

Involvement of intracellular Ca^{2+} stores in 3, 4-diaminopyridine-evoked [^3H]norepinephrine release¹

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KEY WORDS norepinephrine; hippocampus; 3, 4-diaminopyridine; reserpine; BAPTA; dantrolene; desipramine

AIM: To study the mechanism for 3, 4-diaminopyridine (DAP) evoking external Ca^{2+} -independent release of [^3H]norepinephrine ([^3H]NE).

METHODS: Rat hippocampal slices were preincubated with [^3H]NE and superfused with medium. [^3H]NE release was determined.

RESULTS: Under Ca^{2+} -free conditions, DAP evoked [^3H]NE release. In rats pretreated by reserpine, the effect of DAP was no longer detectable. Ca^{2+} chelator BAPTA-AM potently inhibited DAP-evoked [^3H]NE release. Desipramine $100 - 500 \mu\text{mol} \cdot \text{L}^{-1}$ strongly induced [^3H]NE release in a concentration-dependent manner, whereas caffeine $30 - 70 \text{mmol} \cdot \text{L}^{-1}$ was slightly effective on [^3H]NE release. The blocker of Ca^{2+} -induced Ca^{2+} releasable stores, dantrolene sodium did not attenuate DAP-evoked [^3H]NE release. **CONCLUSION:** In the absence of extracellular Ca^{2+} , DAP evokes exocytotic release of [^3H]NE from synaptic vesicles through liberation of internal Ca^{2+} from inositol 1, 4, 5-trisphosphate-sensitive Ca^{2+} stores.

Our previous study found that 3, 4-diaminopyridine (DAP) exhibited particularly potent facilitatory effects on the spontaneous ^3H outflow from rabbit hippocampal slices preincubated with [^3H]norepinephrine ([^3H]NE)⁽¹⁾. This effect was tetrodotoxin (TTX) sensitive and modulated by presynaptic α_2 -adrenoceptors, κ -opioid and adenosine A_1 receptors; It was strongly enhanced after activation of protein kinase C (PKC) with phorbol ester, or reduced by various PKC inhibitors. These observations indicate the

involvement of action potential-evoked exocytotic release of [^3H]NE following DAP application. In the absence of extracellular Ca^{2+} , DAP also induced [^3H]NE release from rabbit and rat hippocampal slices preincubated with [^3H]NE⁽²⁾ and phorbol ester enhanced this evoked [^3H]NE release⁽³⁾. The aim of this study was to study the mechanism of DAP-induced external Ca^{2+} -independent [^3H]NE release from rat hippocampal slices.

MATERIALS AND METHODS

Chemicals 1-(7, 8- ^3H)norepinephrine ([^3H]NE, Amersham, UK); DAP, desipramine, caffeine, dantrolene sodium (Sigma, USA); Tetraacetoxy methyl ester of 1, 2-bis(2-aminophenoxy) ethane- N, N, N', N' -tetraacetic acid (BAPTA-AM, Molecular Probes Inc, USA). Stock solutions of the drugs were prepared in water except BAPTA-AM dissolved in Me_2SO .

Procedure Hippocampal slices (0.35 mm thick), prepared from Sprague-Dawley rats (δ , certificate number 02-35-2, $n = 25$), were preincubated with [^3H]NE $0.1 \mu\text{mol} \cdot \text{L}^{-1}$ and then superfused with medium $0.7 \text{mL} \cdot \text{min}^{-1}$. Samples were collected every 5 min. After 60 min of superfusion the slices were exposed to drugs to induce transmitter release. At the end of superfusion, the slices were solubilized in 0.5 mL Soluene-350 (Packard). The ^3H content of the solubilized slices and the superfusion samples was determined with a liquid scintillation counter. The preincubation medium: 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 $0.03 \text{mmol} \cdot \text{L}^{-1}$ (saturated with 5 % CO_2 in O_2 ; pH 7.4). In Ca^{2+} -free superfusion medium, CaCl_2 was replaced by egtazic acid $1 \text{mmol} \cdot \text{L}^{-1}$. In the experiment with BAPTA-AM, the slices were preincubated with BAPTA-AM $0.4 \text{mmol} \cdot \text{L}^{-1}$ for 2 h (controls with 0.5 % Me_2SO) before incubation with [^3H]NE. Desipramine $1 \mu\text{mol} \cdot \text{L}^{-1}$ was present throughout the superfusion to block reuptake system of NE⁽²⁾.

