Acetylcholine release from guinea pig caudate slices evoked by phorbol ester and calcium

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In guinea pig caudate slices preloaded with [^{3}H]choline, superfusion with medium containing Ca^{2+} and the phorbol ester TPA (12-*O*-tetradecanoyl phorbol 13-acetate) produced an increase in the Ca^{2+}-dependent, depolarization-evoked Ca^{2+} influx. The effect of TPA was antagonized by polymyxin B and H-7, both inhibitors of protein kinase C. The combination of TPA and calcium ionophore (A23187) provoked the release of ACh to a level equal to the maximal response induced by depolarizing stimuli. TPA alone had no significant effect on the release of ACh. These results suggest that protein kinase C probably plays a role in transmembrane signal transduction involved in the release of neurotransmitter from nerve terminals in the brain.

Phorbol ester *Ca^{2+}* *Acetylcholine release* *(Brain slice)*

1. INTRODUCTION

Extracellular signals such as neurotransmitter, hormones and depolarizing stimuli elicit an increase in intracellular free Ca^{2+} levels [1–3]. Ca^{2+} activates protein kinases, following which the signal is thought to be amplified and transduced across the membranes. A number of recent studies provided evidence of phosphorylation linked to secretion processes. At an early stage of cellular response, 1,2-diacylglycerol, which is transiently produced from inositol phospholipids by the signal, activates protein kinase C in the presence of physiological levels of Ca^{2+} and phospholipids [4,5]. The activation of protein kinase C and Ca^{2+} mobilization is essential to induce exocytotic secretion of hormones [6–8], enzymes [9,10] and other bioactive substances [11–14]. Tumor-promoting phorbol esters such as 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) induce exocytosis and mimic the action of hormone and neurotransmitter [15]. It is of interest that TPA activates protein kinase C, as the stable analogue of the physiological lipid metabolite activator diacylglycerol [16]. In peripheral cholinergic nerves, the simultaneous application of Ca^{2+} ionophore and TPA induces a full release of acetylcholine (ACh) from isolated guinea pig ileum [17]. The synergistic effect of Ca^{2+} ionophore and TPA on [^{3}H]dopamine release was noted in PC12 [18] and primary cultured brain cells [19]. As large amounts of protein kinase C and the target protein are present in the brain, especially in synaptosomal fractions [20], we carried out experiments to determine whether protein kinase C is involved in neurotransmitter release from brain slices. We report here that TPA enhances the ACh release evoked by depolarizing stimuli and that Ca^{2+} ionophore and TPA synergistically induce the release of ACh from guinea pig caudate slices.

2. MATERIALS AND METHODS

2.1. *Striatal slices*

Guinea pigs of both sexes weighing 350–450 g were decapitated, the brains quickly removed and cut coronally at the level of the anterior commissure. The caudate slices were dissected with a razor blade and a sliding guide. The caudal part of the caudate nucleus, putamen and globus pallidus

