A phorbol ester and A23187 act synergistically to release acetylcholine from the guinea pig ileum

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Electrical stimulation of guinea pig ileum preloaded with [^{3}H]choline provokes the release of [^{3}H]acetylcholine (ACh) in a Ca^{2+}-dependent manner. This release was markedly increased by the tumor-promoting phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA). The combination of the ionophore A23187 and TPA produced the release of [^{3}H]ACh up to a level equal to or exceeding a maximal response induced by electrical stimulation. A23187 alone gave only a minor response and TPA alone had no apparent effect on the [^{3}H]ACh release. Thus, protein kinase C probably plays a role in cell surface signal transduction related to the release of transmitters from nerve endings.

A23187 *Phorbol ester* *Acetylcholine* *Guinea pig*

1. INTRODUCTION

To trigger the release of a neurotransmitter, increase in intracellular Ca^{2+} concentrations upon depolarization of the membrane of nerve endings is required [1,2]. Depolarization of the membrane, induced by electrical stimulation or appropriate agents, will lead to the turnover of inositol phospholipids [3–6]. One of the breakdown products of this process is 1,2-diacylglycerol through which the calcium-phospholipid dependent protein kinase (protein kinase C) is activated [7,8]. In [9,10] it was postulated that both an increase in intracellular Ca^{2+} concentration and the activation of protein kinase C are essential and act synergistically to produce a full response in stimulus-response coupling, for example, the release of serotonin from platelets. Tumor-promoting phorbol esters such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) mimic the action of hormone and neurotransmitter [11] and activate protein kinase C, in vitro and in vivo [12,13]. Thus, using TPA and the Ca^{2+} ionophore, A23187, the two routes can be separately activated. The synergistic effect of A23187 and TPA in the stimulus-secretion coupling has been demonstrated in various tissues [14–18], but apparently not in nervous tissue. Protein kinase C is present in the brain and peripheral tissues innervated by autonomic nerves such as small intestine, vas deferens, uterus and aorta [19]. In the brain a large amount of the enzyme is present in the synaptosomal fraction as well as the soluble fraction [20]. We carried out experiments to determine whether the two synergistic routes are involved in the release of acetylcholine from parasympathetic nerve endings in the guinea pig ileum, as induced by electrical stimulation.

2. MATERIALS AND METHODS

Guinea pigs of either sex weighing 300–400 g, were decapitated and the ilea were immediately prepared into strips which were incubated with 200 nM [^{3}H]choline (60 Ci/mmol, RCC Amersham) in oxygenated Krebs solution (M: 118 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.19 $MgSO_4$, 25.0 $NaHCO_3$, 1.18 KH_2PO_4, 11 glucose) maintained at 37°C for 1 h. After washing in fresh Krebs medium for 20 min, these strips were incubated in Ca^{2+}-free medium containing 0.1 mM EGTA for 10 min. These tissues were then washed 3 times for 5 min in Ca^{2+}-free medium (without EGTA), mounted in an apparatus and superfused at 37°C at a flow rate

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of 1 ml/min with Ca^{2+}-free medium saturated with 95% O_2 and 5% CO_2, containing 10^{-5} M hemicholinium-3, to prevent the re-uptake of [^{3}H]choline formed from [^{3}H]ACh. Two parallel platinum electrodes were used to stimulate intramural nerves of the strip positioned between these two electrodes. The superfusate was collected every 1 min and the radioactivity of the sample was determined by counting in a liquid scintillation spectrometer. At the end of experiments, the tissue was dissolved in Soluene and the radioactivity was measured in a scintillation counter. The release of [^{3}H]ACh was calculated as the fractional rate which represents the ratio of the amount of tritium leaving the tissue during each 1 min collection period to that present in the tissue at the beginning of the corresponding collection period. A23187 and TPA were dissolved in oxygenated Ca^{2+}-free Krebs solution containing 0.01% dimethyl sulfoxide. The following chemicals were obtained from the indicated firms: A23187 (Calbiochem), dimethyl sulfoxide (Nakarai) and TPA and 4α-phorbol-12,13-didecanoate (4α-PDD) (CCR, Inc.) and Soluene (Packard).

3. RESULTS AND DISCUSSION

The guinea pig ileal strips preloaded with [^{3}H]choline were superfused with Ca^{2+}-free medium and stimulated with A23187 or electrical stimulations in the presence or absence of Ca^{2+}. The application of solvents, 0.01% dimethyl sulfoxide to the perfusion medium had no effect on spontaneous release of [^{3}H]ACh.

