研究会

第36回 理論心電図研究会

Na⁺/H⁺交換輸送体の構造と機能

●活性に必須な因子としてのCHP

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Structure-function of the Na^+/H^+ exchangers—Calcineurin-homologous protein as an essential cofactor

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§ 抄録
細胞形質膜に存在するNa^+/H^+交換輸送体(NHE)は，pH，Na^+濃度，細胞容積の恒常性を維持するのに中心的な役割を演じるトランスポータの一つである[1)2)]．pH，Na^+濃度，および細胞容積の調節は生体にとって根源的な問題であるが，とりわけ拍動を絶えず繰り返している心筋においてこれらのイオン代謝は重要で，その破綻は重篤な疾患につながる．心筋では，心拍ごとのCa^{2+}動員および代謝の亢進によって生ずる酸を速やかに細胞外に排出する必要がある．そのため，NHE 1(普遍型アイソフォームで心筋にも発現するタイプ)を介して絶えずH^+が排出されており，それによって定常的にかなりの量のNa^+が流入することが知られている．NHE 1はまた，ホルモン，増殖因子，高浸透圧などさまざまな刺激によって活性化を受ける[1)2)]．例えば心筋のα1受容体を活性化すると，NHE 1を介する細胞内アルカリ化が起こり，結果として心筋収縮力が増強すると考えられている．さらに，NHE 1は心臓の病態とも密接に関連し，虚血に伴うNHE 1を介したNa^+蓄積が心筋虚血後再灌流障害の成因の一つになることがよく知られている．こうした心筋NHEの生理的・病態生理的な役割を深く理解するために，NHEの構造・機能を分子レベルで解明することは重要である．我々はこれまで，線維芽細胞およびカエル(*Xenopus*)卵母細胞というNHE発現系をモデルシステムとして用いることによって，NHEの構造・機能の問題にアプローチしてきた．最近，NHEと相互作用するCa^{2+}結合タンパク質CHPが，NHEの活性に必須であることを発見したので[3)]，以下詳細に記述したい．(心臓 34：333～340，2002)

I はじめに

Na^+/H^+交換輸送体(NHE)はあらゆる生物・組織に普遍的に存在し，Na^+・H^+のイオン代謝，細胞容積調節に関わる重要なトランスポータである(図1)[1)2)]．動物細胞ではこれまでに7種類のNHE遺伝子が同定されたが，その組織発現パターンは異なっている[1)2)4)5)]：NHE 1，あらゆる組織；NHE 2-4，主として腎臓・小腸・胃の上皮細胞；NHE 5，脳；NHE 6とNHE 7，あらゆる組織の小胞体およびゴルジ小胞．このことから予想されるように，NHEの各アイソフォームはさまざまな組織で異なった生理機能を発揮すると考えられる．実際，NHE 1-3のノックアウトマウスは異なった表現型を示すことが明らかになっている[6)~8)]．

NHEは，ホルモン・増殖因子・高浸透圧など，種々の細胞外刺激によって調節を受けることが知られており，その調節機構もアイソフォームによって異なることが示唆されている．例えば，NHE 1にはカルモデュ

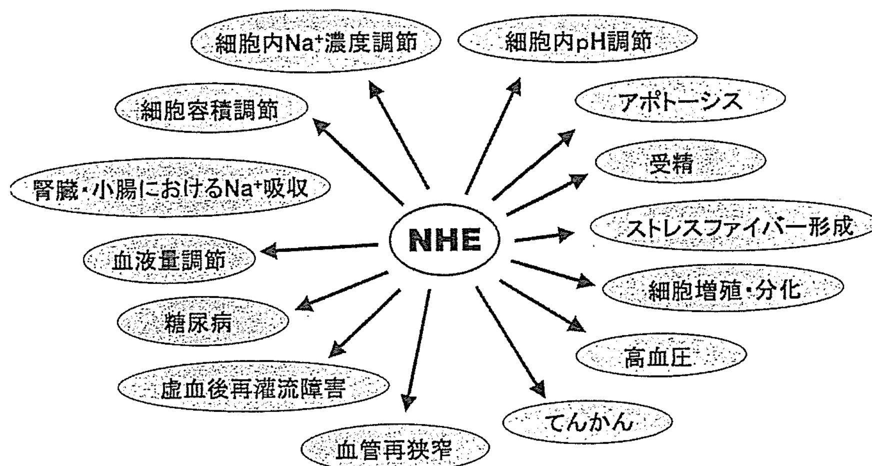

図1 NHEが関与すると考えられる生理機能と病態

リン[9)10)], NHE 3にはNHERF(NHE regulatory factor)が結合し[11)], それぞれCa^{2+}あるいはcAMPによる活性調節に関与することが示唆されている. また, 最近, イノシトールリン脂質(PIP_2)がNHE 1のATPによる調節に関与することが報告された[12)]. 数年前, NHE 1に結合するタンパク質としてカルシニューリン様タンパク質(CHP)がクローニングされた[13)]. あらゆる組織に普遍的に発現するCHPはCa^{2+}結合モチーフ(EF-ハンド)を4個含み, アミノ末端(Gly 2)がミリストイル化されたタンパク質である(図2A). CHPはタンパク質の小胞輸送[14)], 細胞骨格系との相互作用[15)], カルシニューリン活性の阻害[16)]などに関与することが報告されているが, そのNHEにおける作動機構は不明であった.

本研究では, NHEにおけるCHPの機能を詳細に検討し, CHPが種々のNHEアイソフォームの活性に必須の大変重要なタンパク質であることが明らかになった[3)].

II 方法

(1) 細胞培養へのcDNA導入

NHEを持たない変異線維芽細胞PS 120[17)]とそのNHE発現細胞は, 7.5%ウシ胎児血清を含むDulbecco's modified Eagle's mediumを用いて37℃で5% CO_2存在下に培養した. NHE, CHPを含む発現ベクターは, Ca^{2+}/リン酸法によってPS 120細胞にトランスフェクトした. NHEを安定に高発現する細胞は"H^+他殺法"によって選択した. また, GFP標識したCHPタンパク質を安定に発現する細胞クローンは, G 418による選択を行った後, GFPの蛍光をマーカーに選択した.

(2) NHEおよびCHPの発現ベクターの構築

ヒトNHE 1, ラットNHE 2および3はすべて発現ベクターpECEに, CHPはpEGFP-N 1に組み込んだ. NHEおよびCHPの種々の変異導入cDNAは, 変異を含む適当なプライマーのセットを用いてPCRを行い, そのPCR産物を上述のベクターに組み込んで作製した[18)]. また, 変異を含むcDNA塩基配列は自動分析器Applied Biosystems社のモデル377を用いて確認した.

(3) NHEおよびCHP融合タンパク質の大腸菌における発現および精製

MBPおよびGST融合タンパク質の発現と精製は, 種々のNHE, CHPからのPCR産物をベクターpMAL-cおよびpGEX-2 TKに組み込んだ後, 常法に従って行った. また, CHPあるいはミリストイル化されたCHPタンパク質(myr-CHP)(いずれもHis標識)は, cDNAをpET 11に組み込んだのち, それぞれpET 11単独あるいは酵母N-myristoyltransferaseを組み込んだpBB 131とともに大腸菌BL 21にトランスフォームし, 常法に従い精製した.