Calculation The fractional rate of ^3H outflow was calculated as ^3H outflow per 5 min divided by the ^3H content in the slices at the start of the respective 5 min period. The drug-evoked ^3H outflow was estimated by subtracting the basal outflow from the total outflow of the 50

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min after the onset of the drug. The basal outflow of ^3H was assumed to decline linearly from the fraction 55 - 60 min, to the fraction 110 - 115 min of superfusion. The evoked outflow of ^3H was expressed as % of the ^3H content of the slices at the onset of the drug. Results were shown as $\bar{x} \pm s$. The significance of differences between the groups were determined by *t* test⁽⁴⁾.

RESULTS

Effects of reserpine and BAPTA-AM on DAP-evoked [^3H]NE release in the absence of extracellular Ca^{2+} To test whether DAP-evoked external Ca^{2+} -independent release of [^3H]NE is exocytosis from synaptic vesicles, the rats were injected sc with reserpine $10 \text{ mg} \cdot \text{kg}^{-1}$ 12 h before death. When NE stored in synaptic vesicles was depleted by reserpine, DAP-evoked [^3H]NE release was no longer detectable (Tab 1).

Tab 1. Effects of reserpine and BAPTA-AM on DAP-evoked [^3H]NE release in the absence of extracellular Ca^{2+} . Hippocampal slices were preincubated with [^3H]NE and superfused continuously. After 60 min of superfusion the slices were exposed to DAP to induce [^3H]NE release. $n = 4 - 6$ slices from 2 rats, $\bar{x} \pm s$. $^c P < 0.01$.

Drug/ $\mu\text{mol} \cdot \text{L}^{-1}$	Evoked outflow of ^3H / % of tissue ^3H
DAP 300 + reserpine	9.6 ± 0.4 0.13 ± 0.10^c
DAP 200 + BAPTA-AM	4.10 ± 0.22 0.41 ± 0.05^c

The effects of Ca^{2+} chelator BAPTA-AM were studied to examine the necessity of elevation of cytosol free Ca^{2+} for the induction of DAP-evoked [^3H]NE release. BAPTA-AM $0.4 \text{ mmol} \cdot \text{L}^{-1}$ inhibited the DAP-evoked release of [^3H]NE by 90 % of controls (Tab 1).

Effects of desipramine and caffeine on [^3H]NE release in the absence of extracellular Ca^{2+} At 60 min of superfusion, addition of desipramine $100 \mu\text{mol} \cdot \text{L}^{-1}$ for 10 min to the Ca^{2+} -free superfusion medium (with egtazic acid $1 \text{ mmol} \cdot \text{L}^{-1}$) enhanced ^3H outflow over basal values. The outflow reached the maximum at 65 - 70 min. Subsequently the ^3H outflow slowly returned to basal values within 50 min after the addition of

desipramine (Fig 1).

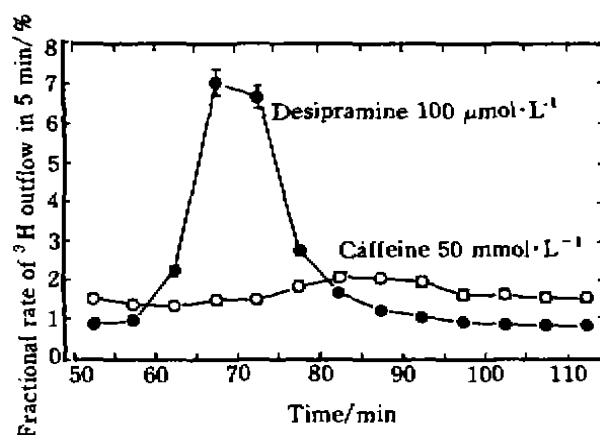


Fig 1. Desipramine- and caffeine-induced release of [^3H]NE from rat hippocampal slices preincubated with [^3H]NE and superfused continuously. After 60 min of superfusion the slices were exposed to desipramine $100 \mu\text{mol} \cdot \text{L}^{-1}$ or caffeine $50 \text{ mmol} \cdot \text{L}^{-1}$ in Ca^{2+} -free medium containing egtazic acid $1 \text{ mmol} \cdot \text{L}^{-1}$. $n = 4$ slices from 2 rats, $\bar{x} \pm s$.

