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External Ca^{2+}-independent norepinephrine release from hippocampal slices and modulation by protein kinase C activation[1]

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ABSTRACT 4β-Phorbol 12, 13-dibutyrate (PDB) enhanced [^{3}H] norepinephrine ([^{3}H]NE) release from rat hippocampal slices evoked by 3, 4-diaminopyridine (3, 4-DAP, 100—400 μmol·L^{-1}), veratridine (1—7 μmol·L^{-1}) or ouabain (100—200 μmol·L^{-1}), but had no effect on those evoked by monensin (0.01—10 μmol·L^{-1}) in the absence of extracellular Ca^{2+}. Tetrodotoxin (TTX, 0.3 μmol·L^{-1}) blocked [^{3}H]NE release evoked by 3,4-DAP, veratridine, or ouabain, but failed to affect that evoked by monensin. Tetraacetoxy methyl ester of 1-2-bis-(2-aminophenoxy) ethane-*N*,*N*,*N*',*N*'-tetraacetic acid (BAPTA-AM), Ca^{2+} chelator, inhibited the [^{3}H]NE release evoked by these substances, but did not inhibit the enhancement of the evoked NE release by PDB. The findings suggest that under external Ca^{2+}-free conditions protein kinase C activation enhances [^{3}H]NE release evoked by the membrane depolarizing agents, but does not affect that evoked by Na^+-induced internal Ca^{2+} release.

KEY WORDS norepinephrine; aminopyridines; veratridine; ouabain; monensin; protein kinase C

Activation of protein kinase C (PKC) enhanced[1,2], whereas inhibition of PKC decreased, the release of various neurotransmitters[3]. A physiological role of PKC in modulation of transmitter release was suggested. The mechanism of the presynaptic enhancement of transmitter release by PKC activation remains obscure. We recently found[4] that under normal conditions 3, 4-diaminopyridine (DAP)-evoked [^{3}H] norepinephrine ([^{3}H]NE) release from hippocampal slices consisted of 2 parts; the first being dependent and the second being independent from the presence of extracellular Ca^{2+}. The antagonist of *N*-type Ca^{2+}-channels ω-conotoxin inhibited the former, whereas PKC activation enhanced mainly the latter[5]. These findings indicated the importance of external Ca^{2+}-independent release and excluded the possible effect of PKC activation on voltage-sensitive Ca^{2+} channels on the presynaptic membrane. The present investigation is to further study the properties of the facilitatory effect of PKC activation on NE release evoked by various agents in the absence of extracellular Ca^{2+}.

MATERIALS AND METHODS

Drugs 1-[7,8-^{3}H]norepinephrine, specific activity 1.6 PBq·mol^{-1} (Amersham, UK); DAP, ouabain, monensin, 4β-phorbol 12, 13-dibutyrate (PDB), tetrodotoxin (TTX), desipramine (Sigma, USA); veratridine (Aldrich, USA); tetraacetoxy methyl ester of 1-2-bis-(2-aminophenoxy) ethane-*N*, *N*, *N*', *N*'-tetraacetic acid (BAPTA-AM, synthesized in Department of Chemistry, East China Normal University). Stock solutions of the drugs to be investigated were prepared in the superfusion medium or water; veratridine and monensin were dissolved in ethanol; PDB and BAPTA-AM were dissolved in dimethylsulfoxide (Me_2SO); TTX was dissolved in sodium citrate buffer (pH 4.85).

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Procedure Hippocampal slices (0.35 mm thick), prepared from ♀ Sprague-Dawley rats, were preincubated in the presence of [^{3}H]NE 0.1 μmol·L^{-1} (1.6 PBq·mol^{-1}) and then superfused at a rate of 0.7 ml·min^{-1} with buffer as described previously[6,7]. After 60 min the slices were stimulated once for 10 min by addition of 3,4-DAP, veratridine, ouabain, or monensin to the superfusion medium. Drugs were added to the medium 15 min before stimulation (if not indicated otherwise). The preincubation medium was composed: NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, disodium edetate 0.03 mmol·L^{-1} (saturated with 5 % CO_2 in O_2; pH 7.4); during superfusion, $CaCl_2$ was replaced by egtazic acid 1 mmol·L^{-1}. In the experiment with BAPTA-AM, the slices were preincubated with BAPTA-AM 0.4 mmol·L^{-1} (controls with 0.5 % Me_2SO) for 2 h before incubation with [^{3}H]NE. As a routine desipramine 1 μmol·L^{-1} was present throughout the superfusion.

