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不可逆激动剂α-CAM与阿片受点内巯基结合的反应[1]

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Irreversible action of the opioid agonist α-CAM and its reaction with SH groups at opioid receptor binding sites[1]

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Abstract 7α-Bis (β-chlorcethyl)amino-methyl-6,14-endoethenotetrahydrooripavine (α-CAM) was found to bind to opioid receptors irreversibly and react directly with sulfhydryl (SH) groups in P_2 preparations of rat brain. The P_2 preparations were pretreated as follows: protection of the SH groups at the opioid receptor binding sites by morphine or etorphine, and inactivation of the SH groups outside the binding sites by *N*-ethylmaleimide (NEM), followed by removal of the morphine or etorphine by washing. α-CAM was still able to bind the pretreated P_2 preparations in an irreversible manner. The results indicate that the formation of covalent bonds between α-CAM and the SH groups of opioid receptor binding sites is possibly one of the biochemical mechanisms of the irreversible action of α-CAM.

Key words endorphin receptors; analgesics; 7α-bis (β-chloroethyl) aminomethyl-6,14-endoethenotetrahydrooripavine (α-CAM); sulfhydryl compounds; binding sites; alkylating agents

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提要 7α-二(β-氯乙基)胺甲基-6，14-乙烯撑基四氢奥利文(α-CAM)能与阿片受点不可逆结合，并直接作用于大鼠脑P_2膜的巯基。将P_2膜用吗啡或依托啡预处理以保护阿片受点内巯基不被后加的NEM烷化，然后冲洗暴露受点内巯基，α-CAM仍能与P_2膜形成不可逆结合，提示α-CAM与阿片受点内巯基结合是其不可逆作用的生化机理之一。

关键词 内啡肽受体；镇痛药；7α-二(β-氯乙基)胺甲基-6,14-乙烯撑基四氢奥利文(α-CAM)；巯基化合物；结合位点；烷化剂

自第一个阿片受体烃化剂合成[1]以来，已有不少关于阿片生物碱类和脑啡肽类不可逆配体的报道，有的已用于阿片受体纯化和亚型的研究[2]。我们报道合成的新化合物α-CAM(7α-二(β-氯乙基)胺甲基-6,14-乙烯撑基四氢奥利文(7α-bis(β-chloroethyl)aminomethyl-6,14-endoethenotetrahydrooripavine)[3]具有超长效镇痛作用，是阿片受体的不可逆激动剂[4]。本文初步研究了α-CAM不可逆作用的生化机理。

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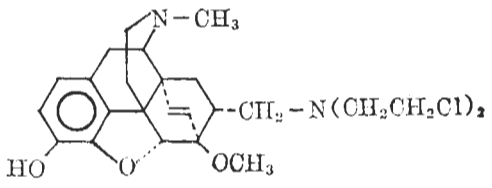

7 α-Bis(β-chloroethyl) aminomethyl-6,14-endoethenotetrahydrooripavine (α-CAM)

Materials and methods

α-CAM, 依托啡(Eto), [^{3}H]Eto(1221 GBq/mmol)和[^{3}H]纳洛酮([^{3}H]Nal,999 GBq/mmol)由上海医科大学药学院合成和标记；吗啡(Mor)由东北第六制药厂生产，经重结晶及氧化铝柱层析纯化；*N*-乙基顺丁烯二酰亚胺(*N*-ethylmaleimide, NEM)由中国科学院生物化学研究所合成；5,5′-二硫双(2-硝基苯甲酸)(5,5′-dithiobis(2-nitrobenzoic acid), DTNB)由防化研究院合成。

放射受点结合试验 Wistar大鼠，110只，体重184±SD 19 g，♀♂不拘。大鼠脑(去小脑)的P_2膜制备和放射受点结合试验方法见文献(5)。不可逆结合试验采用冲洗法[5,6]，将药物与大鼠脑P_2膜一起温孵后，用Tris-HCl 50 mmol/L缓冲液(pH 7.5)冲洗5次，每次加缓冲液混悬后放置5 min，然后离心，16 000×g，4℃，20 min，弃去上清液。

巯基含量测定 大鼠脑P_2膜制备的巯基含量采用DTNB法[7]测定。

Results

α-CAM与大鼠脑阿片受点的不可逆结合 α-CAM(20 μmol/L)与大鼠脑P_2膜一起30℃温孵30 min后，洗涤5次，用[^{3}H]Eto进行放射受点饱和结合试验。结果经计算机Scatchard分析，表明阿片受点的高、低亲和力结合均未恢复(Fig 1)。

NEM对阿片受点结合活性的影响 巯基烷化剂NEM不仅能抑制大鼠脑阿片受点与激动剂[^{3}H]Eto 1.1 nmol/L的结合，也能抑制阿片受点与拮抗剂[^{3}H]Nal 0.8 nmol/L的结合(Fig 2)，表明巯基对阿片受点的结合活性是必需的。