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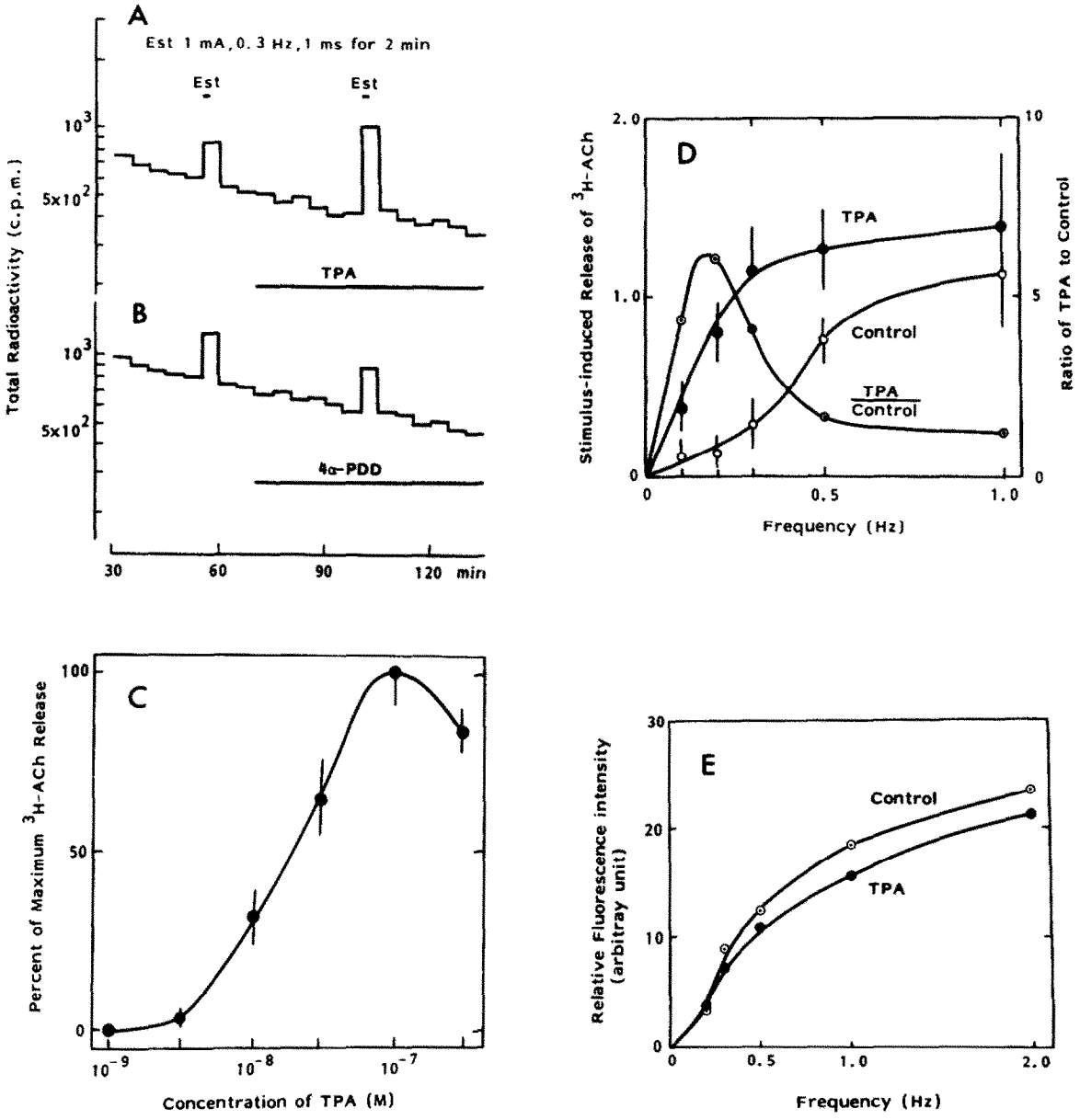

Fig.1. Effect of phorbol ester on electrically stimulated release of [^{3}H]ACh from guinea pig caudate slices preloaded with [^{3}H]choline and $[Ca^{2+}]_i$-dependent fluorescence signal induced by electrical stimulation in the caudate slices preloaded with quin2/AM. (A) 10^{-7} M TPA or (B) 10^{-7} M 4α-PDD was added to the medium 30 min before and during electrical stimulation (Est: 1 mA, 0.3 Hz, 1 ms for 2 min). (C) Concentration dependence of the effect of TPA on the stimulated release of [^{3}H]ACh. Each point is the mean ± SE from 4 experiments of a percentage of the stimulated release of [^{3}H]ACh in the presence of 10^{-7} M TPA. (D) Effect of TPA on frequency-response curve for the release of [^{3}H]ACh induced by electrical stimulation (1 mA, 1 ms, 0.1–1 Hz for 2 min). 10^{-7} M TPA was added to the medium 30 min before and during stimulation. Each point is the mean ± SE from 4 experiments. (E) Effect of TPA on frequency-response curve for $[Ca^{2+}]_i$-dependent fluorescence (quin2-Ca complex fluorescence) signal induced by electrical stimulation (3 mA, 2.5 ms, 0.2–2 Hz train 20 pulses). 10^{-7} M TPA was added to the medium 30 min before and during stimulation.

was excluded. Frontal sections used in the experiments were 350–450 μm thick and weighed 6–8 mg.

