for 9 min. Release of [³H]NA was stimulated in the presence of carbachol (1 mM). Results are expressed as percentage release from which basal release has been subtracted, and are means ± S.E.M. (*n* = 4).

Table 1. Effect of PMA and 4α-PDD on K⁺- and muscarinic-evoked release of [³H]NA by SH-SY5Y cells

SH-SY5Y cells were preincubated with PMA or 4α-PDD for 9 min. Release of [³H]NA was stimulated in the presence of K⁺ (100 mM), carbachol (1 mM), muscarine (2 mM) or arecoline (1 mM). Results are expressed as percentage release from which the basal release (approx. 2.5%, which was unaltered by addition of PMA alone) has been subtracted, and are means ± S.E.M. for the numbers of separate experiments in parentheses: **P* ≤ 0.05 for effect of PMA.

Stimulus	Release (%)		
	No PMA	+PMA	+4α-PDD
K ⁺	6.3 ± 1.2 (6)	14.7 ± 0.2 (6)*	6.6 ± 1.9 (6)
Carbachol	3.5 ± 0.5 (6)	11.6 ± 1.6 (6)*	3.1 ± 0.9 (6)
Arecoline	1.9 ± 0.4 (4)	4.0 ± 0.4 (4)*	—
Muscarine	3.7 ± 0.5 (12)	7.4 ± 0.5 (8)*	—

Fig. 3. Dose-response profiles of muscarinic-agonist-induced release of [3 H]NA in SH-SY5Y neuroblastoma cells

Release was evoked by addition of HBS containing the appropriate concentrations of (a) carbachol (■, ◆) or (b) acetylcholine (□, ◇) in the presence (□, ■) or absence (◇, ◆) of 0.1 μ M-PMA. SH-SY5Y cells were preincubated for 9 min with or without 0.1 μ M-PMA before addition of agonist. Results are expressed as percentage effect (basal release has been subtracted) and are means $\pm$ S.E.M. ($n = 4-6$): 100% effect was 2.32 ± 0.41 with 1 mM-carbachol-PMA and 7.76 ± 0.56 + PMA; 3.1 ± 0.01 with 1 mM-acetylcholine-PMA and 19.25 ± 0.25 + PMA.

Fig. 4. Effect of cholinergic antagonists on PMA-enhanced carbachol-evoked release of [3 H]NA

SH-SY5Y cells were preincubated for 10 min with atropine (◆), HHSD (●) or pirenzepine (▲), followed by a further 9 min incubation with the antagonist in the presence of 0.1 μ M-PMA, before stimulating release with 1 mM-carbachol. K_i values were calculated from the Cheng-Prusoff equation: $K_i = IC_{50}/(1 + [A]/EC_{50})$, where IC_{50} is the concentration of antagonist required to produce 50% inhibition of the carbachol-evoked release, EC_{50} (+PMA) is the concentration of agonist resulting in 50% stimulation of release and $[A]$ is the concentration of agonist (1 mM). Results are expressed as means $\pm$ S.E.M. ($n = 4$).

protein kinase C activation by phorbol esters in neurotransmitter release.

PMA enhanced carbachol (1 mM)-evoked release of [3 H]NA (Fig. 2), with maximum effect at a concentration of 0.1 μ M. This concentration of PMA was therefore chosen to investigate the effect of phorbol esters on both release and intracellular Ca^{2+} .

The phorbol ester PMA (0.1 μ M) alone had no effect on the basal release of [3 H]NA. However, pretreatment with PMA (9 min) enhanced K^+ (100 mM)- and carbachol (1 mM)-evoked release of [3 H]NA by 2.3- and 3.0-fold respectively (Table 1). The inactive isomer 4 α -PDD did not significantly alter the stimulated high- K^+ or carbachol-evoked release of [3 H]NA (Table 1). Furthermore, PMA enhanced muscarine (full agonist)- and

Table 2 Effects of RO 31-7549 and RO 31-8220 on carbachol and K^+ -evoked release of [3 H]NA in the presence and absence of PMA

SH-SY5Y cells were preincubated for 6 min with 10 μ M-RO 31-7549 (RO2) or -RO 31-8220 (RO3), followed by a further 9 min incubation with or without the inhibitors in the absence or presence of PMA (0.1 μ M) as indicated. Release of [3 H]NA was evoked by addition of carbachol (1 mM) or K^+ (100 mM) in the absence or presence of PMA. Results are expressed as means $\pm$ S.E.M. ($n = 4$).

