Clarke, Modet
COLOMBIA

P-2640

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E. S. D.

Re: Expediente No.**10.111.097**
Solicitud de patente a nombre de HALOZYME INC.

Yo, SILVANA MARIA LOPEZ DÍAZ, actuando como apoderada sustituta de acuerdo con la sustitución adjunta e identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **HALOZYME INC**, solicitante de la patente de la referencia, *interpongo recurso de reposición* contra la resolución **No. 2490 emitida el 28 de enero de 2014** por el Superintendente de Industria y Comercio, por medio de la cual se niega el privilegio de patente para la solicitud contenida en el expediente de la referencia.

1. MODIFICACIONES

Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial nuevo pliego reivindicatorio que consta de 8 reivindicaciones la combinación, que comprende: i) una composición que comprende una enzima catepsina L en una cantidad que es 10 mg a 100 mg , en el que la enzima catepsina L consiste en la secuencia de aminoácidos expuesta en la SEQ ID NO: 1 o una forma de dos cadenas de SEQ ID NO : 1 que comprende una cadena pesada que consiste en la secuencia de aminoácidos expuesta como los aminoácidos 1-175 de SEQ ID NO: 1 y una cadena ligera que consiste en la secuencia de aminoácidos expuesta como los aminoácidos 179-220 de la SEC ID N °: 1; ii) un tampón ácido formulado en un intervalo de pH que es de 4,5 a 6; y iii) una composición que comprende uno o más de otros agentes seleccionados de: un agente que es una hialuronidasa soluble consiste en la secuencia de aminoácidos expuesta en una de las SEC ID N °:226 - 231 en una cantidad que es de 10 unidades hasta 500.000 Unidades de dispersión ; un anestésico que es lidocaína en una cantidad que es 10 mg a 1000 mg ; y un vasoconstrictor que es epinefrina en una cantidad que es 10 mg a 5 mg.

En este sentido, ese despacho puede corroborar que las reivindicación 1 modificada es el resultado de la incorporación de características citadas en las reivindicaciones anteriores 2, 7, 8, 9, 11, 14, 15, 16, 17 y 20.

La antigua reivindicación 22 (ahora nueva reivindicación 2) se modifica mediante la incorporación de características citadas en las reivindicaciones anteriores 23 y también se modifica en consonancia con las modificaciones introducidas en la

reivindicación 1. Base adicional se puede encontrar en la página 62 , líneas 3-5 y en la SEC ID N ° 1 en la lista de secuencias , en la página 129 , línea 23 a 29 , y en la página 140 , líneas 15-18 del documento de la correspondiente solicitud internacional.

La reivindicación 24 (ahora nueva reivindicación 4) se modifica mediante la incorporación de las características citadas en las reivindicaciones anteriores 36, 37, 38, 39, 41 y 42.

La Antigua reivindicación 40 (ahora nueva reivindicación 8) se modifica y su sustento se encuentra en la memoria descriptiva como fue presentada, por ejemplo, en la página 74, líneas 1-8 de la correspondiente solicitud internacional WO 2009/111083.

En este sentido informamos que la presente modificación no va más allá del alcance original de la presente solicitud y se proporciona con el ánimo de que se conceda la materia correspondiente.

Sobre este particular nos permitimos decir que no se presenta tasa reivindicaciones adicionales, toda vez que el nuevo pliego reivindicatorio para el caso Original contiene menos de 10 cláusulas.

2. FUNDAMENTOS DEL RECURSO

En relación con los argumentos presentados por ese despacho en la resolución impugnada **No. 2490 emitida el 28 de enero de 2014**, es necesario señalar que carecen de un enfoque adecuado de conformidad con las normas, jurisprudencia y reglas técnicas que existen para evaluar los requisitos de patentabilidad de una invención.

1.1 Falta de Legalidad

En la resolución que se recurre se estableció como estado de la técnica los siguientes documentos:

D1.- ***"US.20030026794 "Selective enzyme treatment of skin conditions"***

D2.- ***"pH-induced conformational transitions of the propeptide of human cathepsin L_ a role for a moten globule state in zymogen activation" Journal of Biological chemistry, Vol 273, page 11498-11504"***

D3.- ***"Acidic pH as a physiological regulator of human cathepsin l Activity" European Journal of Biochemistry, Vol 259, page 926-932."***

Con base en el análisis de estos **tres documentos** se ha negado la patente de invención que ahora se recurre, siendo el caso que tales documentos citados como anterioridades **NO HABIAN SIDO CITADOS COMO ANTERIORIDAD EN EL PROCEDIMIENTO QUE NOS OCUPA**, tal y como se puede apreciar de la simple lectura del examen técnico con número de oficio 431 notificado por fijación en lista el 22 de enero de 2013, dichos documentos ahora fundatorios de la resolución que se impugna, **no fueron considerados en dicho examen**.

Es decir, se ha emitido una resolución final sin que se haya discutido estos documentos en el examen técnico correspondiente, y en esta resolución 2490 son citados como documentos fundatorios de la falta de nivel inventivo de nuestra solicitud. Lo anterior deja al solicitante en completo estado de indefensión debido a que **no fue posible ejercer el derecho de réplica correspondiente** en el momento procesal oportuno, lo cual entonces vulnera lo consignado en el ARTICULO 29 de la Constitución Política de Colombia el cual establece que **"EL DEBIDO PROCESO SE APLICARÁ A TODA CLASE DE ACTUACIONES JUDICIALES Y ADMINISTRATIVAS..."**

Esto en relación con el artículo 45 de la Decisión 486 de la Comunidad Andina de Naciones que otorga el derecho al solicitante de contestar o manifestarse en relación a lo analizado en el examen de patentabilidad.