2.2. *[^{3}H]ACh release from striatal slices*

Slices were preloaded with [^{3}H]choline (10^{-7} M, 80.1 Ci/mmol) for 1 h at 37°C and then rinsed with Krebs-Ringer solution. The preparation was impaled with a pair of parallel platinum electrodes (0.1 mm diameter, distance 0.7 mm, length 1.5 mm), mounted in the superfusion chamber saturated with 95% O_2-5% CO_2 and superfused at a constant flow rate of 0.2 ml/min with Krebs-Ringer solution buffer equilibrated with 95% O_2-5% CO_2 at 37°C. The Krebs-Ringer solution buffer was of the following composition (mM): 118 NaCl, 3 KCl, 2 $CaCl_2$, 1.2 NaH_2PO_4, 23 $NaHCO_3$ and 11 glucose. The superfusate was continuously collected every 5 min and the radioactivity determined in a liquid scintillation spectrometer. Electrical stimulation of slices was performed under conditions of various currents and frequencies. Unchanged [^{3}H]ACh in the superfusate were determined by the method of Potter and Murphy [21]. The amounts of unchanged [^{3}H]ACh in the sample obtained before and during stimulation were about 70 and 80% of total radioactivities, respectively.

2.3. *Calcium influx*

Using the method of Tsien et al. [22], slices were incubated with 1 mM quin2/AM and 0.5% DMSO (v/v) in Krebs-Ringer solution without $CaCl_2$ at 37°C for 1 h and washed 3 times with fresh Krebs-Ringer solution. The preparation was impaled with a pair of parallel platinum electrodes (0.1 mm diameter, distance 2.5 mm, length 3 mm), mounted vertically in the cuvette in 1 ml Krebs-Ringer solution and perfused at a constant flow rate of 3.5 ml/min at 37°C. The fluorescence of the quin2-Ca^{2+} complex was recorded using an Aminco-Bowman spectrophotofluorometer setting with 339 nm excitation, 2 mm slits and 492 nm emission, 3 mm slits.

3. RESULTS AND DISCUSSION

When guinea pig caudate slices preloaded with [^{3}H]choline were superfused with Krebs-Ringer solution containing 2 mM Ca^{2+}, electrical stimulation produced an increase in the release of [^{3}H]ACh, over the spontaneous efflux (fig.1A). The evoked [^{3}H]ACh release was dependent on the frequency between 0.1 and 1.0 Hz (fig.1D) and was Ca^{2+}-dependent and tetrodotoxin (TTX)-sensitive, thereby indicating a release from nerve terminals.

The [^{3}H]ACh release induced by electrical stimulation of 1 mA, 0.2 Hz, 1 ms for 2 min was enhanced by adding TPA in concentrations of 3×10^{-9} to 10^{-7} M, in a concentration-dependent manner, and reached a maximal increase at 10^{-7} M TPA (fig.1C). The frequency-dependent curve for [^{3}H]ACh release from the caudate slices shifted to the right and upwards by 10^{-7} M TPA, as shown in fig.1D. TPA (10^{-7} M) produced a greater potentiation in the [^{3}H]ACh release evoked by stimulations of low frequencies (less 0.5 Hz) and the maximal potentiation was about 6 times that of the control obtained with a stimulation at 0.2 Hz. To determine the intracellular Ca^{2+}-level, under conditions when TPA potentiated the stimulated [^{3}H]ACh release, the fluorescence signal of the quin2-Ca^{2+} complex was measured in the caudate slices preloaded with quin2/AM. A Ca^{2+}-sensitive and frequency-dependent increase in the fluorescence of quin2-Ca^{2+} complex was evoked by electrical stimulation, thereby indicating that increase in the fluorescence intensity was mainly due to increases in intracellular Ca^{2+} induced by the Ca^{2+} influx. No measurable Ca^{2+} influx was obtained by stimulations less than 0.2 Hz. There was a small Ca^{2+} influx at 0.2 Hz at which the [^{3}H]ACh release was maximally potentiated by TPA and the evoked Ca^{2+} influx was not affected significantly by 10^{-7} M TPA (fig.1E).

