Role of Protein Kinase C in the Vesicular Release of Acetylcholine and Norepinephrine from Enteric Neurons of the Guinea Pig Small Intestine

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Abstract—The involvement of protein kinase C in the release of \(^{3}\text{H}\)acetylcholine (ACh) and \(^{3}\text{H}\)norepinephrine (NE) was studied in strips of guinea pig small intestine. 12-\text{O}-\text{tetradecanoyl phorbol 13-acetate} (TPA), but not 4\alpha-\text{phorbol-12,13-didecanoate} (4\alpha-PDD) potentiated the A23187-evoked release of \(^{3}\text{H}\)ACh and \(^{3}\text{H}\)NE from the strips of small intestine preloaded with \(^{3}\text{H}\)choline and \(^{3}\text{H}\)NE, and the potentiating effect of TPA was inhibited by polymyxin B. High K\(^{+}\)-evoked releases of \(^{3}\text{H}\)ACh and \(^{3}\text{H}\)NE in the presence of tetrodotoxin were also potentiated by TPA. These TPA-induced potentiations of the evoked release were greater at a low concentration of external Ca\(^{2+}\) (0.5 mM) than at a high concentration (2 mM). Ouabain induced the release of these neurotransmitters both in the absence and presence of the low concentration of external Ca\(^{2+}\). The ouabain-evoked release was not altered by TPA. These results indicate that the activation of protein kinase C potentiates the vesicular release of ACh and NE at low Ca\(^{2+}\) concentration from the nerve terminals of enteric neurons in the guinea pig small intestine.

Materials and Methods
Adult guinea pigs of either sex, weighing 300 and 500 g, were killed by cervical dislocation. The ilea were immediately excised and cut along the longitudinal axis. A strip about 2 cm in length was prepared from the ileum 10 cm proximal to the ileocaecal sphincter.
Measurements of [3H]acetylcholine release and [3H]norepinephrine release: The strips of ileum were incubated for 60 min with [3H]choline or [3H]NE at a final concentration of 10^{-8} M in Krebs’ solution of the following composition: 118 mM NaCl, 4.8 mM KCl, 2.0 mM CaCl₂, 1.19 mM MgSO₄, 25.0 mM NaHCO₃, 1.18 mM KH₂PO₄ and 11 mM glucose, which was continuously gassed with 95% O₂ and 5% CO₂ and maintained at 34–37°C. After washing in fresh Krebs’ solution for 20 min, the strips were incubated in Ca²⁺-free Krebs’ solution containing 10^{-4} M EGTA for 15 min, then washed 3 times for 5 min in Ca²⁺-free medium without EGTA. The strips were mounted in the apparatus and superfused at a flow rate of 1.2 ml/min with Ca²⁺-free Krebs’ solution maintained at 34–37°C, gassed with 95% O₂ and 5% CO₂. For the experiments on [3H]ACh release, the perfusion medium was Ca²⁺-free Krebs’ solution containing 10^{-5} M hemicholinium-3, to prevent the uptake of [3H]choline formed from [3H]ACh. For [3H]NE release, the incubation and perfusion media were Krebs’ solution containing 10^{-5} M ascorbate and 10^{-4} M pargyline. Ca²⁺-free medium was Krebs’ solution from which CaCl₂ was omitted. Superfusates were collected in a volume of 1.2 ml at 1 min intervals. The radioactivity of 500 µl of superfusates was determined by counting in a liquid scintillation spectrometer. At the end of the experiment, the tissue was dissolved in Soluene, and the radioactivity was measured in a scintillation counter. The release of [3H] was represented as the fractional rate obtained by dividing the amount of [3H] radioactivity in the superfuse by the respective amount of [3H]radioactivity in the tissue. The [3H]radioactivity content in the tissue at each period was calculated by adding cumulatively the amount of each fractional [3H]efflux to the [3H]content of the tissue at the end of the experiment. The proportion of unchanged tritiated ACh and NE to total tritium efflux in the superfusates collected from the stimulated preparations was estimated by the methods described previously (16, 17). The values were over 81% for [3H]-ACh and 89% for [3H]NE. Since the total tritium in the superfusate was considered to approximate the amount of each labeled ligand, it is denoted as the release of tritiated ACh and NE.