In the presence of Ca^{2+}, electrical stimulation (1 ms, 15 V, 0.5–50 Hz, for 30 s) of ileal strips preloaded with [^{3}H]choline produced an increase in the tritium efflux. When perfusion medium containing hemicholinium-3 is used, the efflux of [^{3}H]ACh reportedly accounts for nearly all the tritium released from the tissue preloaded with [^{3}H]choline [21,22]. The stimulus-induced release of [^{3}H]ACh was enhanced with increasing frequencies ranging from 0.5 to 30 Hz, and reached a maximal increase at 30 Hz (fig.1). The perfusion of Ca^{2+}-free medium no longer produced an increase in the [^{3}H]ACh release by electrical stimulation. Tetrodotoxin at 3×10^{-7} M also inhibited the stimulus-induced [^{3}H]ACh release. These results indicate that the ACh formed from choline was

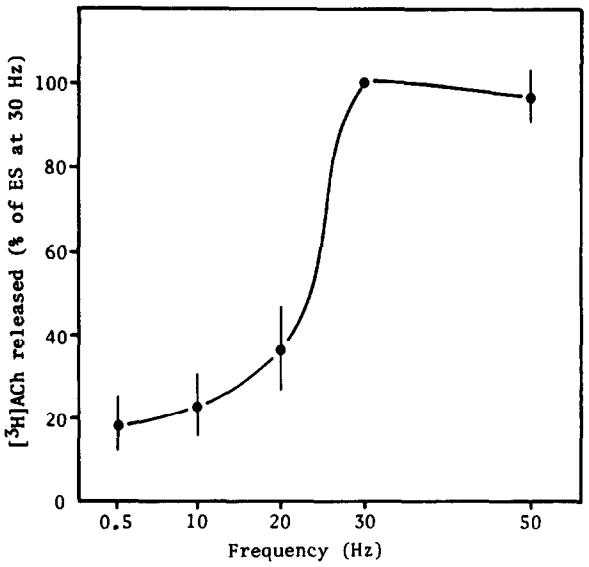

Fig.1. Release of [^{3}H]ACh induced by electrical stimulation from guinea pig ileum preloaded with [^{3}H]choline. The stimulation, which consisted of monophasic pulses (1 ms, 15 V, 0.5–50 Hz), was applied for 30 s. Ileal strips were superfused with Ca^{2+}-free medium and $CaCl_2$ (2.5 mM) was added to the medium for 1 min during application of electrical stimulation. Each point is the mean ± SE from 4 preparations of a percentage of release evoked by electrical stimulation at a frequency of 30 Hz.

released from nerve endings, as tetrodotoxin inhibits neuronal conduction via blocking Na^+ channels [23], and Ca^{2+} is required for the release of neurotransmitters from nerve endings [24]. In the presence but not the absence of 2.5 mM Ca^{2+}, addition of TPA at 10^{-7} M to the perfusate markedly potentiated [^{3}H]ACh release induced by electrical stimulation at 0.5 and 20 Hz, at ED_{20} and ED_{40} values, respectively (fig.2, table 1).

When Ca^{2+} was not included in the superfusate, application of either A23187 or TPA alone at 10^{-7} M, and of the combination of A23187 and TPA for 1 min showed no significant effect on the [^{3}H]ACh release (fig.3, table 1). In the presence of 2.5 mM Ca^{2+} in the superfusate, A23187 at 10^{-7} M produced only a small release of [^{3}H]ACh (fig.3, table 1), while the combination of A23187 and TPA at 10^{-7} M markedly potentiated the [^{3}H]ACh release, but 10^{-7} M TPA alone showed no obvious effect on [^{3}H]ACh release (fig.3, table 1).

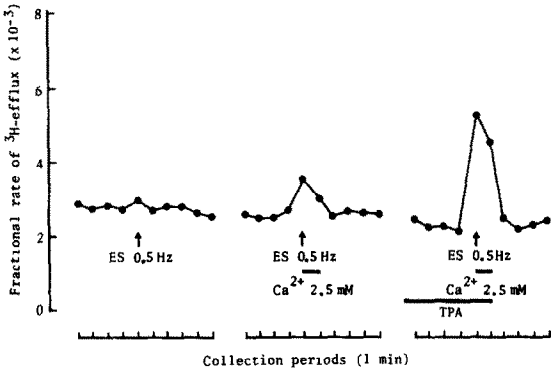

Fig.2. Potentiation by TPA of electrical stimulation-induced [^{3}H]ACh release in the presence of Ca^{2+}. Ileal strips were superfused with Ca^{2+}-free medium. Electrical stimulation (ES) (1 ms, 15 V, 0.5 Hz) was applied for 30 s. $CaCl_2$ (2.5 mM) was added to the medium for 1 min. TPA (10^{-7} M) was added to the medium 60 min before and during ES. Each point represents the mean of 4 preparations. Fractional rate: ratio of the amount of tritium leaving the tissue to that present in the tissue.