El artículo 29 de la Carta Política dispone que el debido proceso debe observarse en toda clase de actuaciones judiciales y administrativas, es decir que obliga no solamente a los jueces sino también a los organismos y dependencias de la administración pública.

El debido proceso administrativo consiste en que los actos y actuaciones de las autoridades administrativas deben ajustarse no sólo al ordenamiento jurídico legal sino a los preceptos constitucionales. Se pretende garantizar el correcto ejercicio de la administración pública a través de la expedición de actos administrativos que no resulten arbitrarios y, en consecuencia, contrarios a los principios del Estado de derecho.

Ello en virtud de que "*toda autoridad tiene sus competencias definidas dentro del ordenamiento jurídico y debe ejercer sus funciones con* ***sujeción al principio de legalidad****, a fin de que los derechos e intereses de los administrados* ***cuenten con la garantía de defensa necesaria*** *ante eventuales actuaciones abusivas, realizadas por fuera de los mandatos constitucionales, legales o reglamentarios vigentes*" [Cfr. Corte Constitucional. Sentencia T-1341 del 11 de diciembre de 2001 (M.P. Álvaro Tafur Galvis)].

De la aplicación del **principio del debido proceso** se desprende que los administrados tienen derecho a conocer las actuaciones de la administración, a

pedir y a controvertir las pruebas, **a ejercer con plenitud su derecho de defensa,** a impugnar los actos administrativos y en fin a gozar de todas las garantías establecidas en su beneficio, lo cual se vio vulnerado en el caso que nos ocupa al no tener en el momento procesal oportuno, los elementos con los cuales la autoridad ahora esta fundamentando la negativa de nuestra solicitud.

Sobre el debido proceso administrativo la Corte se ha manifestado en reiteradas oportunidades [por ejemplo, las sentencias T-442 del 3 de julio de 1992 (M.P. Simón Rodríguez Rodríguez), T-020 del 10 de febrero de 1998 (M.P. Jorge Arango Mejía), T-386 del 30 de julio de 1998 (M.P. Carlos Gaviria Díaz), T-009 del 18 de enero de 2000 (M.P. Eduardo Cifuentes Muñoz) y T-1013 del 10 de diciembre de 1999 (M.P. Alfredo Beltrán Sierra)] y ha precisado que su cobertura se extiende **a todo el ejercicio que debe desarrollar la administración pública** en la realización de sus objetivos y fines estatales, entre los que se encuentran por supuesto el otorgamiento del derecho de patentes.

Al respecto la Corte Constitucional se ha manifestado como sigue:

"*La garantía del debido proceso, plasmada en la Constitución colombiana como derecho fundamental de aplicación inmediata (artículo 85) y consignada, entre otras, en la Declaración Universal de Derechos Humanos de 1948 (artículos 10 y 11), en la Declaración Americana de los Derechos y Deberes del Hombre proclamada el mismo año (artículo XXVI) y en la Convención Americana sobre Derechos Humanos (Pacto de San José de Costa Rica, 1969, Artículos 8 y 9), no consiste solamente en las posibilidades de defensa o en la oportunidad para interponer recursos, como parece entenderlo el juzgado de primera instancia, sino que exige, además, como lo expresa el artículo 29 de la Carta, el ajuste a las normas preexistentes al acto que se imputa; la competencia de la autoridad judicial o administrativa que orienta el proceso; la aplicación del principio de favorabilidad en materia penal; el derecho a una resolución que defina las cuestiones jurídicas planteadas sin dilaciones injustificadas; la ocasión de presentar pruebas y* ***de controvertir las que se alleguen en contra*** *y, desde luego,* ***la plena observancia de las formas propias de cada proceso según sus características***" *[Cfr. Corte* Constitucional. Sentencia T-460 del 15 de julio de 1992 (M.P. José Gregorio Hernández Galindo)].

El derecho de defensa en materia administrativa, el cual ha sido vulnerado por la sentencia recurrida, se traduce en la facultad que tiene el administrado para conocer la actuación o proceso administrativo que se le adelante e impugnar o contradecir las pruebas y las providencias que le sean adversas a sus intereses. La administración debe garantizar al ciudadano interesado tal derecho y **cualquier actuación que desconozca dicha garantía es contraria a la Constitución.**

Por lo tanto, consideramos se deberá declarar procedente el presente Recurso de Reposición y en su momento cumplir con la obligación de correr traslado al solicitante de TODOS y cada uno de LOS

DOCUMENTOS que puedan o no afectar la patentabilidad de la invención solicitada.

No obstante lo anterior, se comentará lo relativo a la sentencia recurrida en su aspecto técnico en cuanto a que se ha considerado como no cumplido el requisito del Nivel Inventivo de nuestra solicitud.

En la resolución impugnada se muestran como nuevos hechos la afectación de la altura inventiva de las antiguas reivindicaciones 1-43 a la vista de las referencias citadas en D1 (publicado EE.UU. App patente US2003/0026794), D2 (Jerala et al (1998) J. Biol. Chem., 273:11498-11504) y D3 (Turk et al. (1999) Eur. J. Biochem., 259:926-932).

