Phorbol ester and calcium act synergistically to enhance neurotransmitter release by brain neurons in culture

Neomi Zurgil and Nava Zisapel*

Department of Biochemistry, The George S Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv 69978, Israel

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Preincubation of intact fetal brain neurons in culture with the phorbol ester TPA (12-O-tetradecanoyl phorbol-13-acetate) in the presence of calcium, resulted in the enhancement of the depolarization-induced, Ca^{2+}-dependent neurotransmitter release by the cells This effect was due to a marked decrease in the concentration of extracellular Ca^{2+} required to provoke the release The concentration of Ca^{2+} needed to produce half-maximal release shifted from approx 0 1 mM in the absence of TPA to 0 018 mM in its presence This activity of TPA was concentration-dependent (half-maximal effect at 4 nM TPA) and was also dependent on the presence of calcium during the preincubation period The TPA-induced enhancement of the stimulated release was also observed when Ca^{2+} entry into the depolarized cells was partially inhibited by Co^{2+} The results suggest that TPA acts synergistically with Ca^{2+} to activate neuronal component(s) involved in Ca^{2+}-dependent neurosecretion

Phorbol ester *Calcium* *Brain neuron* *Neurotransmitter*

1. INTRODUCTION

Neurotransmitter release is triggered by an elevated level of free Ca^{2+} in the cytoplasm of the nerve endings and is thought to proceed by an exocytotic mechanism [1,2]. However, the biochemical processes underlying the coupling of Ca^{2+} stimulation to neurotransmitter release are unknown. The phorbol ester TPA has been shown to enhance or stimulate exocytotic secretion from several cell types, presumably by activating the Ca^{2+}-phospholipid dependent protein kinase C [3–8] There is no evidence, as yet, for the effects of TPA on neurotransmitter release from mammalian neurons.

It has been shown that neural brain cells can survive and mature in chemically defined media, under conditions in which growth of glia and other supportive cells is curtailed [9–13]. The mature cells are able to synthesize endogenous dopamine [11], to accumulate exogenous [^{3}H]dopamine [10] and to release both the endogenous and the preloaded neurotransmitter in a calcium-dependent process upon elevation of the extracellular potassium concentration [10,11]

Here, we report that TPA enhances the stimulated neurotransmitter release from mammalian brain neurons by increasing the apparent affinity of the neurosecretory process to Ca^{2+}. This enhancement takes several minutes to develop and its magnitude depends on the presence of Ca^{2+} during this period.

2. MATERIALS AND METHODS

Fetal rat brain neurons were grown in culture as in [10]. Cells attached to the plastic Petri dish were preincubated with 250 nM [^{3}H]dopamine (9.9 Ci/mmol) (New England Nuclear, Chicago, IL) in culture medium at 37°C for 60 min. The incubation medium was then removed and the cells

* To whom correspondence should be addressed

Abbreviations: TPA, 12-*O*-tetradecanoyl phorbol-13-acetate, EBSS, Earle's balanced salt solution

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washed 3 times by repeated incubations for 20 min at 37°C with EBSS buffer (containing 116 mM NaCl, 5.3 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 26 3 mM $NaHCO_3$, 5.5 mM glucose). The cells were then incubated 4 times for 1 min intervals at 37°C with various concentrations of TPA in EBSS in the absence or presence of different concentrations of Ca^{2+} or Co^{2+}. The efflux of labeled dopamine from the unstimulated cells was determined in the removed incubation buffers. The neurons were then stimulated by incubation with depolarizing isotonic solution (containing 53 mM KCl, 58 mM NaCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$ and 5.5 mM glucose) in the presence and absence of various concentrations of Ca^{2+} and TPA and in the presence and absence of Co^{2+} (0.32 mM). The depolarizing buffer was collected and replaced every 1 min. The amount of [^{3}H]dopamine present in each of the collected fractions was determined by scintillation counting. The cells were then solubilized in 1% SDS, and aliquots of the solubilized samples were taken for determination of the residual radioactivity in the cells. The amount of [^{3}H]dopamine released into the collected fractions was expressed as cpm or as percent of the amount of dopamine present in the cells at the time of stimulation. The cumulative amount of transmitter released within the first 3 min of stimulation was expressed as percent of the total amount of [^{3}H]dopamine present in the cells prior to stimulation. The enhancement of the release was calculated as follows:

$$\% \text{ enhancement} = 100 \left(\frac{\text{amount released in the presence of TPA}}{\text{amount released in the absence of TPA}} - 1\right)$$

3. RESULTS AND DISCUSSION

The effect of TPA on the basal and on the depolarization-induced release of [^{3}H]dopamine from the brain neurons is shown in fig.1 In