Addition of 30 mM K^+ to the superfusion medium produced a Ca^{2+}-dependent increase in the release of [^{3}H]ACh. The high K^+-evoked release of [^{3}H]ACh was reduced to 70% of the control in the presence of 10^{-6} M TTX. TTX blocks nerve conductance by inhibiting the Na^+ channel [23], thus the released [^{3}H]ACh in the presence of 10^{-6} M TTX is mainly due to a direct depolarization of nerve terminals by high K^+. In the presence of TTX, pretreatment with 10^{-7} M TPA for 30 min enhanced by 197% the high K^+-evoked release of [^{3}H]ACh (table 1). This suggests that the site of action of TPA is located on the nerve terminals. 4α-Phorbol 12,13-didecanoate (4α-PDD),

an inactive phorbol analogue, at 10^{-7} M showed no significant changes in spontaneous and depolarization-evoked release of [^{3}H]ACh (fig.1B and table 1).

Inhibition of protein kinase C is considered to be a useful tool to differentiate Ca^{2+}-dependent events regulated by phospholipid or calmodulin. Polymyxin B (a cyclic polycationic peptide antibiotic) and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) are considered to inhibit selectively the protein kinase C mediated reaction rather than the calmodulin-dependent one [24–27]. To confirm whether the TPA-induced potentiation of the high K^+-evoked release of [^{3}H]ACh is mediated by protein kinase C, the effects of polymyxin B and H-7 on [^{3}H]ACh release were examined. The effects of TPA on the high K^+-evoked [^{3}H]ACh release were inhibited by polymyxin B in concentrations ranging from 10^{-9} to 10^{-7} M, in a dose-dependent manner. H-7 at 5×10^{-5} M also inhibited significantly the effect of TPA (table 1). These findings suggest that the depolarization-induced ACh release is potentiated by activating protein kinase C at the nerve endings. It is of interest that the depolarization-induced [^{3}H]ACh release was inhibited significantly by polymyxin B, an inhibitor of protein kinase C. This would suggest the in vivo involvement of protein kinase C in the depolarization-induced ACh release, which is the result of activation of this enzyme by diglyceride generated endogenously, in response to depolarization via catabolism of membrane phospholipids.

When the caudate slices preloaded with [^{3}H]choline were superfused with Ca^{2+}-free medium, stimulation with both A23187 (10^{-6} M) and Ca^{2+} (2 mM) for 2 min produced an increase in [^{3}H]ACh release, which was about half the full release response evoked by depolarizing stimuli such as electrical stimulation and high K^+ (fig.2A). The application of TPA potentiated the release of [^{3}H]ACh induced by A23187 and Ca^{2+} increased linearly in a concentration-dependent manner between 2×10^{-9} and 10^{-7} M TPA, and reached a maximal response at 10^{-7} M (fig.2C). In the absence of Ca^{2+}, addition of A23187 alone and of a combination of A23187 and TPA showed no significant changes. 4α-PDD at 10^{-7} M did not af-

Table 1

Effect of various agents on high K^+-induced [^{3}H]ACh release from striatal slices preloaded with [^{3}H]choline

Agents	30 mM K^+-induced [^{3}H]ACh release	(n)	%
None	0.75 ± 0.08	(24)	100
4α-PDD (10^{-7} M)	0.80 ± 0.12	(4)	107
TPA (10^{-7} M)	1.48 ± 0.15[a]	(4)	197
Polymyxin B (10^{-9} M)	0.74 ± 0.12	(4)	99
Polymyxin B (10^{-8} M)	0.67 ± 0.11	(4)	89
Polymyxin B (10^{-7} M)	0.46 ± 0.10[a]	(4)	60
Polymyxin B (10^{-9} M) + TPA (10^{-7} M)	1.29 ± 0.12	(4)	172
Polymyxin B (10^{-8} M) + TPA (10^{-7} M)	0.90 ± 0.12[b]	(4)	120
Polymyxin B (10^{-7} M) + TPA (10^{-7} M)	0.52 ±0.17[b]	(4)	69
H-7 (5×10^{-5} M) + TPA (10^{-7} M)	0.96	(1)	128

[a] Significant difference from the value in the absence of agent ($P < 0.05$)
[b] Significant difference from the value in the presence of TPA alone ($P < 0.05$)