Stimulus	Release (%)	
	Carbachol	K^+
No PMA	1.6 ± 0.2	3.7 ± 0.8
+ RO2	0.1 ± 0.6	-0.02 ± 0.2
+ RO3	-1.3 ± 0.4	0.5 ± 0.7
+ PMA	9.7 ± 0.1	9.7 ± 0.9
+ RO2	-0.1 ± 0.3	2.4 ± 0.3
+ RO3	1.0 ± 0.2	2.2 ± 0.7

Fig. 5. Effect of protein kinase C inhibitors on PMA-enhanced carbachol-evoked release of [3 H]NA

SH-SY5Y cells were preincubated with RO 31-8220 (□), RO 31-7549 (●), H-7 (■) and polymyxin B (◆) for 6 min, followed by a further 9 min incubation with the inhibitor in the presence of 0.1 μ M-PMA. Release was stimulated by 1 mM-carbachol in the presence of PMA (0.1 μ M). Results are expressed as means $\pm$ S.E.M. ($n = 4$).

arecoline (partial agonist)-evoked release of [3 H]NA by 2.0-fold (Table 1).

Release of [3 H]NA was stimulated with carbachol or acetylcholine in the presence or absence of PMA (0.1 μ M). EC_{50} (concentration required to achieve 50% of maximal stimulation) values of 26.5 ± 1.5 μ M and 1.3 ± 0.01 μ M were observed for carbachol and acetylcholine respectively in the presence of PMA, compared with 100 ± 10 μ M and 5.4 ± 0.9 μ M respectively in its absence (calculated from the data presented in Fig. 3). Hence there was a 4-fold decrease in the EC_{50} values for carbachol- and acetylcholine-evoked release, induced by preincubating with PMA.

The non-selective muscarinic antagonist atropine and the M_3 -selective antagonist HHSD potentially antagonized the carbachol-evoked release of [3 H]NA (Fig. 4), with K_i values of 2.75 ± 0.25 nM and 2.6 ± 0.64 nM respectively in the presence of PMA. In contrast, the M_1 muscarinic antagonist pirenzepine was less potent, displaying a K_i value of 294 ± 17 nM. These values are in a similar range to those reported previously in the absence of PMA (Murphy *et al.*, 1991a) and were consistent with the coupling of an M_3 muscarinic receptor to the release of [3 H]NA in SH-SY5Y cells.

Fig. 6. Effect of PMA on muscarinic-agonist-induced changes in intracellular Ca^{2+}

Fura2-loaded SH-SY5Y cells were preincubated with $0.1 \mu\text{M}$ -PMA (for the times indicated) before addition of (a) 0.5 mM -carbachol (CCH), which increased intracellular $[\text{Ca}^{2+}]$ from a basal value of 143 nM to 457.5 nM , and (b) $10 \mu\text{M}$ -acetylcholine (ACh), which increased intracellular $[\text{Ca}^{2+}]$ from a basal value of 199.4 nM to a peak value of 368.4 nM . Results shown are expressed as an increase in fluorescence and are typical traces from two or three experiments, each performed on separate batches of cells. The time-dependence of the PMA inhibition of the peak carbachol-evoked increases in intracellular Ca^{2+} is illustrated in (c).

Effect of protein kinase C inhibitors on release of $[\text{^3H}]\text{NA}$

The effect of protein kinase C inhibitors on PMA-enhanced release of $[\text{^3H}]\text{NA}$ was investigated. Four different inhibitors were used to try to inhibit the carbachol-evoked release of $[\text{^3H}]\text{NA}$. In the absence of PMA, the carbachol-evoked release was completely inhibited by RO 31-7549 ($10 \mu\text{M}$) and RO 31-8220 ($10 \mu\text{M}$; Table 2). In the presence of PMA, RO 31-7549 and RO 31-8220 potently inhibited the carbachol-evoked response (Fig. 5; Table 2), with IC_{50} (concentration required to achieve 50% inhibition) values of $0.18 \mu\text{M}$ and $0.56 \mu\text{M}$ respectively. H-7 was less potent, causing only 58% inhibition even at a concentration of $10 \mu\text{M}$ (Fig. 5). Polymyxin B was least effective, resulting in a maximum inhibition of only 20% (Fig. 5). On the basis of the observation that RO 31-7549 and RO 31-8220 potently inhibited the carbachol-evoked release of $[\text{^3H}]\text{NA}$, the effect of these two antagonists on K^+ -evoked release in SH-SY5Y was investigated. Release of $[\text{^3H}]\text{NA}$ induced by high K^+ was effectively inhibited by RO 31-7549 ($10 \mu\text{M}$) and RO 31-8220 ($10 \mu\text{M}$; Table 2) in both the presence and the absence of PMA.