(4) 免疫沈降と免疫染色

NHEあるいはCHPの免疫沈降は, コンフルエントに培養したNHE発現細胞を1% Triton X-100を含むバッファーで可溶化したのち, NHE 1, NHE 3, CHP特異的抗体を用いて以前に記述された方法[8)9)]に従って行った. NHEおよびCHPのイムノブロットは, それぞれ免疫沈降したタンパク質を7.5%あるいは12%アクリルアミドを含むゲルで電気泳動したのち, 記述された方法[9)10)]に従って行った. また, NHEの

細胞表層ラベルは，培養細胞を1 mM NHS-ビオチンでインキュベートしたのち，ビオチン化したタンパク質をストレプトアビジンアガロースで回収し，NHEをイムノブロットで検出することによって行った[19)20)].

(5) アフリカツメガエル卵母細胞からの粗膜標品の調製

アフリカツメガエル(*Xenopus*)卵母細胞は，ND 96バッファー(mM：96 NaCl, 2 KCl, 1 $MgCl_2$ and 5 Hepes/NaOH, pH 7.5)中で，室温30分間，1 mg/ml コラゲナーゼで処理して調製した．粗膜標品の調製は，卵母細胞をバッファー(mM：150 NaCl, 10 Mg-acetate, 1 PMSF and 20 Tris-HCl pH 7.6)中，Physcotron(Nition)で60秒間ホモジナイズしたのちショ糖密度勾配遠心を行い，ショ糖20-50％界面を回収することによって行った．また免疫沈降は，粗膜標品を種々の界面活性剤(1％Triton X-100, 0.5％SDS and 1％sodium deoxycholate)を含むバッファー(mM：100 KCl, 5 $MgCl_2$, 1 $CaCl_2$, 1 PMSF and 100 Tris-HCl, pH 8.2)で可溶化したのち，上述した方法に従って行った．

(6) $^{22}Na^+$取り込みの測定

培養細胞に発現したNHE活性は，記述された方法に従い[21)], K^+/nigericin法で細胞内を酸性pH(5.6)に固定した細胞を用いて，NHE阻害薬EIPAで阻害される$^{22}Na^+$取り込み活性を測定することによって求めた．カエル卵母細胞による$^{22}Na^+$取り込みは，卵母細胞をNH_4Cl溶液(mM：80 NH_4Cl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes/Tris, pH 7.4)に1時間あらかじめインキュベートしたのち，1 mM $^{22}NaCl$を含むcholine-Cl溶液(mM：80 choline-Cl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes/Tris, pH 7.4)に15分間インキュベートし，$^{22}NaCl^+$を含まないcholine-Cl溶液で洗ったのち細胞に取り込まれた$^{22}Na^+$放射能を測定することによって行った．

Ⅲ 結果

(1) NHEにおけるCHP結合部位の同定

CHPは普遍的に発現するタンパク質であり，イムノブロットで示されるように，今回発現実験に使用したPS 120細胞にも内在性CHPが存在する(図2B)．CHPおよびNHE 1は共免疫沈降をするので(図2B)，両タンパク質は互いに強く結合する．NHE 1のCHP結合部位を同定するために，NHE 1細胞質ドメイン(図3A)の種々の領域を含むMBP融合タンパク質を作製し(図3A)，GST-CHPタンパク質によるfar-Western解析を行った．図3Bに結果の1例を示す．また，図3Cには共免疫沈降の結果を示す．

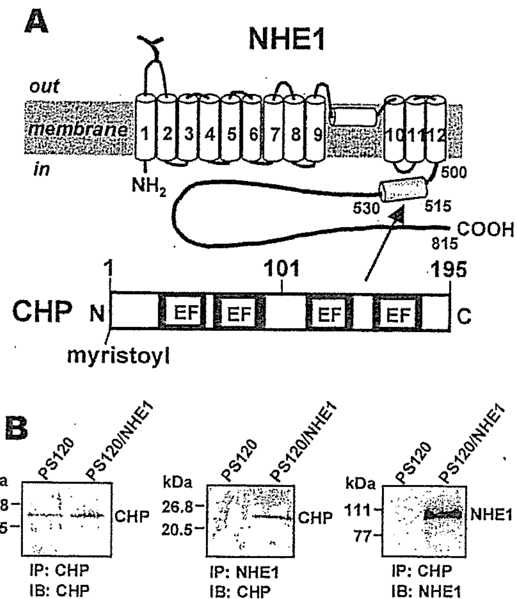

図2 A：NHE 1およびCHPの構造モデル
CHPはNHE 1のC末細胞質ドメインの膜に近い領域(aa 515-530)に結合する．
B：共免疫沈降によるNHE 1-CHP相互作用の検出
詳しくは本文参照．

これらの解析の結果，CHPの結合部位はNHE 1細胞質ドメインの膜に近いaa 515-530の領域であることがわかった(図2A，図3A)．CHP結合部位を含むNHE 1領域aa 503-600をNHE 2および4の相同な領域で置換したキメラタンパク質はCHPと共免疫沈降した(図3D)．したがって，NHE 3の結果(図4D)と併せて，CHPはNHEアイソフォーム1-4いずれとも結合する．次にCHP-NHE相互作用におけるCa^{2+}およびミリストイル化の役割を検討するため，MBP-NHE 1融合タンパク質を用いて，大腸菌より精製したCHPおよびmyr-CHPタンパク質のpull-downアッセイを行った．その結果，相互作用にはCa^{2+}もミリストイル化も必須ではないことがわかった(図3E)．また，myr-CHPタンパク質はCa^{2+}依存的に電気泳動移動度が異なり，Ca^{2+}によって立体構造の変化が起こることを示唆している[22)].

(2) 変異導入したNHEタンパク質の性質

CHP結合部位のアミノ酸配列は各NHEアイソフォームでよく保存され，α-helix構造であると考えられる(図4A)．疎水的相互作用がカルシニューリンサブユニット(AB)間の結合に重要であることが明らか