Stimulation: Experiments were started 60 min after the spontaneous release of tritium had approached a plateau level. The fractional rates of spontaneous release of [3H]ACh and [3H]NE 60 min after superfusion with the Ca²⁺ (2 mM)-containing medium and Ca²⁺-free medium were (3.68±0.21) x 10^{-3}/min (n=7) and (3.76±0.18) x 10^{-3}/min (n=7) for [3H]ACh release and (3.82±0.18) x 10^{-3}/min (n=7) and (3.85±0.25) x 10^{-3}/min (n=7) for [3H]NE release, respectively. Since there were no significant differences in the fractional rate of spontaneous releases of [3H]ACh and [3H]NE between the absence and presence of external Ca²⁺, we examined the effects of 12-O-tetradecanoylphorbol 13-acetate (TPA) on the release induced by the application of stimulant alone or the simultaneous application of stimulant and CaCl₂ to the Ca²⁺-free medium. A23187 dissolved in Krebs’ solution containing 0.01% dimethyl sulfoxide (DMSO) was applied to the superfusion medium for 1 min. High K⁺ medium in which KCl was replaced by isomolar NaCl was superfused for 30 sec, and ouabain was superfused for 1 min. TPA was dissolved in Krebs’ solution containing 0.01% DMSO immediately before use. Data were analyzed by Student’s t-test, and a P value of 0.05 or less was considered statistically significant.

Drugs and chemicals: Substances used were as follows: [3H]choline (60 Ci/mmol, Amersham); [3H]norepinephrine (NE) (43.9 Ci/mmol, New England Nuclear); hemicholinium-3 (Aldrich), tetrodotoxin (TTX) (Sankyo); ouabain, ethyleneglycol-bis(β-aminoethyl ether) N,N’-tetraacetic acid (EGTA) and dimethyl sulfoxide (DMSO) (Nakarai); A23187 (Calbiochem); 12-O-tetradecanoylphorbol 13-acetate (TPA) and 4a-phorbol-12,13-didecanoate (4a-PDD) (CCR); Soluene® (Packard); and polymyxin B and pargyline hydrochloride (Sigma).

Results

TPA-induced potentiation of the release of [3H]ACh and [3H]NE evoked by A23187: Simultaneous application of 10^{-7} M A23187
### Table 1. Effect of phorbol ester on A23187-induced releases of \[^3\text{H}]\text{ACh}\) and \[^3\text{H}]\text{NE}\)

| Agents           | ACh          | NE           |
|------------------|--------------|--------------|
|                  | Fractional rate \(\times 10^{-3}/\text{min}\) | Evoked release | Fractional rate \(\times 10^{-3}/\text{min}\) | Evoked release |
|                  | Spontaneous | Stimulation  |            | Spontaneous | Stimulation  |            |
| A23187+Ca\(^{2+}\) | 3.72±0.29    | 6.06±0.24    | 0.63±0.04  | 4.01±0.33   | 6.15±0.29   | 0.53±0.03  |
| A23187+Ca\(^{2+}\)+TPA | 3.57±0.22   | 7.39±0.41*   | 1.07±0.05* | 3.78±0.27   | 8.17±0.31*  | 1.16±0.04* |
| A23187+Ca\(^{2+}\) | 3.76±0.34    | 6.22±0.27    | 0.66±0.04  | 3.88±0.32   | 6.08±0.38   | 0.57±0.04  |
| A23187+Ca\(^{2+}\)+4\(\alpha\)-PDD | 3.48±0.29   | 5.82±0.34    | 0.67±0.04  | 3.65±0.20   | 5.77±0.32   | 0.58±0.03  |
| A23187+Ca\(^{2+}\) | 3.76±0.33    | 6.41±0.31    | 0.71±0.05  | 4.11±0.28   | 6.52±0.35   | 0.59±0.04  |
| A23187+Ca\(^{2+}\)+PMB | 3.51±0.31   | 5.89±0.26    | 0.68±0.04  | 3.82±0.31   | 5.96±0.28   | 0.56±0.04  |
| A23187+Ca\(^{2+}\)+PMB+TPA | 3.40±0.34   | 5.75±0.34    | 0.69±0.05  | 3.67±0.24   | 5.78±0.33   | 0.58±0.04  |