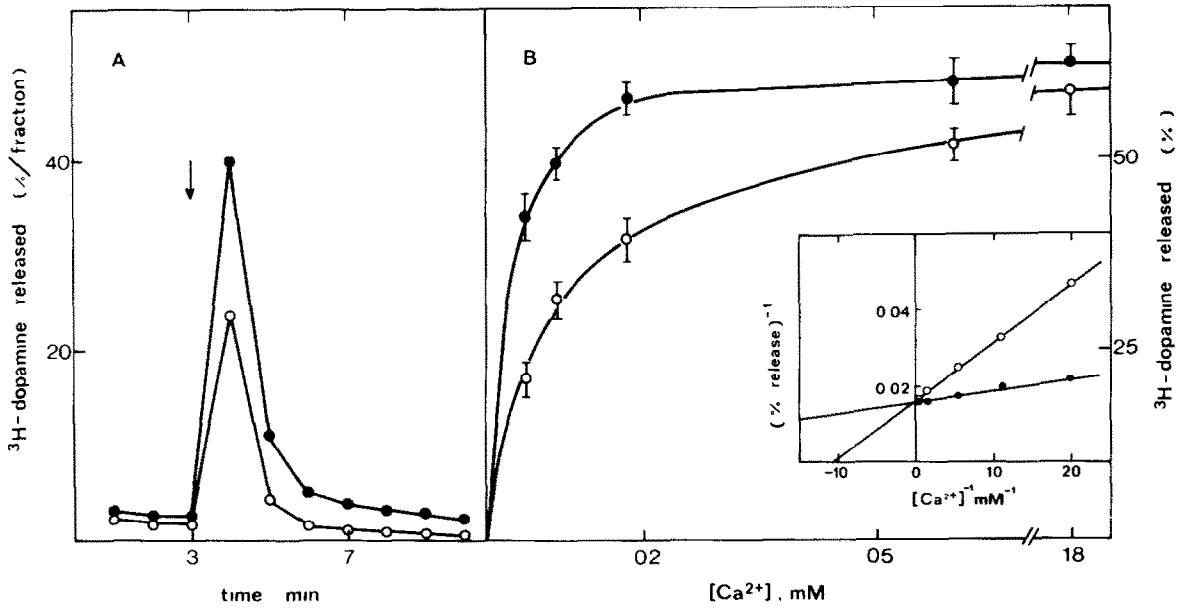

Fig 1 Effects of TPA and calcium on the release of [^{3}H]dopamine from brain neurons in culture Cells preloaded with [^{3}H]dopamine were incubated 4 times for 1 min in EBSS in the absence (○) and presence (●) of TPA (100 ng/ml) (A) Cells were then subjected to depolarization in the presence of 0 09 mM Ca^{2+} in the presence (●) or absence (○) of TPA The depolarizing solution was removed and replaced every 1 min The amount of [^{3}H]dopamine in each of the collected fractions is presented (B) Cells were depolarized as in (A) but in the presence of various concentrations of Ca^{2+} The amount of [^{3}H]dopamine released within 3 min of depolarization is presented as percent of the total [^{3}H]dopamine content in the cells prior to the stimulation. A double-reciprocal plot of the evoked release as a function of extracellular Ca^{2+} concentrations is shown in the inset

unstimulated cells and in the presence of Ca^{2+} (0.09 mM), TPA caused a small enhancement of [^{3}H]dopamine release (fig.1A). This enhancement was somewhat increased with increasing extracellular Ca^{2+} concentrations (e.g., see fig.3A). When the cells were subsequently depolarized in the presence of Ca^{2+} (0.09 mM), the evoked release of neurotransmitter from the TPA-treated cells was markedly enhanced as compared to the untreated controls (fig 1A). Fig.1B illustrates the dependency of the evoked neurotransmitter release on the concentration of extracellular Ca^{2+} In untreated neurons, the cumulative amount of neurotransmitter released upon depolarization increased with increasing Ca^{2+} concentration in the range 0–0.3 mM, and then levelled off Maximal release was obtained above 1 mM Ca^{2+} (fig.1B). In TPA-treated cells, maximal evoked release was obtained at 0.2 mM Ca^{2+}. The apparent affinities of the evoked release for extracellular Ca^{2+} and the maximal release were calculated from the double-reciprocal plots of the data (fig.1B inset). Thus, in untreated cells, the concentration of Ca^{2+} needed to provoke half-maximal release was 0.1 mM. In TPA-treated cells, the apparent affinity of the evoked neurotransmitter release increased (fig.1B), and the concentration of Ca^{2+} needed to provoke half-maximal release was reduced to 0.018 mM (fig.1B inset). TPA did not affect significantly the maximal amount of the evoked release (fig.1B inset). Consequently, the enhancement of neurotransmitter release was higher at lower concentrations of Ca^{2+}, and was less expressed at higher Ca^{2+} concentrations (above 1 mM).