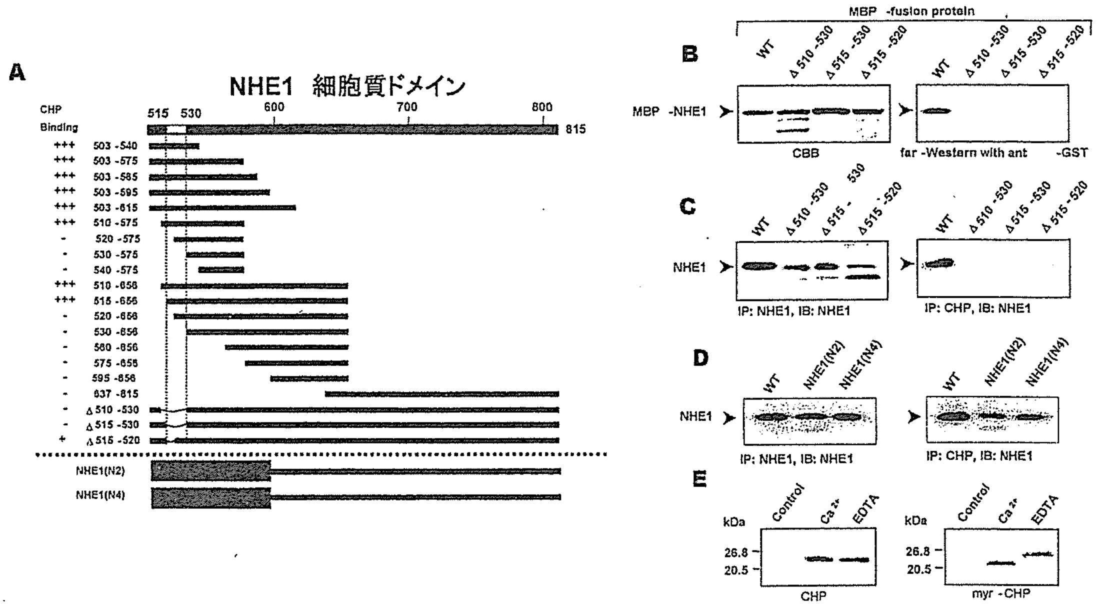

図 3　A：CHP との相互作用を検討するために作製した融合タンパク質の模式図
B-E：NHE-CHP 相互作用の解析
詳しくは本文参照.

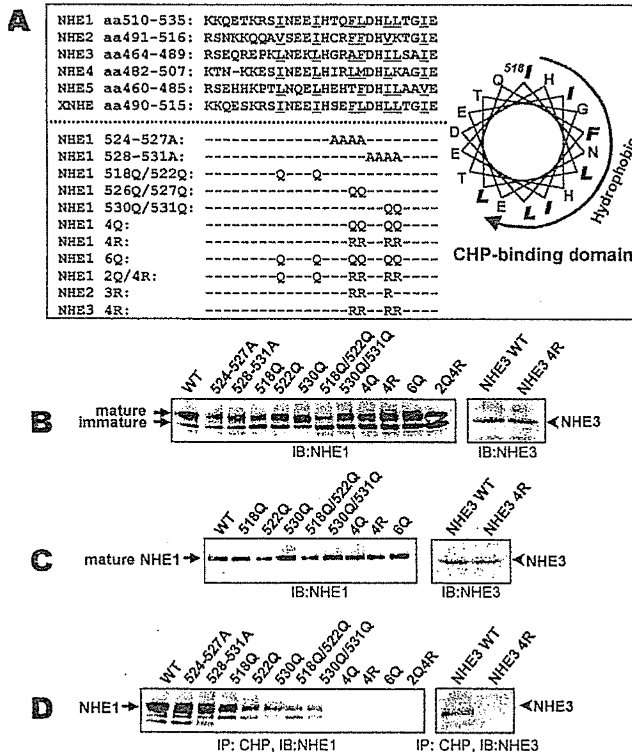

図 4　A：各 NHE アイソフォームにおける CHP 結合ドメインのアミノ酸配列と変異導入
B：NHE1，NHE3 およびそれらの変異タンパク質の発現
C：ビオチンによる細胞表層ラベル
D：免疫沈降によって検討した種々の変異タンパク質の CHP 結合能
詳しくは本文参照.

になっているが[23)24)]，これをもとに，NHE1-3の疎水性アミノ酸残基を親水性残基に置換した変異タンパク質を作製し(図4A)，細胞に発現した．すべてのNHE1変異タンパク質において，高・低分子量の二つのバンドが検出された(図4B)．これらのタンパク質はそれぞれグリコシル化された形質膜に存在するmatureなものと細胞内膜に局在するimmatureなものに相当する[25)]．これら変異NHE1が確かに細胞膜に発現していることを確かめるために，NHS-ビオチンによる表層ラベルを行った．

その結果，すべての高分子量変異タンパク質がビオチンでラベルされることがわかった(図4C，発現量によって標準化した値を図4Aに示す)．また，変異タンパク質のうち，4Q，4R，6Q，2Q/4Rは完全にCHP結合を失っていた(図4)．したがって，CHP結合はNHE1の形質膜発現に必須ではない．興味深いことに，これら4つの変異NHE1タンパク質のNa^+/H^+交換活性は野生型に比べて著しく低かった(図5C)．さらに，種々の変異NHE1のCHP結合能と交換活性はほぼ比例して変化した(図5BおよびC)．このことは，CHP結合がNHE1活性に必須であることを示唆している．同様の結果は，NHE2および3でも得られている(図5C，挿入図)．

(3)　CHP結合部位の注入によって起こるカエル卵母細胞Na^+/H^+交換活性の低下

NHEにおけるCHPの役割をさらに検討するために，アフリカツメガエル卵母細胞の系を使用した．卵母細胞はヒトNHE1と高いホモロジー(78％)を持つ[26)27)]内在性NHE1，および内在性CHPを所有している．これらのタンパク質は，ヒト由来のタンパク質を抗原として得た抗体によって確認することができた(図6B)．そこで，野生型およびそのコントロールとしてCHP結合を失う変異(欠失および4R)を含むHis標識したNHE1タンパク質(aa 503-600)を大腸菌から精製し(図6A)，それを卵母細胞に大量に注入することにより内在性CHPを枯渇させることを考えた．野生型NHE1タンパク質を卵母細胞に注入すると，10時間経過後，注入したHisタンパク質が内在性CHPと結合し(図6B，下)，そのため内在性NHE1はCHPと免疫沈降しなかった(図6B，中)．しかし，変異タンパク質を注入した時には内在性NHE1とCHPの共免疫沈降が観察された(図6B，中)．興味深いことに，野生型Hisタンパク質を注入すると，交換活性は著しく阻害された(図7AおよびB)．活性阻害の比較的ゆっくりとした(>3h)時間経過(図7A)は，内在性NHE1からCHPがゆっくりと遊離することを反映するものと思われる．この活性阻害は，CHP結合能を持たない変異NHE1タンパク質の注入では起こらなかった(図7B)．他方，卵母細胞に発現したヒトNHE1活性はヒトCHPの共発現によって著明に増加した(図なし)．

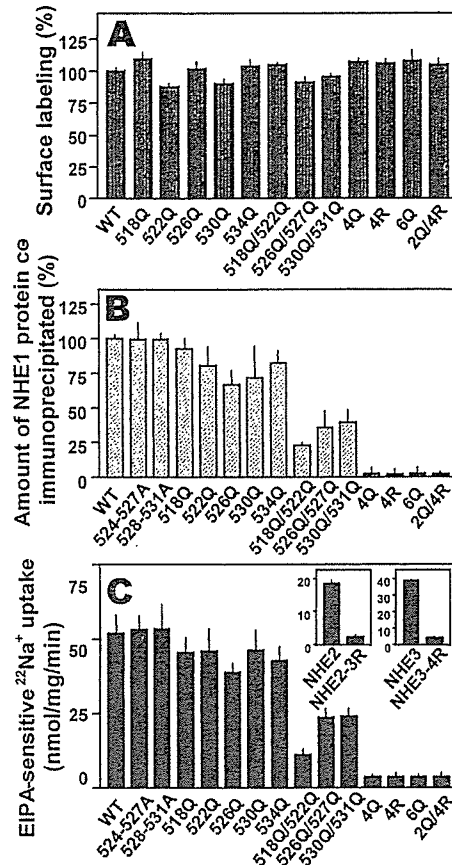

図5　NHE1とその変異タンパク質の細胞表層ラベル(A)，CHP結合能(B)，Na^+/H^+交換活性(C)のまとめ
交換活性はCHP結合能とみごとに相関している．
詳しくは本文参照．