Addition of caffeine $50 \text{ mmol} \cdot \text{L}^{-1}$ to the Ca^{2+} -free medium containing egtazic acid $1 \text{ mmol} \cdot \text{L}^{-1}$ after 60 min of superfusion very slowly and slightly increased ^3H outflow, which reached the maximum at 85 - 90 min and gradually returned to basal level after the drug was withdrawn (Fig 1).

Both the enhancements of ^3H outflow by desipramine and caffeine were concentration-dependent and increased linearly from 100 to 500 $\mu\text{mol} \cdot \text{L}^{-1}$, and from 30 to 70 $\text{mmol} \cdot \text{L}^{-1}$ respectively (Tab 2).

Tab 2. Desipramine- and caffeine-induced [^3H]NE release from rat hippocampal slices. After 60 min of superfusion the slices were exposed to various concentrations of desipramine or caffeine. $n = 4 - 10$ slices from 3 rats, $\bar{x} \pm s$.

Drug	Evoked outflow of ^3H / % of tissue ^3H
Desipramine/ $\mu\text{mol} \cdot \text{L}^{-1}$	
100	22.6 ± 1.0
200	40.2 ± 5.1
300	52.3 ± 2.3
400	61.4 ± 5.0
500	74.4 ± 1.3
Caffeine/ $\text{mmol} \cdot \text{L}^{-1}$	
30	0.45 ± 0.20
50	2.44 ± 0.21
70	6.0 ± 1.0

Effects of dantrolene sodium on DAP-evoked external Ca^{2+} -independent release of $[\text{}^3\text{H}]\text{NE}$ We used dantrolene Na^+ (saturated in medium) to block Ca^{2+} -induced Ca^{2+} release. In the absence of extracellular Ca^{2+} , DAP-evoked ^3H outflow was unchanged (4.8 ± 0.4 % in dantrolene Na^+ group and 4.6 ± 0.4 % in control group).

DISCUSSION

We have previously shown¹¹ that $[\text{}^3\text{H}]\text{NE}$ release was evoked by DAP from rabbit hippocampal slices preincubated with $[\text{}^3\text{H}]\text{NE}$. We proposed that in the presence of extracellular Ca^{2+} , DAP by blocking K^+ currents, depolarizes the neuronal membrane, induces Ca^{2+} influx and exocytosis of NE storage vesicles.

In the present investigation, it was observed that in the absence of extracellular Ca^{2+} , DAP significantly evoked $[\text{}^3\text{H}]\text{NE}$ release, but became ineffective when NE stored in synaptic vesicles was depleted by reserpine pretreated⁶. This indicates that under Ca^{2+} -free conditions, DAP-evoked $[\text{}^3\text{H}]\text{NE}$ release was also a vesicular component. Any possible involvement of extracellular Ca^{2+} in the process of DAP-evoked $[\text{}^3\text{H}]\text{NE}$ release can be excluded by egtazic acid $1 \text{ mmol} \cdot \text{L}^{-1}$ contained in medium⁶. The question arises, by which mechanisms DAP evoked exocytotic release of $[\text{}^3\text{H}]\text{NE}$, whether it is dependent from liberation of intracellular Ca^{2+} stores? This speculation is supported by the experiment with BAPTA-AM.

BAPTA-AM is hydrolyzed after crossing the membrane and dissociates Ca^{2+} chelator BAPTA. The Ca^{2+} liberated from intracellular stores was rapidly buffered by BAPTA, so that no appreciable accumulation of Ca^{2+} occurred during repetitive stimulation⁷. As shown in Tab 1, the release of $[\text{}^3\text{H}]\text{NE}$ induced by DAP was significantly inhibited in the slices pretreated with BAPTA-AM, indicating an involvement of liberation of intracellular Ca^{2+} stores in the process of DAP-evoked release of $[\text{}^3\text{H}]\text{NE}$.