The enhancement of the depolarization-evoked dopamine release increased with increasing TPA concentration in the range 1–20 ng/ml and then levelled off (fig.2) Half-maximal effect was observed at 2.6 ng/ml (4 nM) of the phorbol ester. This concentration was very close to that reported to affect secretion in other cell types and to activate isolated protein kinase C [4–9]. About 3 min preincubation of the neurons with TPA (100 ng/ml) were needed to achieve maximal enhancement of the evoked release (not shown).

The presence of calcium in the preincubation buffers was found to be obligatory for the expression of the TPA-induced enhancement of neurotransmitter release. Preincubation of the brain neurons with TPA in the absence of Ca^{2+}

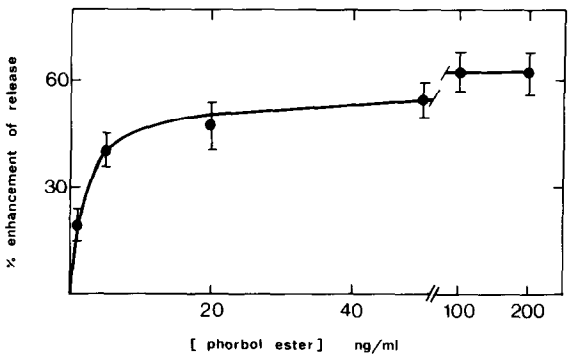

Fig 2. Effect of different concentrations of TPA on the stimulated release of [^{3}H]dopamine from the brain neurons Cells were preloaded with [^{3}H]dopamine, incubated in EBSS containing Ca^{2+} (0 18 mM) in the presence and absence of various concentrations of TPA The cells were then stimulated by depolarization in the presence of Ca^{2+} (0 18 mM). The enhancement by TPA of [^{3}H]dopamine release was calculated as described in section 2

markedly reduced the enhancement of the evoked release (table 1). Conversely, preincubation of untreated neurons in the absence of Ca^{2+} somewhat increased the amount of neurotransmitter release since less depletion of the intracellular [^{3}H]dopamine pool occurred during the prestimulation period (table 1) The concentration of Ca^{2+} in the preincubation buffer needed to achieve half-maximal enhancement of neurosecretion by TPA was estimated to be 6 μM (table 1). It should be noted that this estimate may be lower than the true value, since some activation by TPA could also occur during the stimulation period. Thus, the residual enhancement of evoked release observed in cells treated with TPA in the absence of Ca^{2+} could be due to the Ca^{2+} present in the depolarizing buffer (table 1). Interestingly, the Ca^{2+} requirement of the activation process was considerably lower than that needed to provoke neurotransmitter release (fig 1)

The enhancement of the Ca^{2+}-dependent neurotransmitter release could in theory be achieved by. (i) higher affinity of Ca^{2+}-triggered processes for Ca^{2+}; (ii) increased influx of Ca^{2+} into the depolarized neurons; (iii) a combination of the two possibilities (i) and (ii). To investigate further these possibilities the effect of TPA on the evoked release was assessed in the presence of Co^{2+} (fig.3)

Table 1

Effect of Ca^{2+} in the preincubation period on the enhancement of the stimulated release of [^{3}H]dopamine by TPA

	Ca^{2+} in preincubation (μM)				
	0	1	10	50	100
% release (TPA treated cells)	40 9	42 3	45 0	47 6	49 7
% release (untreated cells)	34.2	34 1	31 5	31 0	31 0
% enhancement by TPA	19	34	43	54	62

Cells preloaded with [^{3}H]dopamine were incubated for 4 min without or with TPA (100 ng/ml) in EBSS containing various concentrations of Ca^{2+} The cells were then depolarized in the presence of Ca^{2+} (0.18 mM) The enhancement of the release was calculated from the corresponding amounts of [^{3}H]dopamine released from TPA treated and from untreated cells as described in section 2