以上の結果は，CHP結合がNHE1活性を発揮するのに必須であることを強く示唆する．

(4)　GFPで蛍光標識したCHPタンパク質の細胞内局在

蛍光(GFP)標識したCHPタンパク質は，NHEを発現していないPS120細胞では細胞質に一様に分布するのに対して，NHE1-3を発現すると形質膜に局在するようになった(図なし)．しかし，CHP結合を失った変異NHE1-3を発現する細胞では，そのような形質膜への局在は起こらなかった．このことは，NHEが

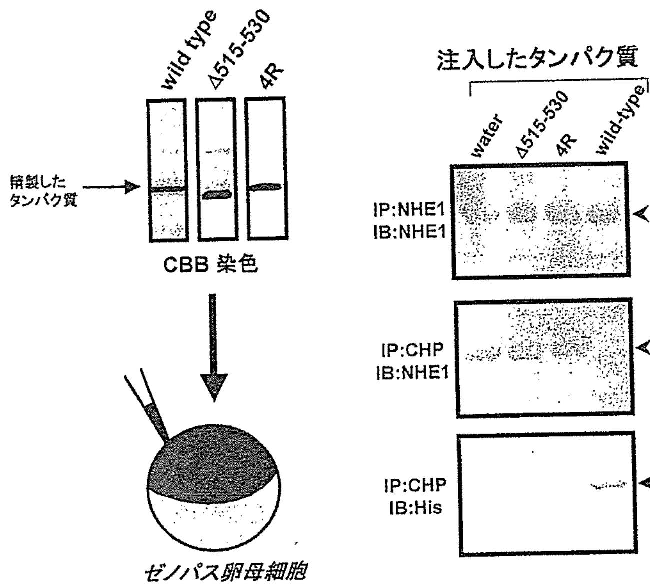

図 6　CHP 結合領域の注入によるアフリカツメガエル(ゼノパス)卵母細胞 NHE 1 からの CHP の枯渇
A：NHE 1 および CHP 結合を失った変異 NHE 1 (Δ 510-530 および 4 R) の領域 aa 503-600 を精製し，アフリカツメガエル卵母細胞に注入した．
B：野生型 NHE 1 タンパク質の注入においてのみ，アフリカツメガエル NHE 1 から CHP 結合が消失する．
詳しくは本文参照．

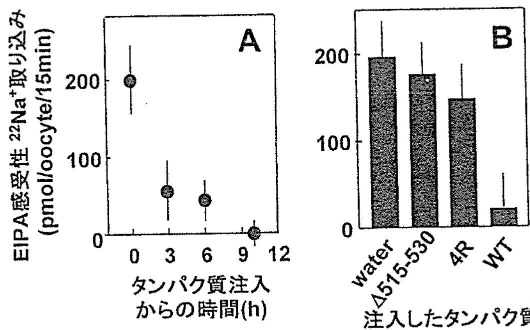

図 7　野生型 NHE 1 タンパク質の注入によるアフリカツメガエル NHE 1 活性の著しい低下
A：注入からの時間経過．
B：注入から 10 時間後の NHE 1 活性．
詳しくは本文参照．

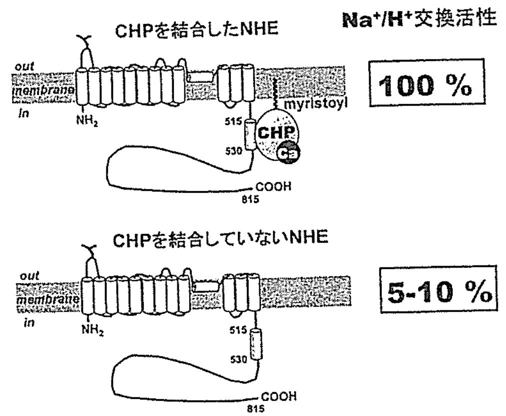

図 8　まとめ
CHP を結合した NHE の活性を 100 %とすると，CHP が結合していない NHE の活性は 5-10 %程度である．このことはNHE 1-5 の各アイソフォームにおいて共通と考えられる．

CHP の形質膜における主要なターゲットであることを示している．また，CHP 結合実験(図 2 E)と一致して，ミリストイル化あるいは Ca^{2+}結合が起こらない変異 CHP タンパク質も，NHE の共発現によって形質膜に局在するようになる(図なし)．こうして，高発現されたこれら変異 CHP タンパク質は内在性 CHP と置換していることが期待できるが，その際，Na^{+}/H^{+}交換活性の阻害は起こらなかった．

この結果は，CHP のこれら翻訳後修飾が，少なくとも CHP による NHE 活性維持機構には必須でないことを示唆している．

Ⅳ　考察

NHE ファミリーの最初の遺伝子 NHE 1 がクローニングされて以来，一般的には単一の NHE ポリペプチドが生理的な交換活性を発揮するのに十分であると考えられてきた．しかしながら，今回の結果は，CHP が NHE 1 の生理活性を発揮するのに不可欠なタンパ

ク質であることを示している(図8). 今回の結果はさらに, CHP が NHE1 だけでなく NHE2-4 でも同様の機能を持つことを示唆している. CHP 結合部位は NHE5 では保存されているが, 内膜系の NHE6, NHE7 では保存されていない. これらの結果は, CHP が形質膜タイプの NHE に共通の内在性タンパク質因子として機能することを示唆する.

NHE によるイオン輸送は膜貫通ドメインを含む N 末側領域(～500 アミノ酸)(図2A)によって起こる. なぜなら, C 末細胞質ドメインを完全に欠失させても, 依然として低い活性が検出されるからである. このことは, CHP 結合のない変異 NHE がきわめて低い活性(5-10%)を示すという今回の結果と合致している. CHP 結合は aa 515-530 で起こるが, 実はサブドメイン I と呼ばれる aa 500-600 の領域は Na^+/H^+交換活性にきわめて重要な領域である. おそらく, サブドメイン I は, CHP 結合によってその立体構造を著明に変化させることで, N 末側膜貫通ドメインとの相互作用を変えてイオン輸送路の構造を“活性化状態”に保持する働きを持つものと考えられる. i) CHP 結合がどのような分子機構で NHE を活性化するのか, ii) CHP の翻訳後修飾がホルモン, 増殖因子, ATP, Ca^{2+}, 高浸透圧などによる NHE の活性化にどのように関与するのか, といった問題に答えるためには, NHE の結晶構造の解析を含めた, より高い次元の研究を推進する必要があるであろう.