Calculation The stimulation-evoked outflow of ^{3}H was estimated by subtracting the basal outflow from the total outflow of ^{3}H during the 50 min after the onset of stimulation; the basal ^{3}H outflow was thereby assumed to decline linearly from the fraction 5 min before, to the fraction 50—55 min after the onset of stimulation[5]. The evoked outflow of ^{3}H was expressed as percentage of the ^{3}H content of the slices at the onset of the stimulation.

Statistics All results were shown as $\bar{x} \pm s$, n = number of single observations in at least 2 rats. Statistical comparisons were carried out with the Mann-Whitney test if Kruskal-Wallis analysis indicated a significance between the group means.

RESULTS

Stimulation-evoked [^{3}H]NE release and its enhancement by PDB Hippocampal slices preincubated with [^{3}H]NA were stimulated for 10 min by addition of DAP (100 — 400 μmol·L^{-1}), veratridine (1 — 7 μmol·L^{-1}), ouabain (100 — 200 μmol·L^{-1}), or monensin (0.01 — 10 μmol·L^{-1}) to the Ca^{2+}-free superfusion medium. [^{3}H]NE release was evoked by these substances in a concentration-dependent manner. PDB 1 μmol·L^{-1} added 15 min before stimulation to the Ca^{2+}-free medium enhanced the [^{3}H]NE release evoked by DAP, veratridine, or ouabain, but not that evoked by monensin (Fig 1).

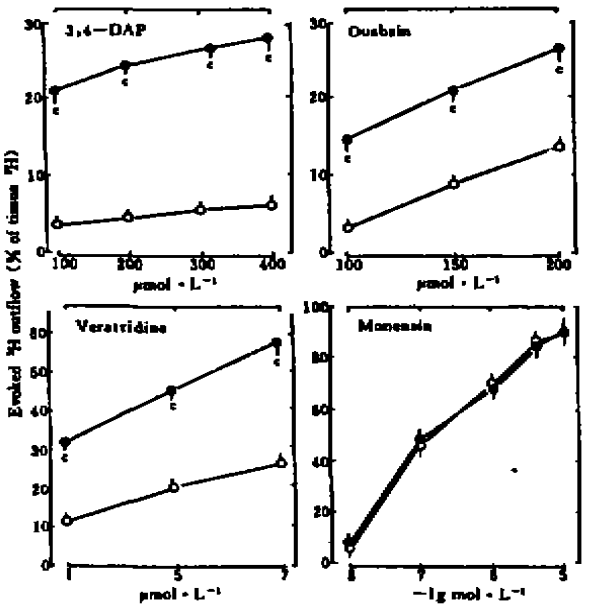

Fig 1. [^{3}H]NE release evoked by DAP, veratridine, ouabain, or monensin from rat hippocampal slices and its enhancement by PDB in the absence of extracellular Ca^{2+}. The slices were stimulated by addition of DAP (100—400 μmol·L^{-1}), veratridine (1—7 μmol·L^{-1}), ouabain (100—200 μmol·L^{-1}), or monensin (0.01—10 μmol·L^{-1}) to the superfusion medium after 60 min of superfusion (○). PDB 1 μmol·L^{-1} (●) was added to the medium from 15 min before stimulation onwards. n=4—20 slices for each concentration from 2—3 rats, $\bar{x} \pm s$. $^c P<0.01$ vs respective controls.

Effects of TTX on stimulation-evoked [^{3}H]NE release TTX (0.3 μmol·L^{-1}), added 15 min before stimulation to the Ca^{2+}-free superfusion medium strongly inhibited the [^{3}H]NE release evoked by DAP 200 μmol·L^{-1} (97% inhibition), veratridine 0.6 μmol·L^{-1} (96% inhibition) or ouabain 100 μmol·L^{-1} (95% inhibition), but showed no significant effect on the [^{3}H]NE release evoked by monensin 0.01 μmol·L^{-1} (Fig 2).