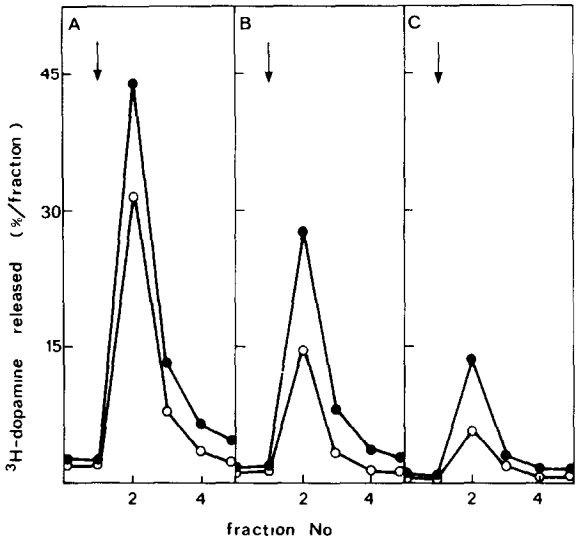

Fig 3. Effects of Co^{2+} on the stimulated release of [^{3}H]dopamine and on its enhancement by TPA (A) Cells preloaded with [^{3}H]dopamine were incubated for 4 min in EBSS containing Ca^{2+} (0 18 mM) in the presence (●) or absence (○) of TPA (100 ng/ml) prior to stimulation The cells were then depolarized in the presence of Ca^{2+} (0.18 mM) and in the presence (●) or absence (○) of TPA (100 ng/ml). The incubation buffers were removed at 1 min intervals The amount of [^{3}H]dopamine released into each of the collected fractions is expressed as a percentage of the cell content (B) As (A) but the depolarizing buffers also contained Co^{2+} (0 32 mM) (C) As (B) but Co^{2+} (0.32 mM) was also present in the preincubation buffers

Cobalt ions are specific blockers of the voltage-sensitive calcium channels [14] and thus inhibit Ca^{2+} influx into the stimulated neurons [11]. As expected, the amount of neurotransmitter released upon depolarization of brain neurons in the presence of Co^{2+} was reduced (fig.3A,B) Under these conditions, the enhancement of the evoked release in the TPA-treated cells was larger than that observed in cells depolarized in the absence of Co^{2+} (fig.3A,B). Preincubation of the cells with Co^{2+} for 4 min prior to stimulation of the cells further reduced the evoked neurotransmitter release (fig 3C), presumably by cancellation of kinetic advantages of Ca^{2+} over Co^{2+}, but the enhancement of the release in the TPA-treated cells was still evident (fig.3C) These results were consistent with the previous ones, and indicated that less extracellular Ca^{2+} is sufficient to elicit the release

The activity of TPA in brain neurons may point to the involvement of protein kinase C in neurosecretion TPA is known to activate isolated protein kinase C in the presence of Ca^{2+} and phospholipid [6]. The synergistic role of protein kinase C and Ca^{2+} mobilization has been demonstrated in several secretory systems such as the release of serotonin from platelets [3], release of catecholamines from leaky adrenal medullary cells [4], granule secretion from human platelets [5], histamine release from rat peritoneal mast cells [6] and from rat basophilic leukemia cells [7] or insulin release from rat hepatic islets [8]. The enhancement by TPA of neurotransmitter release

from the brain neurons may be consistent with the exocytotic nature of neurosecretion.

REFERENCES

[1] Rubin, R P. (1970) Pharmacol Rev 22, 389–428
[2] Miledi, R. (1973) Proc. R. Soc Lond Biol Sci 103, 421–425
[3] Kaibuchi, K , Sano, K , Hoshijama, M , Takai, Y and Nishizuka, Y (1982) Cell Calcium 3, 325–335
[4] Knight, D E and Baker, P F (1983) FEBS Lett 160, 98–100
[5] Rink, T J , Sanchez, A and Hallam, T J (1983) Nature 305, 317–319
[6] Kalakami, Y., Kaibuchi, K , Samamura, M , Takai, Y and Nishizuka, Y. (1984) Biochem Biophys. Res Commun 121, 573–578.
[7] Sagi-Eisenberg, R and Pecht, I (1984) Immunol Lett 8, 237–241
[8] Kojima, I., Lippes, K and Rasmussen, H (1983) Biochem. Biophys. Res Commun 116, 555–562
[9] Yavin, Z and Yavin, E (1980) Dev Biol 75, 454–459
[10] Zurgil, N and Zisapel, N (1981) Life Sci. 29, 2265–2271.
[11] Yarom, M , Zurgil, N and Zisapel, N. (1985) submitted
[12] Zurgil, N and Zisapel, N (1983) FEBS Lett 156, 257–261
[13] Zurgil, N. and Zisapel, N. (1984) Dev Brain Res 13, 293–303
[14] Ginsborg, B.L and Jenkinson, D.H. (1976) in Handbook of Experimental Pharmacology (Zaimis, E ed) vol 42, pp 229–364, Springer, New York.