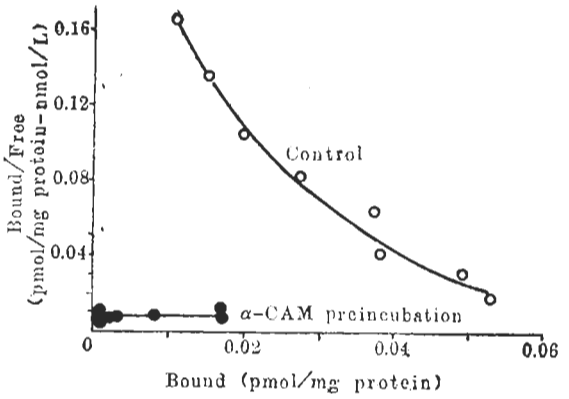

Fig 1. Irreversible binding of 7 α-bis (β-chloroethyl) aminomethyl-6,14-endoethenotetrahydrooripavine (α-CAM) with opiate receptors. P_2 membrane preparations of rat brain (without cerebellum) were preincubated with α-CAM (20 μmol/L) for 30 min at 30℃ and washed 5 times, then the [^{3}H] etorphine (Eto) binding activity of opiate receptors was determined. Scatchard plot with a computer shows that neither high nor low affinity binding activity of opiate receptors was recovered.

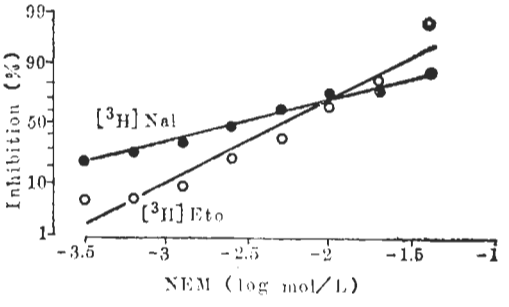

Fig 2. Inhibition of [^{3}H]etorphine-opiate receptor binding and [^{3}H]naloxone-opiate receptor binding by *N*-ethylmaleimide (NEM). Rat brain P_2 membrane preparations(1 mg protein) were incubated with [^{3}H]etorphine (Eto) 1.1 nmol/L (or [^{3}H]naloxone(Nal) 0.8 nmol/L) in the presence (non-specific binding, cpm_n) or absence (total binding, cpm_t) of Eto 5 μmol/L (Nal 4 μmol/L) or NEM of different concentrations (binding in the presence of inhibitor, cpm_i). The % of inhibition of [^{3}H]Eto (or [^{3}H]Nal) binding by NEM were given by $(cpm_t - cpm_i)/(cpm_t - cpm_n)$.

α-CAM 对大鼠脑 P_2 膜巯基的作用 用 DTNB 法测定的半胱氨酸标准曲线来衡量大鼠脑 P_2 膜中巯基的含量，结果表明含 5 mg 蛋白质的 P_2 膜巯基的含量为 22.4±1.9 μmol/L (n = 6)。

α-CAM 和 NEM 对大鼠脑 P_2 膜巯基含量的影响均有剂量依赖性。α-CAM 的作用比 NEM 弱，但两药浓度增大时，均可使 P_2 膜巯基的含量减少到接近于零(Fig 3)。

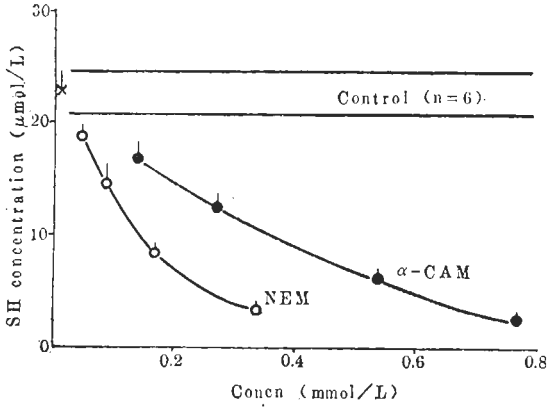

Fig 3. The dose-effect relationship of α-CAM and NEM on sulfhydryl group concentrations in rat brain P_2 membrane preparations (containing 5 mg protein). n = 3, $\bar{x}$±SD.

α-CAM 与阿片受体不可逆结合部位的检定 用大剂量 Mor (12 mmol/L)预先与大鼠脑 P_2 膜一起 30℃ 温孵 20 min，再加入 NEM 20 mmol/L 继续温孵 20 min，然后用 Tris-HCl 缓冲液冲洗 5 次，分别测定洗涤前后 P_2 膜与 [^{3}H]Eto(1.1 nmol/L)的特异结合量。结果表明 Mor 保护的 P_2 膜经冲洗后其结合[^{3}H]Eto 的活性明显恢复(Fig 4)。

用上述受到 Mor 保护的 P_2 膜与 α-CAM (10 μmol/L)一起 30℃温孵 20 min，冲洗 5 次后再测定洗涤前后 P_2 膜对[^{3}H]Eto 的结合活性。结果表明反复冲洗也不能解除 α-CAM 与阿片受点的结合(Fig 5)。