En este sentido, ese despacho ha indicado que D1 es la técnica anterior más cercana a la reivindicación 1 anterior porque enseña una enzima catepsina L, una formulación farmacéuticamente aceptable y uno o más de otros agentes que tienen actividad para la piel.

Aunado a lo anterior, también se afirma que D1 difiere de las reivindicaciones 1 a 43 negadas porque no enseña una combinación que contiene un tampón ácido que proporciona una condición de activación ácida para la enzima. El examinador, sin embargo, indica que tanto D2 y D3 enseñan que la catepsina L se activa a pH ácido de manera que un experto en la materia sería capaz de incluir un tampón ácido en la formulación de D1 que contiene la catepsina L y uno o más agentes que tienen actividad para la piel, y de ese modo llegar de manera evidente a la combinación reivindicada en la presente solicitud.

En cuanto a estos argumentos, respetuosamente solicitamos la reconsideración de este rechazo a la luz del nuevo pliego reivindicatorio dado que según las reivindicaciones enmendadas 1 y 22 (nueva reivindicación 2) se establece ahora que el agente adicional es un agente específico que es la **lidocaína**, **epinefrina** o **hialuronidasa soluble**. Estos agentes no son agentes que se enseñen en D1, ni tampoco son agentes que un experto en la materia habría empleado en combinación con un catepsina L en vista de las enseñanzas de D1. Por lo tanto, las reivindicaciones ahora modificadas cumplen con el requisito de nivel inventivo y no son evidentes a la vista de las referencias citadas D1 a D3, tomada de manera independiente o en cualquier combinación.

Adicionalmente, resulta importante señalar que las reivindicaciones ahora modificadas están dirigidas a una sola cadena o dos formas de cadena de la catepsina L expuesta en la SEC ID N °:1 que están formuladas en composiciones o contenedores con un tampón ácido de pH 4,5 a 6, y para su uso en administración sub-epidérmica a un entorno de pH neutro.

En este orden de ideas, como se describe en la presente solicitud, las combinaciones, composiciones y recipientes de los mismos utilizan formulaciones para las administraciones de tipo sub-epidérmica para entregar la enzima y otros agentes en el intersticio debajo de la capa epidérmica de la piel para tratar enfermedades o afecciones que implican componentes de la matriz extracelular, tales como el colágeno. En la sub-epidermis de la piel, la piel tiene un pH neutro (véase, por ejemplo en la página 72, líneas 1-7 de la memoria descriptiva de la correspondiente solicitud internacional de la invención colombiana).

Mediante **la formulación de catepsina L en un tampón ácido**, la enzima es activa cuando se administra primero a la sub-epidermis; debido al pH neutro en el intersticio, sin embargo, el ambiente de pH se neutraliza dejando la enzima inactiva con el tiempo. Esto es una ventaja de las presentes formulaciones, ya que pueden ser utilizadas para lograr la actividad temporal de la catepsina L para evitar la degradación no controlada y prolongada de colágeno y otros sustratos (véase, por ejemplo en la página 70 , líneas 14-29 ; en la página 71 , línea 21 a la página 76 , línea 2 de la correspondiente solicitud internacional de la presente invención).

Por lo tanto, las reivindicaciones ahora modificadas se dirigen a los productos y artículos o fabricación que facilita el uso de la enzima catepsina L activa-ácido para el medio ambiente de pH neutro de la sub-epidermis. Por ejemplo, las reivindicaciones dirigidas a los contenedores son artículos de fabricación que contienen dos compartimentos (por ejemplo, una jeringa de dos compartimentos que puede incluir una aguja para inyección) que incluyen 1) el agente activo maduro catepsina L en forma liofilizada, y 2) un tampón ácido en una composición separado a un pH de 4,5 a 6 que se puede utilizar para la reconstitución de la catepsina L inmediatamente antes de su uso (por ejemplo, para inyección a la sub -epidermis) de modo que la catepsina L es activa cuando se administra primero. Del mismo modo, la composición y combinaciones incluyen la formulación con un tampón ácido que tiene un pH de 4,5 a 6, de manera que la catepsina L es activa cuando se administra para el medio ambiente de pH neutro de la sub-epidermis.

Como se discute más adelante, esto resulta ser todo lo contrario de las enseñanzas de D1, que enseña la formulación de una enzima para el tratamiento de la piel (no por debajo de la piel). Dado que la catepsina L es inactiva en pH neutro el experto nunca seleccionaría la catepsina L entre la larga lista de posibles agentes en D1 para el tratamiento de un objetivo que tiene un **pH neutro**, y nunca seleccionaría la catepsina L de la formulación para la sub-epidermis.

Aunado a lo anterior, para la formulación de la enzima catepsina L en un **tampón ácido para el tratamiento de la sub-epidermis en un tampón ácido**, las combinaciones y composiciones reivindicadas también incluyen otros agentes

que facilitan la entrega o administración de la enzima a la sub-epidermis. Por ejemplo, las reivindicaciones enumeran que un anestésico tal como lidocaína, solo o en combinación con epinefrina, se puede proporcionar en combinación con una enzima catepsina L que se formula en un tampón ácido. La memoria descriptiva enseña que la inclusión de lidocaína y/o epinefrina se suministra con el fin de proporcionar alivio del dolor, por ejemplo, causada por la administración del tampón ácido y administración a la sub-epidermis (véase, por ejemplo en la página 127, líneas 23-26 de la correspondiente solicitud internacional de la patente en estudio). Por ejemplo, la descripción enseña en la página 128, líneas 2-4 de la solicitud internacional: "

"Por ejemplo, cuando se proporciona el pH como la condición de activación, se desea la administración de una anestesia local para minimizar el dolor asociado con la exposición a pH ácido."