The strips of ileum were superfused with Ca\(^{2+}\)-free medium. TPA (10\(^{-7}\) M), 4\(\alpha\)-PDD (10\(^{-7}\) M) and polymyxin B (PMB) (10\(^{-6}\) M) were treated 30 min, 30 min and 40 min before and during the simultaneous application of A23187 (10\(^{-7}\) M) and CaCl\(_2\) (2 mM) for 1 min, respectively. *Significantly different from the control \(P\leq0.05\). Values are the means±S.E. of 7 animals. Evoked release was calculated as: \(\frac{\text{Stimulation release} - \text{Spontaneous release}}{\text{Spontaneous release}}\).
and 2 mM Ca\(^{2+}\), but not 10\(^{-7}\) M A23187 alone and Ca\(^{2+}\) alone, to the Ca\(^{2+}\)-free medium evoked the release of [\(^3\)H]ACh and [\(^3\)H]NE from the strips of small intestine preloaded with [\(^3\)H]choline and [\(^3\)H]NE, respectively (Table 1). Application of 0.01% DMSO, the vehicle, to the superfusion medium had no effect on the spontaneous release of [\(^3\)H]ACh and [\(^3\)H]NE. Pretreatment with 10\(^{-7}\) M TPA for 30 min potentiated the release of [\(^3\)H]ACh and [\(^3\)H]NE evoked by the combination of A23187 and Ca\(^{2+}\), without changing the spontaneous release of [\(^3\)H]ACh and [\(^3\)H]NE (Table 1). Since the potentiation by TPA at 10\(^{-7}\) M A23187 was maximal 30 min after the treatment and leveled off thereafter for at least 45 min (data not shown), in the following studies, the strips were pretreated with various concentrations of TPA for 30 min before the simultaneous application of A23187 and Ca\(^{2+}\).

Fig. 1. Potentiation by TPA of the release of [\(^3\)H]acetylcholine (ACh) (●) and [\(^3\)H]norepinephrine (NE) (○) evoked by simultaneous application of A23187 and Ca\(^{2+}\) (2 mM). Each point is the mean±S.E. from 7 animals of the ratio of TPA to the control. Ratio of TPA to the control was calculated as:

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\text{Ratio of TPA to control} = \frac{\text{Evoked release in the presence of TPA}}{\text{Evoked release in the absence of TPA}}
\]

TPA at the concentration range of 10\(^{-9}\) M to 3\(\times\)10\(^{-7}\) M potentiated the release of [\(^3\)H]ACh and [\(^3\)H]NE evoked by the combination of 10\(^{-7}\) M A23187 and 2 mM Ca\(^{2+}\), in a concentration dependent manner, and the maximal response was obtained at 3\(\times\)10\(^{-8}\) M (Fig. 1). The potentiation by TPA was significantly greater in the release of [\(^3\)H]NE than in the release of [\(^3\)H]ACh. The effect of TPA was not mimicked by 10\(^{-7}\) M 4α-PDD, a non-promotor analog of TPA, and it was antagonized by 10\(^{-6}\) M polymyxin B (Table 1).

As shown in Fig. 2, the A23187-evoked release of [\(^3\)H]ACh was increased with increasing external Ca\(^{2+}\) concentrations. TPA potentiated the A23187-evoked release of [\(^3\)H]ACh, and the potentiation was significantly greater at an external Ca\(^{2+}\) concentration of 0.5 mM than at the concentration of 2 mM.

**TPA-induced potentiation of the release**
of [3H]ACh and [3H]NE evoked by high K⁺: The simultaneous application of 30 mM K⁺ and 2 mM Ca²⁺, but not 30 mM K⁺ alone, to the Ca²⁺-free medium containing 10⁻⁶ M tetrodotoxin produced increases in the release of [3H]ACh and [3H]NE. The pretreatment with 10⁻⁷ M TPA for 30 min potentiated the release of [3H]ACh and [3H]NE evoked by the combination of 30 mM K⁺ and 2 mM Ca²⁺ in the presence of 10⁻⁶ M tetrodotoxin (Table 2). The potentiating effect of TPA was significantly greater on the evoked release of [3H]NE than on the release of [3H]ACh.

Since a significant difference in the potentiation by TPA of A23187-evoked release of [3H]ACh was observed at external Ca²⁺ concentrations between 0.5 mM and 2 mM, the effect of external Ca²⁺ concentration was also examined on the high K⁺-evoked release of [3H]ACh and [3H]NE. When the concentration of 0.5 mM Ca²⁺ was used instead of 2 mM Ca²⁺ in the stimulation by the combination of high K⁺ and Ca²⁺, 10⁻⁷ M TPA potentiated the stimulation-evoked release of [3H]ACh and [3H]NE (Table 2). The potentiation by TPA at the low concentration of external Ca²⁺ (0.5 mM) was significantly greater than that at the high concentration of external Ca²⁺ (2 mM).

Effect of TPA on the ouabain-induced release of [3H]ACh and [3H]NE: Ouabain at 2 x 10⁻⁵ M alone and the combination of ouabain and 0.5 mM Ca²⁺ evoked the release of [3H]ACh and [3H]NE (Table 3). The ouabain-evoked release of [3H]ACh was significantly greater than that of [3H]NE. In the presence or absence of Ca²⁺, pretreatment with TPA at 10⁻⁷ M for 30 min had no significant effect on the ouabain-evoked releases of [3H]ACh and [3H]NE (Table 3).