V 結論

今回の研究において, 我々は CHP が種々の NHE 活性に必須の共通タンパク質であることを明らかにした. おそらく, この研究は, Ca^{2+}結合タンパク質が活性に必須なタンパク質因子として二次性能動輸送体に強固に結合することを示した最初の仕事であると思われる. 今回, CHP の機能を線維芽細胞などの発現系を用いて一般化した形で証明したが, CHP は心筋を含むすべての組織において NHE の必須因子として機能していると思われる. NHE は心筋虚血後再灌流障害の成因の一つであり, NHE 活性を抑制することによって障害がかなり軽減されることが知られている. 今回の研究は, CHP もまた再灌流障害予防を考える際考慮されるべき一つの重要なターゲットになり得ることを示唆している.

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Altered Emotional Behaviors in Mammalian Bombesin Receptor Knockout Mice: Implication for the Molecular Pathogenesis of Stress-Induced Psychiatric Disorders in Humans

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1. Introduction

Neuropeptides are thought to play important roles in the pathogenesis of various psychiatric disorders, including posttraumatic stress disorder (PTSD) (Merali et al. 2002). To elucidate the pathophysiological role of neuropeptides for the disorders in vivo, we have employed behavioral analyses of gene-manipulated mice lacking their receptors. Among various neuropeptides, we have focused on bombesin, because its receptors are highly expressed in the hippocampus and the amygdala. Both regions are known to mediate memory and emotional behaviors.

Bombesin (BN) is a tetradecapeptide originally purified from the skin of the European frog *Bombina bombina* (Anastasi et al. 1971). Two BN-like peptides, gastrin-releasing peptide (GRP) and neuromedin B (NMB), have been identified in mammalian tissues (McDonald et al. 1979; Minamino et al. 1983). These peptides exert their effects by binding to G-protein coupled receptors on the cell surface; these are the GRP-preferring receptor (GRP-R) and the NMB-preferring receptor (NMB-R). In addition, a third subtype of mammalian bombesin receptor (BRS-3) has been cloned; however, high affinity natural ligand(s) specific to BRS-3 have not yet been identified (Battey and Wada 1991; Fathi et al. 1993). BN-like peptides and their receptors are widely distributed in the mammalian central nervous system and modulate many aspects of behavior such as spontaneous activity and feeding behavior, as well as learning and memory (Flood and Morley 1988; Flynn 1991; Kirkham et al. 1993; Santo-Yamada et al. 2001). We previously produced three strains of BN-like peptide receptor knockout mice using a gene-targeting method (Ohki-

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Hamazaki et al. 1997, 1999; Wada et al. 1997). The resulting mice, deficient in GRP-R, NMB-R, and BRS-3, were used in studies designed to clarify and distinguish the functional properties of BN-like peptides in the brain. As a result, we found that the NMB/NMB-R system may work as a risk factor for stress vulnerability and that the GRP/GRP-R system may play some role in learning and memory.

2. NMB and NMB-R

We investigated the effect of restraint stress on the behavior of NMB-R-deficient mice. We first examined the maternal behavior of the mice before and after the stress for 30 min (Yamada et al. 2002a). Near the end of the stress, three pups were laid individually in corners of each home cage. Immediately following the stress treatment, each experimental virgin female mouse was introduced into the vacant corner of the home cage. The essential maternal indices (licking, pup retrieval, grouping, nesting, and crouching) were measured in the initial and final 5-min observation periods during a 35-min test session. As shown in Fig. 1, the overall ma-

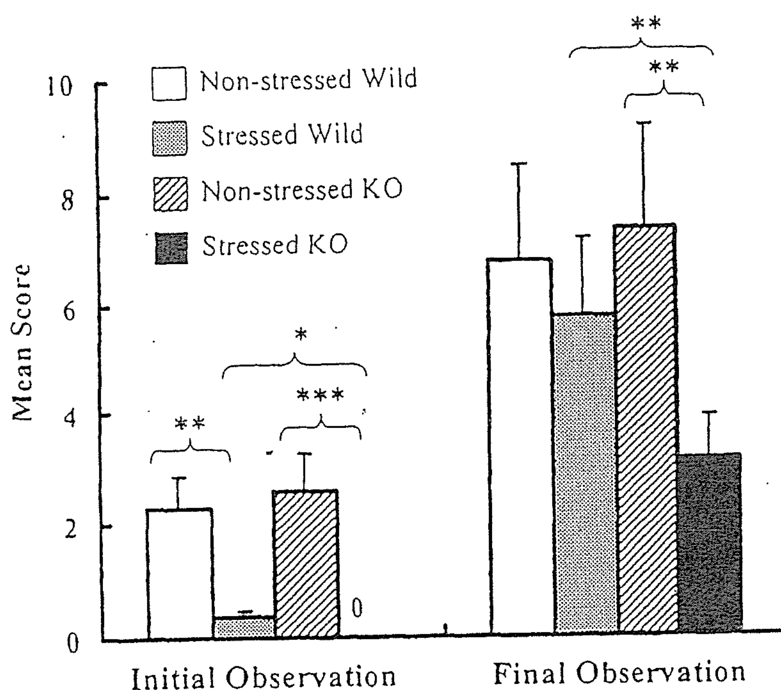

Fig. 1. Overall maternal behavioral performance of neuromedin B-preferring receptor (NMB-R)-deficient mice (*KO*) and wild-type mice with/without 30-min restraint-induced stress. The overall scores represent the sum of the scores of the individual components: pup retrieval, grouping, crouching, and nesting. Data given as mean + quartile deviation. *Asterisks* represent statistical significance between the indicated groups: *** $P < 0.0001$; ** $P < 0.005$; * $P < 0.03$

ternal indices at the final observation were significantly lower in stressed NMB-R-deficient mice compared with those of stressed wild-type control mice. At the initial observation, we did not find significant difference of the indices between the two genotypes with stress. The maternal behaviors of wild-type and NMB-R-deficient mice are both suppressed in the initial observations. Furthermore, nonstressed mice show similar indices between the two genotypes. These results suggest that restraint-induced stress can impair maternal behavior in mice, and NMB-R-deficient mice may suffer more severely from the stress than wild-type mice.

Similar vulnerability of NMB-R-deficient mice to the restraint stress was observed in the inhibitory avoidance learning (Yamada et al. 2003). Using a one-trial passive avoidance test, stressed NMB-R-deficient mice exhibited a marked reduction in memory performance (Fig. 2). In the test trial, we did not observe any difference in the mean step-through latency between nonstressed NMB-R-deficient and wild-type mice; however, stressed NMB-R-deficient mice showed significantly shorter latency than stressed wild-type mice and nonstressed NMB-R-deficient mice (Fig. 2). Although NMB-R-deficient mice exhibited elevated spontaneous activity in a novel environment (open field) compared with nonstressed mutant mice after 30 min of stress, a similar difference was also observed between stressed/non-stressed wild-type mice. An elevated plus maze test did not show any effect of the stress stimulus on anxiety in either wild-type or NMB-R-deficient mice. Furthermore, pain response of wild-type and NMB-R-deficient mice induced by electric