In the present study, using high concentration desipramine⁸ to stimulate the inositol 1, 4, 5-trisphosphate (IP_3)-sensitive Ca^{2+} stores⁹, we observed a strong external Ca^{2+} -independent release

of $[\text{}^3\text{H}]\text{NE}$ from the slices, whereas the effect of caffeine¹⁰ by stimulating Ca^{2+} -induced Ca^{2+} releasable stores¹¹ on inducing $[\text{}^3\text{H}]\text{NE}$ release was very slight even in very high concentration of the drug. This suggests that in the absence of external Ca^{2+} , the elevation of cytosol free Ca^{2+} , which is necessary for stimulation-evoked $[\text{}^3\text{H}]\text{NE}$ release from adrenergic terminals of hippocampal slices, is mostly from the IP_3 -sensitive Ca^{2+} stores. It has been reported that dantrolene sodium blocked Ca^{2+} -induced Ca^{2+} release from endoplasmic reticulum in neurons¹². In the present investigation, dantrolene Na^+ did not show any inhibitory effect on DAP-evoked release of $[\text{}^3\text{H}]\text{NE}$, suggesting that in the absence of extracellular Ca^{2+} , Ca^{2+} -induced Ca^{2+} release might not be involved in the mechanism of DAP-evoked $[\text{}^3\text{H}]\text{NE}$ release. Conclusion, in the absence of extracellular Ca^{2+} , DAP by liberation of intracellular Ca^{2+} from IP_3 -sensitive Ca^{2+} stores evokes exocytotic release of $[\text{}^3\text{H}]\text{NE}$ from synaptic vesicles.

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胞内储备钙参与 3, 4-二氨基吡啶 诱发去甲肾上腺素释放¹

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关键词 去甲肾上腺素; 海马; 3, 4-二氨基吡啶;
利血平; BAPTA; 丹曲林; 地昔帕明

目的: 用大鼠海马脑片研究 3, 4-二氨基吡啶 (DAP) 诱发去甲肾上腺素胞外钙-不依赖释放的机制 方法: 大鼠海马脑片用 [^3H]NE 孵育后, 进行表面灌流, 测 [^3H]NE 释放. 结果: 在胞外无钙条件下, DAP 能显著加强 [^3H]NE 释放, 当用利血平使囊泡 [^3H]NE 排空, 则 DAP 作用消失, 用高浓度地昔帕明刺激 IP_3 -敏感的胞内 Ca^{2+} 储备库, 能有力地增强 [^3H]NE 释放, 而高浓度咖啡因对 [^3H]NE 释放只有很微弱的作用, 丹曲林钠对 DAP 诱发 [^3H]NE 释放无任何抑制作用. 结论: 在胞外无钙条件下, DAP 通过 IP_3 -敏感的 Ca^{2+} 储备库释放 Ca^{2+} , 从而诱发囊泡内的去甲肾上腺素释放.

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Effects of methylflavonolamine on free intracellular calcium in isolated embryonic rat brain cells

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KEY WORDS methylflavonolamine; Fura-2; calcium; flunarizine; glutamate; brain; cultured cells

AIM: To observe the effects of methylflavonolamine (MFA) on free intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) of isolated embryonic rat brain cells in presence and absence of high extracellular potassium and *L*-glutamate. **METHODS:** $[\text{Ca}^{2+}]_i$ was measured in a spectrofluorophotometer by preloading the cells with calcium sensitive fluorescent indicator Fura 2-AM. **RESULTS:** Resting $[\text{Ca}^{2+}]_i$ was $197 \pm 20 \text{ nmol} \cdot \text{L}^{-1}$ ($n = 44$) in the presence of $\text{Ca}^{2+} 1.3 \text{ mmol}$

$\cdot \text{L}^{-1}$ in Hanks' solution. MFA $0.15 \text{ mmol} \cdot \text{L}^{-1}$ had no effect on the resting $[\text{Ca}^{2+}]_i$. When extracellular Ca^{2+} was $1.3 \text{ mmol} \cdot \text{L}^{-1}$, MFA ($0.03 - 0.3 \text{ mmol} \cdot \text{L}^{-1}$) concentration-dependently inhibited the $[\text{Ca}^{2+}]_i$ elevation induced by high extracellular potassium, with an IC_{50} value of 0.14 (95 % confidence limits: $0.05 - 0.42$) $\text{mmol} \cdot \text{L}^{-1}$. At higher concentration ($0.15 - 0.30 \text{ mmol} \cdot \text{L}^{-1}$), MFA decreased *L*-glutamate-induced $[\text{Ca}^{2+}]_i$ elevation, with an IC_{50} of 0.20 (95 % confidence limits: $0.01 - 3.40$) $\text{mmol} \cdot \text{L}^{-1}$. **CONCLUSION:** MFA inhibited Ca^{2+} influx through voltage-dependent calcium channel and, at higher concentration, through receptor-operated calcium channel in the embryonic rat brain cells.