Tissues were superfused with Krebs Ringer solution, in the presence of 10^{-6} M tetrodotoxin. High K^+ (3×10^{-2} M) medium containing $CaCl_2$ (2×10^{-3} M) was applied for 2 min. 4α-PDD or TPA was added 30 min before and during application of high K^+ medium. Polymyxin B or H-7 were applied for 5 min before start of TPA superfusion

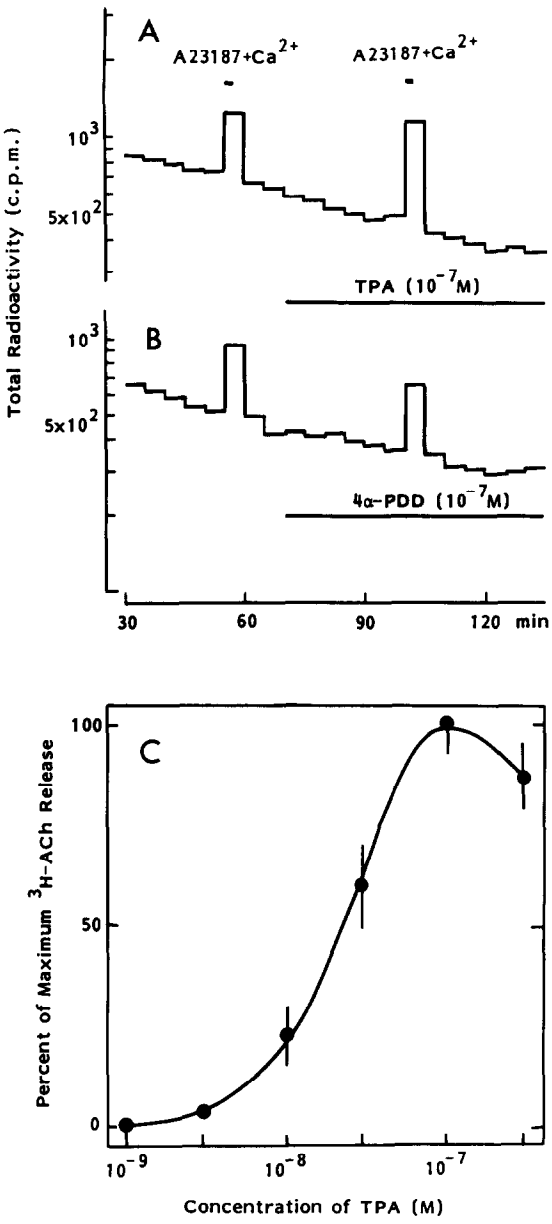

Fig.2. Effect of phorbol ester on A23187-induced release of [^{3}H]ACh from caudate slices. Caudate slices were superfused with Ca^{2+}-free medium. 10^{-6} M A23187 and 2×10^{-3} M $CaCl_2$ were added to the medium for 2 min. (A) 10^{-7} M TPA and 10^{-7} M 4α-PDD were added to the medium 30 min before and during application of A23187. (C) Concentration dependence of the effect of TPA on A23187-induced release of [^{3}H]ACh. Each point is the mean ± SE from 4 experiments of a percentage of A23187-induced release of [^{3}H]ACh in the presence of 10^{-7} M TPA.

fect the [^{3}H]ACh release induced by A23187 (10^{-6} M) and Ca^{2+} (2 mM) (fig.2B). This finding shows that protein kinase C is involved not only in peripheral cholinergic transmission in the guinea pig ileum but also in the central release of ACh from the nerve terminals. Although it is generally assumed that neurotransmitter release occurs through exocytosis, recent evidence [28] indicates that exocytosis may be one of several release mechanisms. In the resting synapse, ACh is likely to be mainly released from the non-vesicular store, in a Ca^{2+}-dependent manner [28,29]. The present results show that the spontaneous release of [^{3}H]ACh was not significantly affected by the activation of protein kinase C. Therefore, the activation of protein kinase C appears to increase the affinity for Ca^{2+}, at least in cases of vesicular ACh release from central cholinergic nerve terminals, as well as in cases of exocytotic secretion of hormones and other substances.

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