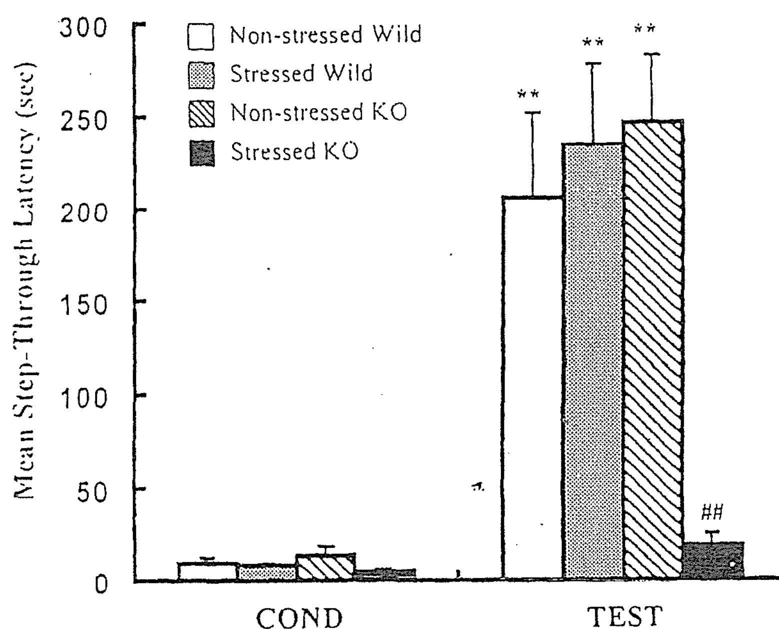

Fig. 2. One-trial passive avoidance test. Data given as mean + SEM. *Asterisks* indicate statistical significance between the acquisition trial and the test trial ($P < 0.01$); *hashes* indicate statistical significance between stressed NMB-R-deficient mice and stressed wild-type mice or nonstressed NMB-R-deficient mice in the test trial ($P < 0.01$). *COND*, acquisition trial; *TEST*, test trial

footshock was not affected under either stressed or nonstressed conditions. These results indicate that impaired memory performance in stressed NMB-R-deficient mice is not a consequence of changes in spontaneous activity, anxiety, or pain response, and suggest that the NMB/NMB-R pathway plays a role in regulating the stress response via the neural system that controls learning and memory.

We then evaluated the risk assessment behavior of the mice using two representative behavioral paradigms, the light-dark (L-D) box test and the elevated plus maze test (Yamada et al. 2002b). In the L-D box test, there were no significant differences between mutant mice and their wild-type littermates with respect to the conventional parameters such as dark-light (D-L) latency and the duration of staying in the light compartment. However, in the analysis of risk assessment behavior (stretched attend posture), NMB-R-deficient mice exhibited a significant decrease in risk assessment behavior relative to the wild-type cohort (data not shown). Similar to the results of the L-D box test, the analyses of risk assessment behavior from the elevated plus maze test revealed that NMB-R-deficient mice displayed a relative decrease in the frequency of this posture (data not shown). Although risk assessment behavior has yet to gain broad consent as a behavioral index of anxiety, there are reports that this behavior may reflect some emotional state of animals (Yamada et al. 2002b).

Our results suggest that the NMB/NMB-R pathway modulates some forms of emotion (perhaps including anxiety) and constitutes one of the risk factors of stress vulnerability. NMB-R-deficient mice should provide useful information for molecular pathogenesis of stress-induced disorders. Because the NMB/NMB-R system possibly interacts with 5-hydroxytryptamine (5-HT) neurons (Yamada et al. 2002c), further studies should reveal the neural circuits responsible for stress-induced mental disorders including PTSD.

3. GRP and GRP-R

We investigated the role of the GRP/GRP-R system in memory and learning. We first examined the effect of GRP peptide in wild-type mice with drug-induced amnesia. GRP was administered following training in a one-trial passive avoidance test. When scopolamine was used to induce amnesia prior to training, GRP (32 nmol/kg, ip) improved memory performance when the dosage of scopolamine was relatively low (1 mg/kg, ip). We then examined the role of GRP for the acquisition of inhibitory avoidance learning in mice using GRP-R antagonists. An administration of [Leu^{13}-(CH_2NH)-Leu^{14}]BN (antagonizes GRP-R > NMB-R) impaired the performance of inhibitory avoidance learning in all doses (16, 32, 64 nmol/kg). These results suggest that the GRP/GRP-R system plays an important role in memory and learning. Recently, GRP was shown to be important for inhibiting memory specifically related to learned fear (Shumyatsky et al. 2002). We generated specific antibody against the receptor, and found that the antibody is an excellent tool for investigating the expression of GRP-R in the brain (Kamichi et al. 2005). Double-

labeling immunohistochemistry demonstrated that subpopulations of GRP-R are present in GABAergic neurons in the amygdala. Consequently, GRP-R immunoreactivity was observed in the GABAergic neurons of the limbic region. These anatomical results support the idea that the GRP/GRP-R system mediates memory performance by modulating neurotransmitter release in the local GABAergic network.

4. Conclusion

Our results indicate that the NMB-R-deficient mouse is an important tool for investigating the molecular mechanism of stress-induced disorders and developing therapeutic drugs for the disorders. Besides the NMB/NMB-R system, the GRP/GRP-R system is likely to be involved in fear memory. Thus, it is likely that the mammalian bombesin system plays a role in regulating stress response through the neural system that controls learning and memory. Further investigation of the involvement of the mammalian bombesin system in PTSD should provide useful information for the treatment of the disease.

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13
Protein–Lipid Interactions in the Formation of Raft Microdomains in Biological Membranes

Akihiro Kusumi, Kenichi Suzuki, Junko Kondo, Nobuhiro Morone, and Yasuhiro Umemura

13.1
Many Plasma Membrane Functions are Mediated by Molecular Complexes, Microdomains and Membrane Skeleton-based Compartments

The biological membrane has been considered as a two-dimensional liquid, with the membrane-constituent molecules, i.e. the lipids and proteins, diffusing within the membrane more or less freely without affecting the overall morphology of the membrane. However, in the actual cellular plasma membrane, the situation may be quite different, as seen in Fig. 13.1a, which shows schematic diagrams of the plasma membrane of the neuron and the intestine epithelial cell. If all the proteins and lipids in the membrane moved freely in the plasma membrane, then the plasma membrane would become featureless. However, the plasma membrane actually contains a variety of specialized regions, such as synapses, coated pits, caveolae, and cell–cell and cell–substrate adhesion structures, in which specific proteins and lipids are assembled to carry out specialized functions. Therefore, the cell must have some means to regulate the movement and assembly of specific membrane proteins and lipids in/on the plasma membrane, and to induce the recruitment of a variety of proteins from the cytoplasm, thus facilitating the interactions of assembled molecules to have them work. Understanding such mechanisms is one of the key issues in cell biophysics.

The examples given above are large structures with an average size greater than 50 nm. However, the plasma membrane contains a variety of smaller structures, perhaps with briefer lifetimes or with shorter residency times for the constituent molecules. These may include the clusters of signaling molecules and scaffolding proteins, and possibly raft domains in the plasma membrane, as well as the plasma membrane compartments formed by the membrane skeleton-based partitioning.