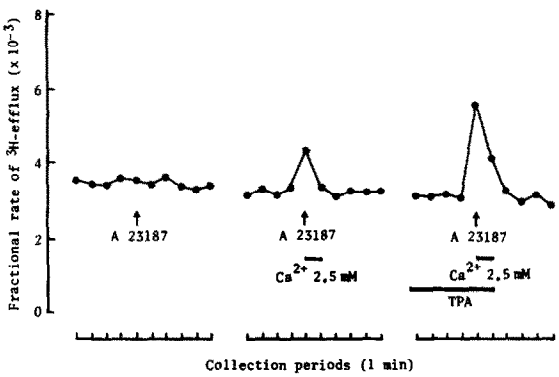

Fig.3. Potentiation by TPA of A23187-induced [^{3}H]ACh release in the presence of Ca^{2+}. Ileal strips were superfused with Ca^{2+}-free medium. A23187 (10^{-7} M) and $CaCl_2$ (2.5 mM) were added to the medium for 1 min. TPA (10^{-7} M) was added to the medium 60 min before and during application of A23187. Each point represents the mean of 4 preparations. Fractional rate: ratio of the amount of tritium leaving the tissue to that present in the tissue.

Table 1

Effect of TPA on release of [^{3}H]ACh from guinea pig ileum

Stimulus	Ca^{2+} (2.5 mM)	[^{3}H]ACh release (% of ES-evoked release)	
		In the absence of TPA	In the presence of TPA
None	–	0	2.1 ± 0.2
A23187 (10^{-7} M)	–	0	1.1 ± 0.1
ES (0.5 Hz)	–	1.5 ± 0.1	1.3 ± 0.1
(20 Hz)	–	0.4 ± 0.4	2.7 ± 0.9
(30 Hz)	–	0.7 ± 0.1	–
None	+	0.4 ± 0.6	2.8 ± 0.4
A23187 (10^{-7} M)	+	17.2 ± 1.8	47.8 ± 4.5[a]
ES (0.5 Hz)	+	17.4 ± 1.6	58.8 ± 5.5[a]
(20 Hz)	+	35.9 ± 3.3	115.9 ± 10.5[a]
(30 Hz)	+	100	–

[a] Significantly different from the value in the absence of TPA

Ileal strips were superfused with Ca^{2+}-free medium. A23187 and electrical stimulation (ES) (1 ms, 15 V) were applied for 1 min and 30 s, respectively. $CaCl_2$ (2.5 mM) was added to the perfusion medium for 1 min during application of stimulus. TPA (10^{-7} M) was added 60 min before and during application of stimulus. Each value is the mean ± SE from 4 preparations of percent of maximal response (30 Hz-evoked release)

To determine whether TPA functions through the activation of protein kinase C, the ileal strip was superfused with medium containing a non-promoter analog of TPA, 4α-PDD [25]. This agent showed no significant effect either alone or in combination with A23187.

Thus, A23187 plus TPA in the presence of external Ca^{2+} (2.5 mM) mimics the release of ACh induced by electrical stimulation, and neither A23187 nor TPA alone will produce a complete stimulation. The protein kinase C–phospholipid complex appears to be the phorbol ester binding site itself [26,27]. The divalent ionophore in the presence of external Ca^{2+} induces an increase in intracellular Ca^{2+} concentrations. These findings indicate that the effect of TPA on [^{3}H]ACh release is dependent on the intracellular Ca^{2+} concentration, a result to be expected if TPA acts via protein kinase C.

In the presence of Ca^{2+}, TPA potentiated [^{3}H]ACh release induced by electrical stimulation at ED_{40} to a maximal response. The present results may be explained by a plausible model of information flow via two routes of the Ca^{2+} messenger system. First, depolarization of the synaptic membrane induced by electrical stimulation produces an increase in intracellular Ca^{2+} concentration mainly by an increased influx of Ca^{2+}. The transient rise of Ca^{2+} concentration leads to the activation of calmodulin protein kinase and other Ca^{2+}-dependent processes [28]. Second, depolarization leads to an increase in diacylglycerol content in the synaptic membrane as a result of breakdown of inositol phospholipids and the affinity of Ca^{2+} for the protein kinase would thus be increased [9,10].

Our observations support the idea that protein kinase C plays a role in cell surface signal transduction related to release of transmitters from the nerve endings.

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