Effect of PMA on muscarinic-evoked increases in intracellular Ca^{2+}

The increase in intracellular Ca^{2+} induced by carbachol in normal Ca^{2+} -containing medium is biphasic, consisting of an initial Ca^{2+} transient followed by a decline to a plateau (Lambert

& Nahorski, 1990; Murphy *et al.*, 1991b). Carbachol increased the intracellular Ca^{2+} from a basal value of 143 nM to a peak value of 457.5 nM in less than 2 s (Fig. 6a). Preincubation of SH-SY5Y cells with PMA ($0.1 \mu\text{M}$) had no effect on the basal Ca^{2+} level, but dramatically decreased both the carbachol (Fig. 6a)- and acetylcholine (Fig. 6b)-induced rise in intracellular Ca^{2+} . The inhibition of the initial Ca^{2+} transient was time-dependent (Fig. 6c). The simultaneous addition of carbachol and PMA resulted in an inhibition of 19% of the initial Ca^{2+} transient (results not shown). Longer preincubations (5 min) resulted in an inhibition of 85%. PMA also inhibited the plateau response to carbachol in a time-dependent manner (Fig. 6a), but did not appear to alter the plateau resulting from acetylcholine stimulation to the same extent (Fig. 6b).

DISCUSSION

Activation of protein kinase C by the phorbol ester PMA enhanced K^+ -, carbachol-, acetylcholine-, muscarine- and arecoline-evoked release of $[\text{^3H}]\text{NA}$ in SH-SY5Y cells, suggesting a regulatory role of protein kinase C in neurotransmitter release in this cell line. This enhancement of neurotransmitter release has been demonstrated previously in several secretory systems, including the release of $[\text{^3H}]\text{NA}$ in rat brain synaptosomes (Nichols *et al.*, 1987), PC12 cells (Harris *et al.*, 1986) and rabbit hippocampal slices (Allgaier *et al.*, 1986), release of $[\text{^3H}]\text{dopamine}$

from brain neurons in culture (Zurgil & Zisapel, 1985) or acetylcholine release in guinea-pig caudate slices (Tanaka *et al.*, 1986) and cholinergic synaptosomes from *Torpedo* electric organ (Guitart *et al.*, 1990).

The involvement of protein kinase C in signal transduction may be supported by the observation that phorbol esters that fail to activate protein kinase C (Castagna *et al.*, 1982) do not induce neurotransmitter release (Allgaier *et al.*, 1986; Murphy & Smith, 1987). The observation that PMA enhanced NA release, together with the lack of enhancement of release by the inactive phorbol ester 4 α -PDD, suggested that PMA enhanced release by activation of protein kinase C. Similarly, the inactive phorbol ester 4 α -PDD has previously been reported to have no significant effect on acetylcholine release in cholinergic synaptosomes (Guitart *et al.*, 1990) and guinea-pig caudate slices (Tanaka *et al.*, 1986).

PMA alone had no effect on the basal release of [3 H]NA in SH-SY5Y cells, suggesting that protein kinase C activation alone was not sufficient to stimulate release. These results were in contrast with earlier studies, whereby phorbol esters have been shown to increase the basal release of [3 H]dopamine in PC12 cells (Mel-dolesi *et al.*, 1988) or of acetylcholine in synaptosomes (Guitart *et al.*, 1990) and cause a small enhancement of [3 H]dopamine release in cultured neurons (Zurgil & Zisapel, 1985). The enhancement of carbachol-evoked release of [3 H]NA was both time- and concentration-dependent, having maximum effect at approx. 10 min at a concentration of 0.1 μ M. Furthermore, there was a 4-fold decrease in the EC₅₀ values for carbachol- and acetylcholine-evoked release of [3 H]NA in the presence of PMA, supporting the observation that activation of protein kinase C enhances the affinity of exocytosis for Ca²⁺, as previously reported for chromaffin cells (Knight & Baker, 1983).