Note that in this chapter the term "membrane domains" is used in a very broad sense, covering a large variety of non-random assemblies of membrane molecules that may exhibit a wide range of sizes and lifetimes of assembly. These assemblies

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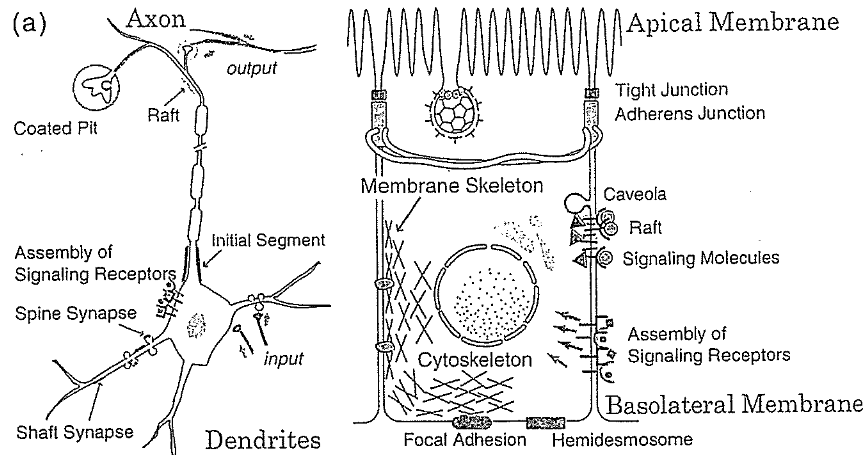

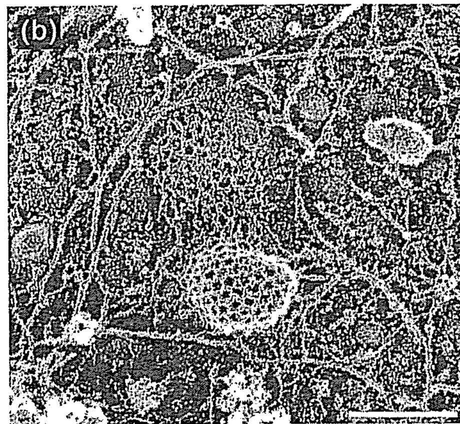

Fig. 13.1 (a) Schematic figures of the plasma membranes of a neuron (left) and an epithelial cell (right). The plasma membranes of these cell types are totally polarized into the axonal (left)–apical (right) membrane and the somatodendritic (left)–basolateral (right) membrane. These are some of the largest plasma membrane compartments. The plasma membrane contains a variety of specialized regions and molecular complexes with various time–space scales, collectively called membrane domains in this chapter (a broad definition is used here).
(b) Electron micrograph showing a forming clathrin-coated pit linked to the actin-based membrane skeleton. Such an electron micrograph gives the impression that clathrin-coated pits are stable structures, but in reality they form and dissociate from the membrane with a half-life of 40 s, on average.

include very small, transient domains or molecular complexes made of several proteins or lipids, on the one hand, as well as large, stable assemblies such as cell–cell adhesion domains and the apical membranes of epithelial cells, on the other hand. Between these two extreme cases are the raft domains: their sizes and lifetimes are likely to be between these two extreme cases, and they may be

greatly enhanced upon signaling or by the clustering of raft-associating proteins (Paladino et al. 2004), i.e. their sizes and lifetimes may vary greatly.

In this chapter, we feature the raft domains; however, before we concentrate on this specific type of membrane domain, we will first place the raft domain structure and dynamics in a larger perspective, to better understand the mechanisms by which they are formed in the non-ideal two-dimensional fluid mixture of many components, and are stabilized to become visible by optical and electron microscopic techniques. By placing the raft concept in the greater perspective of all kinds of membrane domains, the important structural, dynamic and functional characteristics of the raft domains that distinguish them from other membrane domains and protein assemblies may be clarified.

Then, we will discuss raft domains in steady-state cells (without stimulation) and in cells after extracellular or intracellular stimulation. Throughout this chapter, we emphasize the importance of bringing the concept of timescales into the research of membrane domains. The lifetime of the domain and the residency time of a molecule in a domain might be much shorter than generally assumed (of the order of seconds or less rather than on the order of minutes), and thus these timescales must always be considered to understand the membrane domains and their functions.

13.2
Timescales, Please!

Take a look at the ultrafine structure of the clathrin-coated pit as observed by electron microscopy (Fig. 13.1 b). Due to its large size and highly structured morphology, the clathrin-coated pit may appear to reside in the membrane for a long time. However, in reality, their half-lives are only 40 s long on average (Gaidarov et al. 1999). Another point to be noted is that even if the overall structure remains in the membrane, each individual molecule in the structure may be exchanging with the bulk pool or newly synthesized molecules much more rapidly. Consider the desmosome – a cellular organelle shared by two adhering cells. The desmosome is responsible for a strong type of cell–cell adhesion, described in the previous section as an example of a large, stable membrane domain. It can be as large as 10 μm in diameter in differentiated keratinocytes and requires 4 M guanidinium chloride, a very potent protein denaturant, for its disassembly *in vitro*. The lifetime of the desmosome may be as long as (or even longer than) the doubling time of the cell (over 60 h in some keratinocytes in culture), but the half-lives of the proteins that form the desmosome may be of the order of several hours (Pasdar and Nelson 1988 a, b, 1989).

These two points, the *lifetime* and the *residency time* (or the exchange rate), are the key issues addressed in this chapter, because they are the keys to understanding the structures and functions of all kinds of biomolecular systems in living cells, including membrane domains. The bottom line is that we always have to consider these membrane domains and structures as very dynamic entities, even when they are

large and clearly visible by light and electron microscopy, and this timescale viewpoint is even more important with smaller structures, like raft domains. Researchers have to apply these timescale concepts for understanding the mechanisms of the formation and function of microdomains, and for critically evaluating the limitations of various methods (time resolutions) used to study membrane domains. However, membrane researchers have been slow to adopt the timescale and time-resolution concepts, and this has caused major confusion in the membrane research field. In this chapter, we would like to address the timescales of molecular processes in the formation and the function of various membrane microdomains.

13.3
Four Types of Membrane Domains

First, we will briefly review the various membrane domains (as noted above, this term is used in a very broad sense here). The plasma membrane domains may be generally categorized into four classes.

Category 1: Large, stable structures that can be visualized by both electron microscopy and immunofluorescence microscopy, like synapses, desmosomes, clathrin-coated pits, etc., as described in Section 13.1.

Category 2: Partitioning of the plasma membrane into small compartments (Fig. 13.2a), due to the presence of the actin-based membrane skeleton (fence, Fig. 13.2b) and a variety of transmembrane proteins anchored to and lined up along the membrane skeleton (pickets, Fig. 13.2c). This is likely to occur throughout the plasma membrane, except for the large, stable domains of Category 1, although the membrane skeleton is likely to associate intimately with these structures. Virtually all of the molecules incorporated in the plasma membrane (e.g. even the phospholipids residing in the outer leaflet of the plasma membrane) are affected by these pickets and fences (Fig. 13.2a). They tend to undergo short-term confined diffusion within a compartment and long-term hop diffusion between these compartments. The compartment sizes are generally between 30 and 230 nm (cell-type dependent). The residency time of individual transmembrane proteins and phospholipids in a compartment may be of the order of 1–1000 ms (depending on the molecule and the cell type). The movement of the compartment itself or the membrane skeleton mesh has not been studied extensively. This model requires a paradigm shift of the plasma membrane concept. The traditional fluid-mosaic model of Singer and Nicolson (1972) may be true in the space scale of 10 nm (the size of the original cartoon published in their paper), but for longer-scale diffusion over 10 nm, the partitioning of the plasma membrane has to be considered (Fig. 13.2a).