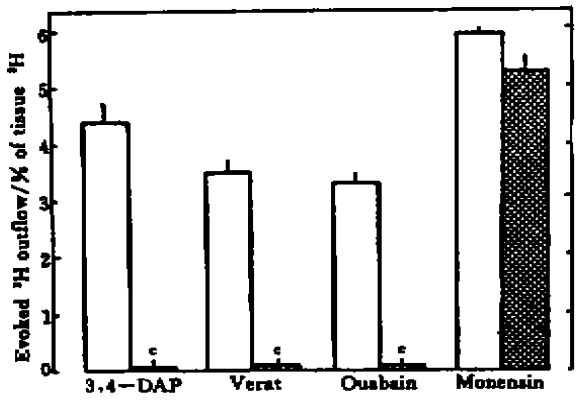

Fig 2. Effects of TTX on [³H]NE release evoked by DAP, veratridine, ouabain, or monensin from hippocampal slices in the absence of extracellular Ca^{2+}. The slices were stimulated by addition of DAP 200 μmol·L^{-1}, veratridine 0.6 μmol·L^{-1}, ouabain 100 μmol·L^{-1}, or monensin 0.01 μmol·L^{-1} to the superfusion medium (□). TTX 0.3 μmol·L^{-1} (■) was added to the superfusion medium from 15 min before the stimulation onwards. n=4—10 slices per treatment, $\bar{x}\pm s$. $^cP<0.01$ vs respective controls.

Effects of BAPTA-AM on stimulation-evoked [³H]NE release and its enhancement by PDB Under Ca^{2+}-free conditions hippocampal slices were preincubated with BAPTA-AM (0.4 mmol·L^{-1}) for 2 h (controls were preincubated with medium containing 0.5% Me_2SO) before incubation with [³H]NE. BAPTA-AM pretreatment inhibited the [³H]NE release evoked by DAP 200 μmol·L^{-1}, veratridine 0.6 μmol·L^{-1}, ouabain 100 μmol·L^{-1}, or monensin 0.02 μmol·L^{-1} (Tab 1).

When PDB (0.5 μmol·L^{-1}) was added to the Ca^{2+}-free superfusion medium 15 min before stimulation, the relative enhancement by PDB (by subtracting the control value without PDB) of [³H]NE release evoked by 3,4-DAP, veratridine, or ouabain was not significantly decreased in the slices preloaded with BAPTA-AM (Tab 1).

Tab 1. Effects of BAPTA-AM on [³H]NA release evoked by 3,4-DAP, veratridine, ouabain, or monensin and on its enhancement by PDB. n=4—6 slices for each treatment from 2—3 rats, $\bar{x}\pm s$. $^bP<0.05$ vs respective controls (without BAPTA-AM); $^cP<0.01$ vs respective controls (without PDB).

μmol·L^{-1}	Control	BAPTA-AM
3,4-DAP 200	4.10±0.22	0.41±0.05[b]
+ PDB 0.5	26.89±1.18[c]	20.82±1.65[c]
Veratridine 0.6	5.57±0.30	3.57±0.26[b]
+ PDB 0.5	28.72±2.14[c]	27.86±2.05[c]
Quabain 150	16.30±1.04	5.31±0.32[b]
+ PDB 0.5	44.32±3.03[c]	30.13±2.52[c]
Monensin 0.02	5.02±0.45	0.47±0.04[b]

DISCUSSION

In 2 previous papers[3,5] we proposed that DAP by blocking K^+ currents occuring in hippocampal tissue[8], depolarizes the terminal membrane, induced and propagated Na^+-influx-carried action potentials under Ca^{2+}-free conditions. The increase in intracellular Na^+ concentrations was the triggering event causing an increase in cytoplasmic free Ca^{2+} necessary for exocytosis. In the present study although we did not directly measure the intracellular free Ca^{2+} concentration, the experiments with TTX and BAPTA-AM did provide important information supporting this conclusion.

Very similar results were obtained in experiments with veratridine. The sensitivity to TTX and the inhibition by BAPTA-AM of this evoked release model further indicated the importance of Na^+ entry during depolarization and of the liberation of intracellular Ca^{2+} stores in the mechanism of action of veratridine[9].

Ouabain by inhibiting Na^+, K^+-ATPase elevated the intracellular Na^+ concentrations and by facilitating the Na^+-dependent Ca^{2+} influx increased the NE release[10]. In the

present study, the inhibition of ouabain-evoked NE release by TTX and BAPTA-AM further supports the involvement of Na^+-carried action potentials and mobilization of internal Ca^{2+} stores in this evoked NE release process.