In a previous report, we demonstrated that carbachol-evoked release of [3 H]NA in SH-SY5Y was inhibited by atropine, HHSD and pirenzepine, with K_i values of 0.33 nM, 18 nM and 1183 nM respectively, consistent with the role of an M₃ muscarinic receptor (Murphy *et al.*, 1991a). The effect of selective antagonists on PMA-potentiated carbachol-evoked release was also investigated, to determine whether activation of protein kinase C altered the pharmacology of the muscarinic-induced response. Although the K_i values for the inhibition of carbachol-evoked release were altered in the presence of PMA, the order of potency of HHSD versus pirenzepine did not alter the pharmacological characterization of the muscarinic receptor coupled to [3 H]NA release in SH-SY5Y cells.

Protein kinase C inhibitors were used to determine whether the phorbol ester enhancement of the carbachol- and K⁺-induced release of [3 H]NA from SH-SY5Y cells was mediated by protein kinase C. The compounds RO 31-7549 and RO 31-8220, based on the structure of the protein kinase C inhibitor staurosporine (Davis *et al.*, 1989), proved to be potent inhibitors of carbachol- and K⁺-evoked release of [3 H]NA, suggesting that activation of protein kinase C was clearly involved in release. The involvement of protein kinase C activation in the release of [3 H]NA was also supported by the observation that both carbachol- and K⁺-evoked releases in the absence of PMA were inhibited by these antagonists.

Whereas RO 31-7549 and RO 31-8220 effectively inhibited carbachol-evoked release of [3 H]NA, polymyxin B and H-7, which have been reported to inhibit protein kinase C (Kawamoto & Hidaka, 1984; Hidaka *et al.*, 1984), were shown to be less effective in antagonizing carbachol-evoked release enhanced by PMA. In contrast, however, polymyxin B (0.1 μ M) has been reported to inhibit the effects of PMA (0.1 μ M) on depolarization-evoked (30 mM-K⁺) release of [3 H]acetylcholine by approx. 65% in guinea-pig caudate slices, whereas H-7 was less potent in this

preparation (35% at 50 μ M; Tanaka *et al.*, 1986). In a recent study by Guitart *et al.* (1990), H-7 was reported to be a potent inhibitor of PMA-induced acetylcholine release in cholinergic synaptosomes. These conflicting reports suggested that the potency of these inhibitors may depend on the preparation under investigation, with the possibility of different protein kinase C isoenzymes being involved.

Activation of the diacylglycerol/protein kinase C pathway may have a negative-feedback effect on Ca²⁺ signalling that results in the inhibition of agonist-induced increases in intracellular Ca²⁺ (Berridge, 1987). Pretreatment with phorbol esters has been shown to inhibit Ca²⁺ signalling, presumably either by stimulating the removal of Ca²⁺ from the cytosol or by inhibiting the hydrolysis of phosphatidylinositol 4,5-bisphosphate and hence InsP₃ formation (Berridge, 1987). The latter has been demonstrated in several preparations, such as hippocampal slices (Labarca *et al.*, 1984), vascular smooth muscle (Brock *et al.*, 1985) and cultured human 1321N1 astrocytoma cells (Orellana *et al.*, 1985). In the present study, activation of protein kinase C by PMA significantly inhibited the elevation of intracellular Ca²⁺ evoked by the cholinergic activation of the M₃ muscarinic receptor. Vicentini *et al.* (1985) similarly reported a time-dependent decrease in the carbachol-evoked Ca²⁺ transient in quin2-loaded PC12⁺ cells (induced to differentiate with nerve growth factor). Maximum inhibition of the carbachol-evoked elevation of intracellular Ca²⁺ and inhibition of phosphatidylinositol hydrolysis in these cells resulted after a 3 min preincubation with PMA (0.1 μ M; Vicentini *et al.*, 1985). Furthermore, they demonstrated that, in the absence of extracellular Ca²⁺, the Ca²⁺ transient was inhibited to the same extent as that observed in Ca²⁺-containing medium, thereby concluding that PMA effected both Ca²⁺ influx and intracellular mobilization. Our results are also consistent with such an interpretation, although with acetylcholine the effect on the plateau appears to be less than with carbachol.