**Discussion**

The present results emphasize that the activation of protein kinase C potentiates the
Table 2. Effect of TPA on high K⁺-induced releases of [³H]ACh and [³H]NE

| Agents          | ACh | NE |
|-----------------|-----|----|
|                 | Fractional rate (×10⁻⁹/min) | Ratio of TPA to control | Fractional rate (×10⁻⁹/min) | Ratio of TPA to control |
|                 | Spontaneous | Stimulation | Evoked release | | Spontaneous | Stimulation | Evoked release | |
| Ca²⁺+ 2 mM      | 3.76±0.29 | 6.00±0.25 | 0.60±0.03 | 4.15±0.32 | 6.86±0.42 | 0.65±0.04 |
| K⁺+Ca²⁺ (Control) | 3.58±0.34 | 7.75±0.33* | 1.17±0.04* | 2.02±0.11 |
| K⁺+Ca²⁺+TPA     | 3.58±0.34 | 7.75±0.33* | 1.17±0.04* | 2.02±0.11 |
| Ca²⁺+ 0.5 mM    | 3.89±0.32 | 5.21±0.34 | 0.34±0.04 | 4.20±0.26 | 5.69±0.35 | 0.36±0.04 |
| K⁺+Ca²⁺ (Control) | 3.73±0.25 | 6.92±0.40* | 0.85±0.04* | 2.59±0.19 |
| K⁺+Ca²⁺+TPA     | 3.73±0.25 | 6.92±0.40* | 0.85±0.04* | 2.59±0.19 |

The strips of ileum were superfused with Ca²⁺-free medium containing 10⁻⁶ M tetrodotoxin. KCl (30 mM) and CaCl₂ (0.5 mM or 2 mM) were applied simultaneously for 30 sec. TPA (10⁻⁷ M) was treated 30 min before and during application of the combination of KCl and CaCl₂. Values are the means±S.E. of 7 animals. *Significantly different from the control (P<0.05). Evoked release was calculated as: Stimulation release - Spontaneous release. Ratio of TPA to the control was calculated in the same preparation as: Evoked release in the presence of TPA / Evoked release in the absence of TPA.

Table 3. Effect of TPA on ouabain-induced releases of [³H]ACh and [³H]NE in the absence and presence of external Ca²⁺

| Agents          | ACh | NE |
|-----------------|-----|----|
|                 | Fractional rate (×10⁻⁹/min) | Ratio of TPA to control | Fractional rate (×10⁻⁹/min) | Ratio of TPA to control |
|                 | Spontaneous | Stimulation | Evoked release | | Spontaneous | Stimulation | Evoked release | |
| Ca²⁺-free       | 3.58±0.31 | 4.54±0.27 | 0.27±0.03 | 3.94±0.23 | 4.51±0.27 | 0.15±0.03 |
| Ouabain (Control) | 3.39±0.22 | 4.17±0.24 | 0.23±0.03 | 3.79±0.29 | 4.35±0.32 | 0.15±0.03 |
| Ouabain+TPA     | 3.39±0.22 | 4.17±0.24 | 0.23±0.03 | 3.79±0.29 | 4.35±0.32 | 0.15±0.03 |
| Ca²⁺+ 0.5 mM    | 3.67±0.25 | 4.78±0.31 | 0.30±0.03 | 3.82±0.21 | 4.68±0.27 | 0.23±0.03 |
| Ouabain (Control) | 3.55±0.30 | 4.71±0.26 | 0.33±0.03 | 1.11±0.09 |
| Ouabain+TPA     | 3.55±0.30 | 4.71±0.26 | 0.33±0.03 | 1.11±0.09 |