Monensin, is a highly effective factor in transporting Na^+ and inducing membrane hyperpolarization rather than depolarization[11]. Since voltage-sensitive Na^+ channels are not involved in the action of monensin, [^{3}H]NE relaese evoked by monensin was not affected by TTX (Fig 2). BAPTA-AM inhibited the monensin-evoked [^{3}H]NE release in the absence of extracellular Ca^{2+}, suggesting that the effects of monensin on catecholamine secretion were due to elevation of the intracellular Na^+ level and subsequent release of Ca^{2+} from intracellular stores.

The facilitatory effect of phorbol ester-mediated activation of PKC on NE release evoked by 3,4-DAP has been shown in the absence of extracellular Ca^{2+}[5]. In the present study we observed that PDB also enhanced NE release induced by veratridine or ouabain, but did not show any significant effect on that induced by monensin. As mentioned above, monensin causes Na^+-induced internal Ca^{2+} liberation and membranal hyperpolarization, whereas 3, 4-DAP, veratridine and ouabain cause membrane depolarization in addition to Na^+-induced internal Ca^{2+} release, suggesting that PKC activation modulates the NE release probably by affecting the event subsequent to membrane depolarization rather than by interaction with Na^+-induced internal Ca^{2+} release process.

To conclude, under external Ca^{2+}-free conditions there are 2 independent pathways of evoking the NE release from distinct NE pools: 1) Na^+-induced liberation of internal Ca^{2+} stores triggers NE release; and 2) At resting level of internal Ca^{2+}, membrane depolarization induces NE release. Monensin evokes NE release by the first pathway. DAP, veratridine, and ouabain evoke NE release by both the first and second pathway. BAPTA-AM blocks only the first. PKC activation modulates stimulation-evoked NE release by affecting the second, but not by interaction with the first.

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海马胞外钙不依赖去甲肾上腺素释放及蛋白激酶C的调制作用

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摘要 在细胞外无钙时, 佛波醇基酯能加强3,4-二氨基吡啶、藜芦定或哇巴因所诱发的去甲上腺素(NE)释放, 但对莫能星(Mon)诱发的NE释放无作用. 河豚毒素能阻断前3种物质诱发的NE释放, 但对Mon诱发的释放无作用. 钙螯合剂BAPTA-AM能抑制这4种物质诱发的NE释放. 结果提示蛋白激酶C仅调制由膜去极化因素诱发的NE释放.

关键词 去甲肾上腺素; 氨基吡啶; 藜芦定; 哇巴因; 莫能星; 蛋白激酶C

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Pentoxifylline attenuates platelet activating factor-induced permeable edema in isolated perfused guinea pig lungs[1]

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ABSTRACT The effect of pentoxifylline (Pen) on platelet activating factor (PAF)-induced pulmonary injury was studied in isolated guinea pig lungs perfused with cell-free Tris buffered Ringer solution. PAF (1.0 nmol·L^{-1}) increased lung weight and pulmonary filtration coefficient (K_f), which indicated the formation of high permeable pulmonary edema. Pen (0.5 and 1.0 mmol·L^{-1}) markedly attenuated the PAF-induced increment of lung weight and vascular permeability, but not the increment of pulmonary capillary pressure and venous resistance. There was no correlation between the severity of lung edema and the number of leukocytes in the perfusates. These results suggest that Pen has direct anti-permeability effect on pulmonary microvessels.

KEY WORDS pentoxifylline; pulmonary edema; platelet activating factor

Pentoxifylline [1-(5-oxohexyl)-3, 7-dimethylxanthine, Pen] can increase intracellular cAMP by inhibiting cyclic nucleotide phosphodiesterase[1] and attenuate the formation of high permeable pulmonary edema in several models of acute lung injury including sepsis after *Escherichia coli* administration[2,3]. The ability of Pen to reduce lung injury was attributed to its inhibitory effects on neutrophil functions[4,5].

Platelet activating factor (PAF) is a potent lipid mediator involved in endotoxin-induced acute lung injury[6]. Our previous study showed that PAF increased albumin flux across cultured pulmonary endothelial cell

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