También, las reivindicaciones enumeran que la enzima catepsina L se puede formular en combinación o como una composición con una hialuronidasa soluble con el fin de causar la degradación de los glicosaminoglicanos en el espacio intersticial para aumentar la dispersión de la enzima catepsina L (o lidocaína o epinefrina) siguiente de su administración subepidermal (por ejemplo, la administración subcutánea) (véase, por ejemplo en la página 127, líneas 26-32, y en la página 130, líneas 5-33 de la correspondiente solicitud internacional).

Como se discute más adelante, las referencias citadas, por separado o en combinación, **no enseñan o sugieren a la persona medianamente versada en la materia cualquier combinación, composición o recipiente que contenga la enzima catepsina L formulada en tampón ácido para uso en la entrega a la sub-epidermis**, ni ninguna combinación o composición que incluya, además uno o más de lidocaína, epinefrina y/o una hialuronidasa soluble. Además, estos documentos no proporcionan ninguna razón para combinar estos agentes en una combinación u otra formulación como actualmente se reivindica.

En efecto, las enseñanzas de las referencias citadas difieren de las reivindicaciones ahora modificadas.

1 . D1 (Patente US. No. US2003/0026794)

D1 enseña métodos de tratamiento de enfermedades de la piel usando las composiciones que contienen una enzima o enzimas que afectan selectivamente una o más capas de la piel. De hecho, entre la larga lista tipo-lavandería de enzimas como se enseña en D1 se encuentra la catepsina L (CATL). Es más, D1 enseña que las enzimas se pueden proporcionar en cualquier formulación farmacéuticamente aceptable adecuada y ejemplifica enzimas formuladas como una solución en solución salina tamponada con fosfato a pH 7,4, **pero no enseña o sugiere cualquier enzima formulada a un pH ácido, y, ciertamente, no de pH 4,5-6 como se reclama en la presente invención.**

Como se señaló anteriormente, D1 enseña la formulación de una enzima para el tratamiento de la piel (no por debajo de la piel) en una formulación farmacéuticamente adecuada, e incluye una lista de posibles enzimas. **D1 no ofrece alguna razón para la selección de la catepsina L entre la larga lista de los agentes y la formulación en un tampón de pH ácido**.

En este sentido, debe quedar claro que **no** hay enseñanza o sugerencia en el estado del arte de que un tampón de pH ácido sea un tampón farmacéuticamente aceptable, ni que empleando tal tampón sea requerido para cualquier enzima de administración tal como se enseña allí para las capas más superiores de la piel. Además, no hay ninguna enseñanza en D1 de la formulación de la enzima por separado del tampón ácido en un recipiente de dos compartimentos, tal como una jeringa.

D1 también enseña que las composiciones pueden contener otros compuestos o agentes como co-aditivos "que tienen propiedades terapéuticas, cosméticas y / o estéticas deseables. " En particular, D1 enseña que los co-aditivos pueden ser un exfoliante, droga inmunomoduladora o fármaco citotóxico. El propósito del co-aditivo es para su uso como otro tratamiento de la condición de la piel basado en la actividad del otro agente como un agente terapéutico, cosmético y/o la propiedad estética.

Por lo tanto, es claro que **no** hay enseñanza de sugerencia en D1 de la combinación de cualquiera de las enzimas que se enseñan en ella con lidocaína, epinefrina y/o una hialuronidasa soluble, los cuales **no** son agentes que tengan de por sí "propiedades terapéuticas, cosméticas y/o estéticas" para el tratamiento de enfermedades de la piel. Es más, **no** hay enseñanza o sugerencia en D1 que sus enzimas requieran formulación en combinación o composición con otro agente que facilite la entrega o administración de la enzima.

Así las cosas, D1 falla en enseñar o sugerir varias limitaciones de las reivindicaciones ahora modificadas en la presente invención . En primer lugar, D1 no enseña o sugiere cualquier enzima, y ciertamente no la catepsina L, según se reivindica, como una combinación o composición o en un recipiente con un tampón ácido que tiene un pH de 4,5 a 6, y tampoco enuncia o sugiere alguna razón para hacerlo de dicha manera. En segundo lugar, D1 tampoco enseña o sugiere cualquier enzima, y ciertamente no la catepsina L, según se reivindica, como una combinación o composición con lidocaína, epinefrina o una hialuronidasa soluble, ni ninguna razón para generar una combinación o composición de este tipo. Por lo tanto, las reivindicaciones ahora modificadas no carecen de actividad inventiva en vista de D1, individualmente o en combinación , con D2 o D3 .

2 . D2 (Jerala et al. (1998) J. Biol. Chem., 273:11498-11504)

D2 se refiere a las propiedades estructurales de la porción de propéptido de la catepsina L al disminuir el pH. Para evaluar las propiedades estructurales del propéptido, esta anterioridad enseña la expresión recombinante y la producción de la longitud completa de la catepsina L de propéptido o aminoácidos 1-78 del propéptido. Como se enseña en la presente solicitud, el propéptido es de 96 aminoácidos de longitud y corresponde a los residuos 18-113 de la actual SEQ ID NO: 62. **En contraste, la SEQ ID NO:1 es la catepsina L y madura que no contiene el propéptido**.