The strips of ileum were superfused with Ca²⁺-free medium. Ouabain (2×10⁻⁶ M) alone or together with CaCl₂ (0.5 mM) was applied for 1 min. TPA (10⁻⁷ M) was treated 30 min before and during application of ouabain alone or the combination of ouabain and CaCl₂. Values are the means±S.E. of 7 animals. Evoked release was calculated as: Stimulation release - Spontaneous release. Ratio of TPA to the control was calculated in the same preparation as: Evoked release in the presence of TPA / Evoked release in the absence of TPA.
vesicular release of ACh and NE from the enteric nerve terminals of guinea pig small intestine. When two routes of the Ca\(^{2+}\) messenger system were separately activated using A23187, a calcium ionophore, and TPA, an activator of protein kinase C (3, 4), the release of ACh and NE evoked by the combination of A23187 and Ca\(^{2+}\) were potentiated by TPA. This indicates that TPA activates protein kinase C in the neuronal tissue, resulting in the potentiation of releases of ACh and NE. This idea may be supported by the findings of no effect of 4\(\alpha\)-PDD on the release evoked by the combination of A23187 and Ca\(^{2+}\) and an antagonism of polymyxin B on the potentiating effect of TPA, although it cannot be excluded that polymyxin B exerts a nonspecific effect, since the agent has been reported to inhibit protein kinase C by competing for a hydrophobic region on the enzyme to which the phospholipid cofactor binds (18, 19). The effect of H7 on the TPA-induced potentiation was not examined in the present experiment; however, the compound possibly antagonizes the TPA effect, because H7 has been shown to inhibit protein kinase C activity via a direct interaction on the catalytic site of the enzyme (20), although H7 has been shown to inhibit cyclic AMP- and cyclic GMP-dependent protein kinases as well as protein kinase C (20). Therefore, both polymyxin B and H7 are considered to be less specific as inhibitors of protein kinase C, as compared with TPA as an activator.

TPA also potentiated the release of ACh and NE evoked by the combination of high K\(^{+}\)-depolarization and Ca\(^{2+}\) in the presence of tetrodotoxin; under such conditions, the release of neurotransmitter is induced by the direct depolarization of nerve terminals (21, 22), thereby indicating that TPA activated the protein kinase C within the nerve terminals, and the activation of protein kinase C led to the potentiation of the evoked release of neurotransmitters. TPA-induced potentiation of the evoked release of ACh and NE was greater at a low concentration of external Ca\(^{2+}\) than at the high concentration, which is in agreement with the case of ACh release from the caudate slices (6) and NE release from the sinus node (7). The results imply that the activation of protein kinase C may induce a sufficient response at the low concentration of Ca\(^{2+}\).

It has been considered that NE is released mainly in a vesicular manner (14) and about half of ACh is in a vesicular release (12). Ouabain was found to evoke the release of ACh and NE from the enteric neurons both in the absence and presence of external Ca\(^{2+}\), and the amount of ACh release was more than that of NE release. Ouabain, a Na\(^{+}\),K\(^{+}\)-ATPase inhibitor is known to induce the Ca\(^{2+}\)-independent, non-vesicular release of neurotransmitters (12–15, 23); therefore, the results may support the concept that part of non-vesicular release is larger in the ACh release than in the NE release. In addition to the result that the potentiating effect of TPA was greater in the release of NE than that of ACh, no effect of TPA on the ouabain-evoked release indicates that the activation of protein kinase C is involved in the vesicular, but not the non-vesicular release of ACh and NE. Na\(^{+}\),K\(^{+}\)-ATPase has been reported to be activated by protein kinase C (24); however, TPA did not significantly contradict the ouabain effect, and thus ouabain might induce the release through a mechanism other than the inhibition of Na\(^{+}\),K\(^{+}\)-ATPase.

Some concepts have been proposed concerning the mechanism of action of protein kinase C in the neurotransmitter release. The activation of protein kinase C has been shown to block the Ca\(^{2+}\)-activated K\(^{+}\) current (25, 26). The blockade of K\(^{+}\) channel may lead to a prolongation of the action potential of the nerve terminal and then increases the release of neurotransmitter. An enhancement of Ca\(^{2+}\) currents in an invertebrate neuron (27) may lead to an increase in the release of neurotransmitter. Protein kinase C-induced phosphorylation of microtubule-associated proteins (28) and 87 kDa protein (9, 29) present in the nerve terminals have been documented. A dramatic increase in 87 kDa phosphorylation has been found under conditions corresponding to those used for assaying neurotransmitter release from the brain synaptosomal preparations (9). It is likely that the phosphorylation of these proteins by protein kinase C is involved in the exocytosis of neurotransmitter release. There
is a possibility that phosphorylation of such proteins is involved in the vesicular release of ACh and NE from the enteric neurons, although no evidence has been obtained that such proteins are present in the enteric nerve terminals. Sequence analysis of brain complementary DNA libraries demonstrated the presence of multiple subspecies of protein kinase C (α, βI, βII, γ, δ, ε and ξ) (30–34). γ-Type protein kinase C has been demonstrated to be localized on the nerve terminals surrounding the neuronal cell bodies in the deep cerebellar nucleus (35); however, there is no documentation on the presence of protein kinase C in the peripheral neurons. Which type of protein kinase C participates in the exocytotic release of neurotransmitters remains to be determined.

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