In conclusion, pretreatment with the phorbol ester PMA enhanced K⁺- and muscarinic-evoked release of [3 H]NA. The involvement of protein kinase C in triggering release was firmly supported by the observation that inhibitors of this enzyme were effective in antagonizing this effect. In contrast, pretreatment with PMA for 5 min decreased the initial peak and plateau in intracellular Ca²⁺ formation, suggesting that protein kinase C activation by PMA may uncouple muscarinic receptors and inositol phosphate formation. Noradrenaline release was therefore enhanced under conditions that led to a drastic decrease in changes in intracellular Ca²⁺, suggesting that the release mechanism may become much more sensitive to increases in cytosolic Ca²⁺ when PKC is activated. These observations together suggested that noradrenaline release evoked by muscarinic agonists was triggered not only by relatively small changes in cytosolic Ca²⁺ but also by protein kinase C activation.

J.G.McC. was a Lister Institute Research Fellow. N.P.M. was in receipt of a British Heart Foundation studentship.

REFERENCES

- Allgaier, C., Von Kugelgen, O. & Hertting, G. (1986) *Eur. J. Pharmacol.* **129**, 389–392.
- Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193.
- Bonner, T. I. (1989) *Trends Pharmacol. Sci.*, July suppl. 11–15.
- Brock, T. A., Rittenhouse, S. E., Powers, C. W., Ekstein, L. S., Gimbrone, M. A. & Alexander, R. W. (1985) *J. Biol. Chem.* **260**, 14158–14162.
- Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851.
- Cobbold, P. H. & Rink, T. J. (1987) *Biochem. J.* **248**, 313–328.

- Davis, P. D., Hill, C. H., Keech, E., Lawton, G., Nixon, J. S., Sedgwick, A. D., Wadsworth, J., Westmacott, D. & Wilkinson, S. E. (1989) *FEBS Lett.* **259**, 61–63
- Fisher, S. K., Domas, K. L. M. & Roland, R. M. (1989) *Mol. Pharmacol.* **35**, 195–204
- Gryniewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Guitart, X., Marsal, J. & Solsona, C. (1990) *J. Neurochem.* **55**, 468–472
- Hallam, T. J., Jacob, R. & Merritt, J. E. (1988) *Biochem. J.* **255**, 179–184
- Harris, K. M., Kongsamut, S. & Miller, R. J. (1986) *Biochem. Biophys. Res. Commun.* **134**, 1298–1305
- Hidaka, H., Inagaki, M., Kawamoto, S. & Sasaki, Y. (1984) *Biochemistry* **23**, 5036–5041
- Kawamoto, S. & Hidaka, H. (1984) *Biochem. Biophys. Res. Commun.* **125**, 258–264
- Knight, D. E. & Baker, P. F. (1983) *FEBS Lett.* **160**, 98–100
- Labarca, R., Janowsky, A., Patel, J. & Paul, S. M. (1984) *Biochem. Biophys. Res. Commun.* **123**, 703–709
- Lambert, D. G. & Nahorski, S. R. (1990) *Biochem. J.* **265**, 555–562
- Meldolesi, J., Gatti, G., Ambrosini, A. & Pozzan, T. (1988) *Biochem. J.* **255**, 761–768
- Merritt, J. E. & Hallam, T. J. (1988) *J. Biol. Chem.* **263**, 6161–6164
- Murphy, N. P., Ball, S. G. & Vaughan, P. F. T. (1991a) *J. Neurochem.* **56**, 1810–1815
- Murphy, N. P., Ball, S. G., Vaughan, P. F. T. & McCormack, J. G. (1991b) *J. Neurochem.* **57**, 2116–2123
- Murphy, R. L. W. & Smith, M. E. (1987) *Br. J. Pharmacol.* **90**, 327–334
- Nichols, R. A., Haycock, J. W., Wang, J. K. T. & Greengard, P. (1987) *J. Neurochem.* **48**, 615–621
- Nishizuka, Y. (1984a) *Nature (London)* **308**, 693–697
- Nishizuka, Y. (1984b) *Science* **225**, 1365–1370
- Orellana, S. A., Solski, P. A. & Brown, J. H. (1985) *J. Biol. Chem.* **260**, 5236–5239
- Tanaka, C., Fujiwara, H. & Fujii, Y. (1986) *FEBS Lett.* **195**, 129–134
- Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 324–334
- Vicentini, L. M., Di Virgilio, F., Ambrosini, A., Pozzan, T. & Meldolesi, J. (1985) *Biochem. Biophys. Res. Commun.* **127**, 310–317
- Zurgil, N. & Zisapel, N. (1985) *FEBS Lett.* **185**, 257–261

Received 15 July 1991/30 August 1991; accepted 25 September 1991