En este orden de ideas, es necesario señalar que D2 enseña análisis estructural del propéptido con base en los cambios en el pH utilizando espectroscopía de emisión de fluorescencia, dicroísmo circular o de resonancia magnética nuclear.

Además, D2 enseña que a pH neutro el propéptido se pliega en una estructura compacta con estructura secundaria y terciaria definida (primer párrafo de la discusión), pero que sufre un cambio conformacional al disminuir el pH (véase la mitad de la página 11502). D2 sugiere que este cambio conformacional que se produce en el propéptido en cuanto a las disminuciones de pH, puede explicar la activación zimógena ácida de la catepsina L. **Los estudios en D2 no se dirigen a la forma madura de la catepsina L que carece de la propéptido**.

D2 no enseña o sugiere cualquier composición de un catepsina L de enzima madura, dado que D2 se dirige únicamente a los estudios de la porción de propéptido de la catepsina L zimógena. Por lo tanto, D2 no puede enseñar o sugerir cualquier combinación o composición o recipiente del mismo de una enzima madura catepsina L en un tampón ácido de pH que tiene un pH de 4,5 a 6.

También es necesario dirigir la atención de ese despacho sobre el hecho que D2 tampoco enseña o sugiere la combinación o composición de la catepsina L con lidocaína, epinefrina o una hialuronidasa soluble. Por lo tanto, D2 no subsana las deficiencias en las enseñanzas de D1, solo o en combinación adicional con D3.

3 . D3 (Turk et al. (1999) Eur . J. . Biochem , 259:926-932)

D3 se dirige a los estudios que evalúan la inactivación irreversible de la catepsina L humana con un pH de 3 a 3,75. D3 enseña que la inactivación se acompaña de despliegue de la proteína a un pH bajo. D3 enseña que la presencia de sustrato puede retrasar la inactivación mediante la estabilización de su estructura, pero no impide la inactivación irreversible.

Aunado a lo anterior, es importante señalar que D3 no menciona, ni sugiere cualquier combinación o composición o del mismo recipiente de la catepsina L en un tampón ácido como se reivindica, que incluya otro agente que es lidocaína, epinefrina y/o una hialuronidasa soluble. De hecho, no hay enseñanza o

sugerencia en D3 de una enzima catepsina L, tal como se reivindica en la combinación o como una composición con un tampón ácido que tiene un pH de 4,5 a 6 y que contenga uno o más de lidocaína, epinefrina y/o una hialuronidasa soluble. Por lo tanto, D3 no subsana las deficiencias en las enseñanzas de D1 o D2.

Análisis - Las referencias citadas, solas o en combinación, no enseñan o sugieren las reivindicaciones ahora reclamadas

Teniendo en cuenta la discusión anterior, es claro que las enseñanzas combinadas de las referencias citadas no dan lugar a reivindicaciones de la presente invención, y por lo tanto, no destruyen la actividad inventiva de las reivindicaciones pasadas o actualmente modificadas. En D1, no hay ninguna razón proporcionada, y no hay ejemplificación, mediante las cuales un experto en la materia podría formular una enzima en un tampón ácido para la administración a la dermis. En particular, no hay ninguna enseñanza en D1 relacionada con que una solución tamponada ácida sea una formulación fisiológicamente aceptable adecuada para que un experto en la materia emplease en cualquier formulación con una enzima. Los únicos ejemplos que se enseñan en D1 incluyen la formulación de una enzima en una solución salina tamponada con fosfato a pH 7,4. Por lo tanto, D1 no proporciona ninguna razón para combinar sus enseñanzas con las enseñanzas de D2 o D3.

En este orden de ideas, en vista de D1, ó en vista de D1 con D2 y/o D3, el experto en la materia no habría seleccionado catepsina L, que es inactiva a pH neutro, y formular como reivindicada con un tampón ácido de pH para la administración subepidérmica, ya que tal objetivo tiene un pH neutro.

En cuanto a los argumentos nuevos presentados en la resolución impugnada, respetuosamente nos permitimos decir que el examinador está empleando retrospectiva mediante la presente solicitud como una guía en la combinación de las referencias citadas ya que es claro que no hay ninguna indicación en D1, solo o visto en el contexto de D2 o D3, que hubiera conducido a un experto en la técnica a formular la catepsina L de manera diferente a las otras enzimas tal como se enseña allí.

Además, aun suponiendo, en gracia de discusión que un experto en la técnica hubiera combinado las enseñanzas de D1, ya sea con D2 o D3, un experto en la materia no habría llegado a una formulación que contuviera la catepsina L como se reivindica. Por ejemplo, D2 no se refiere a un catepsina L de enzima **madura** como reivindicada, sino que se dirige tan sólo a la forma de propéptido de la enzima.

Además, D3 está dirigida a los procesos de inactivación de la catepsina L a un bajo pH de 3 a 3,75 . En vista de las enseñanzas de D2 o D3, cuando se

consideren en vista de las enseñanzas de D1, un experto en la materia no habría llegado a una combinación o composición o recipiente de los mismos como se reivindica en donde la combinación contiene una catepsina L **madura** y un tampón ácido de pH que tiene un pH de 4,5 a 6.

También, las combinaciones y composiciones reivindicadas incluyen uno o más de lidocaína, epinefrina y/o una hialuronidasa soluble facilitando o ayudando en el tratamiento de la sub-epidermis. Ninguna de las referencias citadas menciona, enseña o sugiere la lidocaína, epinefrina y/o una hialuronidasa soluble, ni ninguna razón para incluir tales agentes en combinación o composición con un catepsina L.