用大剂量 Eto (2.5 mmol/L) 代替 Mor，也得到相同结果。

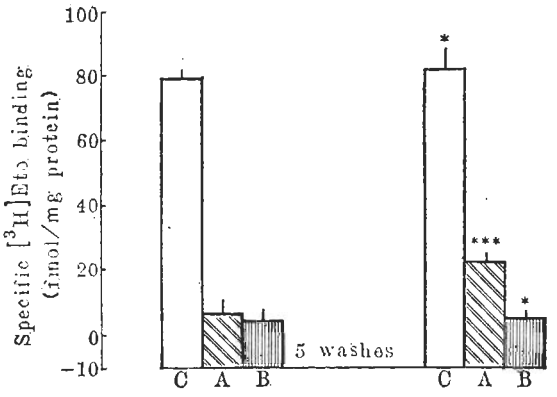

Fig 4. The protection of morphine (Mor) from inactivation of [^{3}H]Eto-opiate receptors binding by NEM. n = 3, $\bar{x}$±SD. A) (Mor + NEM): Rat brain P_2 membrane preparations were preincubated with Mor (12 mmol/L) for 20 min at 30℃ and then incubated with NEM (20 mmol/L) for 20 min at 30℃, and washed 5 times, finally the ability of specific binding (cpm_t − cpm_n) of opiate receptors with [^{3}H]Eto (1.1 nmol/L) was determined. B) (NEM): Tris-HCl buffer (50 mmol/L, pH 7.5) plus NEM. C) (Control): The Tris-HCl buffer only. *P>0.05, ***P<0.01 *vs* C or A or B before washes.

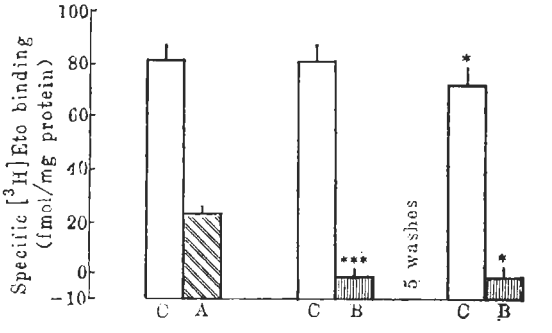

Fig 5. Irreversible binding of α-CAM with opiate receptors protected by Mor from inactivation by NEM. n = 3, $\bar{x}$±SD. A) (Mor + NEM): See legend of Fig 4. B) (Mor + NEM + α-CAM): Rat brain P_2 membrane preparations were pretreated with Mor and NEM, and then washes. This P_2 membrane preparations in which Mor was removed by 5 washes and NEM bound with sulfhydryl groups out of opiate receptor binding sites existed were incubated with α-CAM (10 μmol/L) for 20 min at 30℃ and washed 5 times again, finally the [^{3}H]Eto (1.1 nmol/L) binding activity of opiate receptors was determined. C) (Control): See legend of Fig 4. *P>0.05 *vs* C or B before washes; ***P<0.01 *vs* A.

Discussion

能与受点(即受体分子的特异结合部位)或与受点附近的基团形成共价键结合的配体称为不可逆配体。α-CAM 是带有氯乙胺氮芥基团的阿片受体不可逆配体，其氯乙胺基团在一定条件下可氯解形成吖丙啶鎓(aziridinum)离子，后者化学性质活泼，能够与受体上多种亲核基团如巯基、羟基、氨基等共价结合。Portoghese 等[8]曾推测带有富马酰基团的 β-funaltrexamin(β-FNA)的不可逆生化机理可能与阿片受体的巯基有关，但未见实验证据。本文用实验方法证明了 α-CAM 与阿片受点内巯基结合。我们还研究了 α-CAM 类似物，阿片受点不可逆部分激动剂 A-α-CAM 的不可逆生化机理，也取得相同的结果(待发表资料)。

阿片受体上的巯基与受点的结合活性有关已得到公认，本文的结果也支持这一结论。

药理实验已证明 α-CAM 是阿片受体不可逆激动剂[3,4]，并且当 α-CAM 剂量增大时，可使 P_2 膜巯基的含量减少到接近于零，这都提示 α-CAM 使 P_2 膜巯基含量减少的主要原因是其与巯基共价结合。NEM 是一种对阿片受体无特异性的巯基烷化剂，因此，它对 P_2 膜巯基的作用比 α-CAM 强。

为了检定 α-CAM 是与阿片受点内的巯基还是受点外(或邻近)的巯基结合，我们先用大剂量的可逆性阿片受体激动剂 Mor 或 Eto 将受点内巯基保护起来，再用 NEM 烷化受点外的巯基，然后冲洗掉 Mor 或 Eto，使受点内巯基重新暴露。由于 α-CAM 仍能与这种预处理的 P_2 膜不可逆结合，这提示 α-CAM 可能与阿片受点内的巯基形成共价结合。

目前对阿片受体的其他亲核基团如羟基、氨基等的研究报道尚少，因此 α-CAM 的不可逆生化机理除与阿片受点内的巯基形成共价结合外，是否与其他活性基团也有关，尚待进一步研究。

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