Category 3: Microdomains, called raft domains, where the lipid–lipid interaction plays a major role in their formation. Here, the "lipid" includes the alkyl chains that anchor the protein to the plasma membrane, like those for glycosylphosphatidylinositol (GPI)-anchored proteins, and the signaling molecules on the

cytoplasmic surface of the plasma membrane, such as some of the Src family kinases (SFKs) [note that in this chapter we do not differentiate between whether the alkyl chain is linked to other parts of the molecule via ester (acyl chains) or other bonds, like an ether bond, and we call all these chains alkyl chains]. In addition, we would like to emphasize the tendency of cholesterol to be excluded, if given a choice, from the bulk membrane domain enriched in unsaturated alkyl chains, and to partition into the domains rich in saturated alkyl chains and cholesterol (Crane and Tamm 2004). Thus, this situation is analogous to a hydrophobic interaction. In fact, cholesterol seems to form transient clusters of several cholesterol molecules with a lifetime of 1–100 ns in liposomes containing L-α-dioleoylphosphatidylcholine (DOPC) and cholesterol (Subczynski et al. 1990). The reason for the exclusion of cholesterol from the bulk domain enriched in unsaturated alkyl chains may be due to the lateral non-conformability between the rigid tetracyclic ring structure of cholesterol and the bent structure of the *cis* double bond in unsaturated lipids. In spite of the extensive and intensive efforts to understand raft domains in the plasma membrane of steady-state cells (in the absence of extracellular stimuli), their sizes and lifetimes are essentially unknown. One of the major reasons for the difficulty of determining these key figures may be their small size and instability in the steady state (without extracellular or intracellular stimulation). They are hard to detect *in situ* using optical and electron microscopy techniques (Glebov and Nichols 2004; Prior et al. 2003; Sharma et al. 2004; Varma and Mayor 1998). It seems only after extracellular stimulation, crosslinking of raft molecules or lowering the temperature that the raft domains become stabilized, which makes them visible by the concentration of raft-associated molecules (however, see Brugger et al. 2004).

Category 4: Oligomers of proteins in the membrane. These include dimers of G-protein-coupled receptors (Jordan and Devi 1999) and the ligand-induced signaling complexes of receptor-type tyrosine kinases, such as the ligand-induced epidermal growth factor dimers complexed with Shc, Grb2 and SOS. Such protein clusters are the smallest class of membrane domains. This type of cluster is becoming more important as the involvement of scaffolding proteins that stabilize the interaction of two functional molecules becomes clear.

"Scaffolding" may be a key term for all four of the domains described above. The large, stable domains of Category 1 are certainly important platforms for the functions that require large structures, e.g. internalizing various membrane molecules (clathrin-coated pits) or coping with large macroscopic mechanical stress (cell adhesion structures). The partitioning of the plasma membrane described in Category 2 would induce the strong confinement of molecules within a partitioned compartment upon their oligomerization or molecular complex formation (oligomerization-induced trapping within the compartment, see Fig. 13.3 and its legend; this is a good case of the cooperative action of membrane domains in different categories, Categories 2 and 4 in this particular case), which may be important for the short-term memory of where the external signal was received for the cellular chemotactic responses or localized/polarized

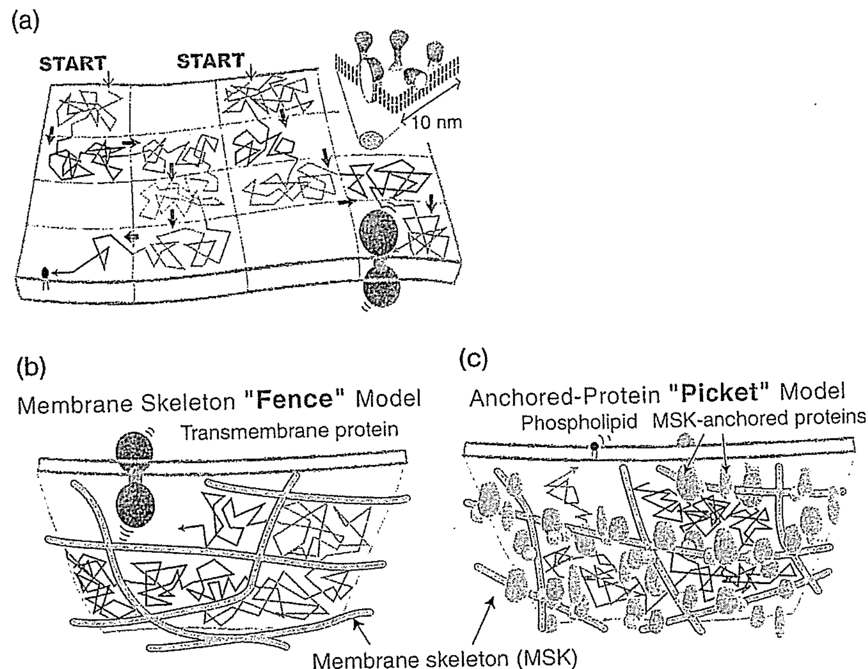

Fig. 13.2 Paradigm shift for the concept of the plasma membrane structure, from the two-dimensional continuum fluid to the partitioned fluid, due to the membrane skeleton 'fence' and the transmembrane protein 'pickets' anchored and aligned on the membrane skeleton fence.
(a) A paradigm shift for the concept of the plasma membrane structure in spatial scales larger than 10 nm may be required, from the two-dimensional continuum fluid to the partitioned fluid, in which its constituent molecules undergo short-term confined diffusion and long-term hop diffusion between the partitioned compartments. The fluid-mosaic model of the plasma membrane of Singer and Nicolson (1972) (see inset) is perfectly suitable on spatial scales less than 10 nm (incidentally, this is about the size of the original cartoon model in Singer and Nicolson's classical paper), but on spatial scales greater than 10 nm, one must consider the influence of the partitioning of the plasma membrane.
(b) Membrane skeleton 'fence' model. The membrane skeleton is chiefly made of actin filaments, which are also bound by many actin-associated proteins. The membrane skeleton is a part of the plasma membrane, since it plays important roles in many membrane functions, as well as a part of the cytoskeleton, since it is continuous with the cytoskeleton. The part of the cytoskeleton associated with the membrane has a different structure from that of the bulk cytoskeleton and contains specific proteins for interactions with the plasma membrane, and thus is called the membrane skeleton to distinguish it from the bulk cytoskeleton. Transmembrane proteins protrude into the cytoplasm, and in the fence model, the cytoplasmic domains of transmembrane proteins collide with the membrane skeleton, which induces temporary confinement of the transmembrane proteins within the membrane skeleton mesh.
(c) Anchored protein 'picket' model. About 15% of the transmembrane proteins are thought to be bound to the membrane skeleton and although their off-rates may be very high, they continually bind and re-associate rapidly. These bound transmembrane proteins form rows of pickets lined up along the membrane skeleton fence, which