Por ejemplo, mientras que D1 enseña que otros agentes pueden ser incluidos en las formulaciones con su enzima para el tratamiento de enfermedades de la piel de la dermis o la epidermis, D1 enseña agentes que en sí, son otros agentes que tienen aplicaciones cosméticas, terapéuticas o estéticas para el tratamiento de la condición de la piel. En contraste, la lidocaína, epinefrina y/o una hialuronidasa soluble no son, ni podrían ser, empleados en combinación o composición con una catepsina L como agentes activos para el tratamiento de una condición de la piel, pero en su lugar, se emplean para facilitar la entrega o administración de la catepsina L cuando se formula con un tampón ácido para la administración sub - epidérmica.

Sumado a lo anterior, D2 y D3 también fallan en enseñar o sugerir estos agentes, y fallan además en proporcionar alguna razón para incluir estos agentes de una combinación o composición con una enzima catepsina L. No hay enseñanza o sugerencia en D1, solo o en combinación con D2 o D3, que habría conducido a un experto en la técnica a emplear lidocaína, epinefrina y/ o una hialuronidasa soluble en combinación o composición con la catepsina L como se reivindica y un tampón ácido que tiene un pH de 4,5 a 6. Por lo tanto, las reivindicaciones actuales no carecen de actividad inventiva en vista de las referencias citadas D1-D3.

Violación del artículo 30 de la Decisión 486 de la Comunidad Andina por una indebida interpretación de la norma.

El examinador declara en la resolución impugnada que el objeto reivindicado utiliza expresiones (por ejemplo, "la enzima ", "un catepsina ", etc ...) que hacen que sea difícil determinar el alcance del objeto para el que se solicita la protección.

Sobre este particular, nos permitimos solicitar respetuosamente la reconsideración de este rechazo a la vista de la modificación de las reivindicaciones de este documento. En su versión modificada, las reivindicaciones enumeran los componentes específicos de las combinaciones y

composiciones, incluyendo referencia a la SEC ID N ° para los componentes de polipéptidos y la cantidad de cada agente en la composición o combinación.

En relación con las características estructurales, el examinador indica que la materia reivindicada carece de concisión, porque una proteína debe caracterizarse por su secuencia de aminoácidos, y otros agentes caracterizados a través de sus características esenciales o estructurales.

Sobre este particular, nos permitimos decir que en su versión modificada, las reivindicaciones enumeran referencias a la secuencia de aminoácidos de proteínas componentes reclamadas (por ejemplo, la catepsina L o hialuronidasa soluble). Además, lidocaína y epinefrina son conocidos compuestos químicos cuyas estructuras eran conocidas por un experto en la materia mucho antes de la primera fecha de presentación (véase, por ejemplo Remko et al (1996) Chem. Papers, 50:35-40.) y Liapakis et al. (2004) Molecular Pharmacology, 65:1181-1190). (Anexo 1)

Objeto de las reivindicaciones. El examinador también afirma que el capítulo reivindicatorio no es clara debido a que el preámbulo de las clausulas se inicia como una "combinación ", que en un principio el objeto de las reclamaciones correspondió a 'una composición'. Sobre este particular, es deseo del solicitante manifestar que este rechazo no es claro, ya que no está claro cuál es la diferencia entre el "encabezado" y el "objeto de las reivindicaciones". El solicitante manifiesta que estaría más que dispuesto a modificar las reivindicaciones conforme a la práctica colombiana, pero no entiende la naturaleza de este comentario específico. En este caso, las reivindicaciones se encuentran basadas y sustentadas en lo presentado inicialmente durante la fase internacional PCT. No es evidente cómo el orden de las reivindicaciones debe afectar a la claridad de la materia reivindicada.

Por ejemplo, la actual reivindicación 1, dirigida a una combinación, es modificada para consolidar la materia reivindicada y encuentra fundamentada en las reivindicaciones originales del documento presentado ante el PCT, principalmente las reivindicaciones 57, 60, 61, 65, 66, 68, 69, 70, 73 y 74 y la reivindicación 22 (reenumerada como reivindicación 2), dirigida a una composición, encuentra fundamento en las reivindicaciones del documento PCT originales 77, 78 y 79.

Finalmente, es deseo del solicitante, sin apartarse del principio de independencia entre las oficinas de propiedad intelectual en el mundo, informar que la correspondiente solicitud han sido concedida en Taiwán la cual comparte la misma reivindicación de la prioridad de EE.UU. provisional 61/068,667, presentada el 6 de marzo 2008 y provisional de EE.UU. 61/127,725, presentada el 14 de mayo 2008. Las reivindicaciones traducidas que se otorgan en el N ° de Patente de Taiwán I395593 en 11 de mayo 2013 se adjuntan a manera de información de la oficina colombiana.

folio 13

2. Teniendo en cuenta lo dicho anteriormente, solicito a usted lo siguiente:

a) Que de conformidad con lo previsto por los artículos 3 (inciso 3); 35 (inciso 2) y 59 (inciso 2) del Código Contencioso Administrativo se consideren y se resuelvan TODOS los argumentos y TODAS las cuestiones planteadas así como las que aparezcan con motivo de este recurso.

b) Que se revoque la resolución **No. 2490 emitida el 28 de enero de 2014** por el Superintendente de Industria y Comercio, y se proceda a conceder el privilegio de patente de invención a la solicitud de mi mandante.

Señor Superintendente,

SILVANA MARIA LÓPEZ DÍAZ
C.C. 22468787 de Barranquilla
T.P 147463 del CSJ
notificaciones@clarkemodet.com.co

4. Datos del trámite

14

Corrección:

Expediente No. 10-111.097

Aparece _____

_____

Debe ser _____

_____

Modificación:

Presentación Solicitud Divisional.

_____

Modificación al capítulo descriptivo.

☐ _____

_____

■ Modificación al capítulo Reivindicatorio
Presento junto con este memorial nuevo pliego reivindicatorio, el cual consta de 8 reivindicaciones.

Modificación al resumen.

☐ _____

_____

Modificación a los dibujos.

☐ _____

_____

Modificación al solicitante.

5. Anexos

■ Comprobante de pago de la tasa para la presentación de la corrección y/o modificación

☐ Poder, si fuere el caso.

■ Documento que contiene las modificaciones o correcciones.

☐ Copia de la solicitud y sus anexos en formato magnético.

6. Firma	
SILVANA MARIA LÓPEZ DÍAZ	
Nombre y apellidos	Firma
C.C. 22.468.787	Tarjeta Profesional T.P. 147463 CSJ

Industria y Comercio
SUPERINTENDENCIA

Espacio reservado para el adhesivo de radicación

DIRECCIÓN DE NUEVAS CREACIONES
MODIFICACIONES Y/O CORRECCIONES

1. Identificación del Tramite

[ ] **Error material**

[X] **Modificación a una Solicitud en trámite**

[X] Patente de Invención
[ ] Patente de Modelo de Utilidad
[ ] Registro de Diseño Industrial
[ ] Esquema de Trazado de Circuitos Integrados

2. Datos del solicitante

Persona Natural [ ] Persona Jurídica [X]

HALOZYME, INC.

Nombre o denominación / Nombre o razón social completa
Nombre representante legal

Tipo de empresa Micro [ ] Pequeña [ ] Mediana [ ] Otra [ ]

Documento de identificación: [ ] C.C. [ ] C.E. [ ] NIT [ ] Otro Número _____

Nacionalidad/País de constitución	Dirección y domicilio del titular	Ciudad
Estados Unidos de América.	2523 Carmelita Avenue, Belmont	California 94002,
Dirección electrónica	No. Fax	Número telefónico

[ ] **Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico**

3. Datos del apoderado:

SILVANA MARIA LÓPEZ DÍAZ	. C.C. 22.468.787	147463 CSJ
Apellidos y Nombre	**Documento de identificación**	**Tarjeta profesional**

Dirección y domicilio		Ciudad
Carrera 11 No. 86-53, Piso 6, Bogotá D.C., Colombia		Bogotá D.C.
Dirección electrónica	**No. Fax**	**Número telefónico**
notificaciones@clarkemodet.com.co	6350824	6181088

En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sírvase indicar el número de su radicación o protocolo de poder general _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
NIT : 800.176.089-2
- / -

15

Industria y Comercio
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RECIBO DE CAJA

No. 14 - 005984

Bogotá D.C., Enero 20 de 2014 - 16:06:50

RECIBIDO DE : CLARKE MODET NI 83.008.643

*** Soporte del Pago ***

TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR.PAGO
RECIBO DE CA	999999999	5924	20/01/2014		20.149.800.00

*** Conceptos Pagados ***

CANT.	RENTISTICO	CONCEPTO	Vr.UNDITARIO	Vr.CONCEPTO
1	50005-01-03	CAMBIO DE MODALIDAD Y MODIFICACIONES D N.CREACION CORRECCION A SOLICITUD EN TRAMITE / MODOFICA	128.000.00	128.000.00
				$128.000.00

SON: **CIENTO VEINTIOCHO MIL PESOS MONEDA CORRIENTE***

Responsable: _____
-******-
Recibo de Caja Aplicado al Expediente No. _____

12

P-2640
Rvs modificadas
Recurso

REIVINDICACIONES

1. Una combinación , que comprende:

i) una composición que comprende una enzima catepsina L en una cantidad que es 10 mg a 100 mg , en el que la enzima catepsina L consiste en la secuencia de aminoácidos expuesta en la SEQ ID NO: 1 o una forma de dos cadenas de SEQ ID NO : 1 que comprende una cadena pesada que consiste en la secuencia de aminoácidos expuesta como los aminoácidos 1-175 de SEQ ID NO: 1 y una cadena ligera que consiste en la secuencia de aminoácidos expuesta como los aminoácidos 179-220 de la SEC ID N °: 1;

ii) un tampón ácido formulado en un intervalo de pH que es de 4,5 a 6; y

iii) una composición que comprende uno o más de otros agentes seleccionados de: un agente que es una hialuronidasa soluble consiste en la secuencia de aminoácidos expuesta en una de las SEC ID N °:226 - 231 en una cantidad que es de 10 unidades hasta 500.000 Unidades de dispersión ;

un anestésico que es lidocaína en una cantidad que es 10 mg a 1000 mg ; y

un vasoconstrictor que es epinefrina en una cantidad que es 10 mg a 5 mg.

2. Una composición farmacéutica , en el que la composición se formula como una sola forma de dosificación en un volumen de 10 ml - 50 ml para la entrega a la sub- epidermis ; y la composición comprende:

i) una enzima catepsina L en una cantidad que es 10 mg a 100 mg, en el que la enzima catepsina L consiste en la secuencia de aminoácidos expuesta en la SEC ID N °: 1 o es una forma de dos cadenas de la SEC ID N °: 1 que comprende una cadena pesada que consiste en la secuencia de aminoácidos expuesta como los aminoácidos 1-175 de SEQ ID NO : 1 y una cadena ligera que consiste en la secuencia de aminoácidos expuesta como los aminoácidos 179-220 de la SEC ID N °: 1;

ii) un tampón ácido que tiene un pH de 4,5 a 6, que activa la catepsina L ; y

iii) un agente seleccionado de entre lidocaína en una cantidad que es 10 mg a 1000 mg , epinefrina en una cantidad que es 10 mg a 5 mg , y una hialuronidasa soluble que consiste en la secuencia de aminoácidos expuesta en cualquiera de las SEC ID NOS :226 -231 en una cantidad que es 10 unidades a 500.000 unidades.

3. La composición de la reivindicación 2, en el que la cantidad de catepsina L en la composición es de 500 mg a 10 mg.

4. Un recipiente que comprende dos compartimentos , en el que un primer compartimento contiene la catepsina L en una cantidad que es 10 mg a 100 mg , en el que la enzima catepsina L consiste en la secuencia de aminoácidos expuesta en la SEC ID N °: 1 o es una forma de dos cadenas de la SEC ID N °: 1 que comprende una cadena pesada que consiste en la secuencia de aminoácidos expuesta como aminoácidos 1 -175 de SEQ ID NO : 1 y una cadena ligera que consiste en la secuencia de aminoácidos expuesta como los aminoácidos 179 a 220 de SEQ ID NO : 1 ; y

la catepsina L se liofiliza ; y

un segundo compartimento que contiene un tampón ácido que proporciona un intervalo de pH que es de 4,5 a 6.

5. El recipiente de la reivindicación 4, que comprende además un compartimiento de mezcla.

6. El recipiente de la reivindicación 4 o la reivindicación 5 que es un tubo, botella o jeringa.

7. El recipiente de cualquiera de las reivindicaciones 4-6, que comprende además una aguja para inyección.

8. El recipiente de cualquiera de las reivindicaciones 4-7, en el que el tampón contiene un ácido seleccionado de entre 2-(N-morfolino) etanosulfónico (MES), ácido succínico , ácido cítrico , fosfato cítrico e histidina.

ANEXO 1

ARTÍCULOS Y COPIA REIVINDICACIONES EN INGLES ACEPTADAS EN TAIWAN

0026-895X/04/6505-1181–1190$20.00
MOLECULAR PHARMACOLOGY
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Synergistic Contributions of the Functional Groups of Epinephrine to Its Affinity and Efficacy at the β_2 Adrenergic Receptor

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ABSTRACT

The structural basis of ligand affinity can be approached by studying the interactions between a drug and receptor residues; the basis for efficacy is more complex and must involve activation-associated conformational changes. We have used wild-type (WT), a constitutively active mutant (CAM), and a "constitutively inactive" mutant β2 adrenergic receptor (β_2AR) to investigate changes in the binding site that accompany binding and activation. The active state (R*) probably involves repositioning of at least some of the agonist-contact residues, thereby optimizing their interactions with agonist and resulting in a higher affinity for agonist. A comparison of the binding affinities of a series of phenethylamine derivatives for WT revealed a remarkable synergism between the various functional groups present in epinephrine. Binding affinity was essentially unchanged with addition of β-OH, N-CH_3, or catechol OHs to phenethylamine. In contrast, when each of these same groups was added to the appropriate compound, already containing the other two groups, to make epinephrine, the increase in affinity was quite large (60- to 120-fold). An initial interaction between two or more contacts may stabilize an intermediate conformation of β_2AR, R′, either by altering amino acid side chain rotamer conformations or by a more global conformational change involving the repositioning of transmembrane segments. The pattern of these effects was different in the CAM in that fewer interactions were required to observe the synergistic effect, consistent with the hypothesis that the CAM mutation enriches the proportion of receptors in R* or in R′ from which R* is more readily assumed.

The biological activity of an agonist is dependent on both its affinity and its efficacy. The structural basis for affinity can be approached by studying the interaction between a drug and critical contact residues in the receptor. Many of these contact sites for catecholamines have been identified in the β_2AR, the best-studied neurotransmitter G-protein-coupled receptor (GPCR). The protonated amine of epinephrine interacts with Asp113$^{3.32}$ (see *Materials and Methods* for a description of the indexing of residues) in the third transmembrane segment (TM3) of β_2AR (Strader et al., 1988); the catechol OHs interact with Ser203$^{5.42}$, Ser204$^{5.43}$, and Ser207$^{5.46}$ in TM5 (Strader et al., 1989; Liapakis et al., 2000); and the β-OH interacts with Asn293$^{6.55}$ in TM6 (Wieland et al., 1996). The catecholamine aromatic ring interacts with a cluster of aromatic residues in TM6 (Strader et al., 1994; Cho et al., 1995; Javitch et al., 1998). Based on a model of β_2AR, the N-CH_3 of epinephrine has been proposed to interact with TM7 (Gouldson et al., 1997); however, this has not yet been experimentally verified.

In contrast to affinity, the structural basis for efficacy is more complex, because the detailed conformational changes associated with receptor activation are not known. Fluorescence spectroscopy (Ghanouni et al., 2001), electron paramagnetic spectroscopy (Resek et al., 1993), and alterations in the accessibility of charged sulfhydryl reagents (Javitch et al., 1997; Rasmussen et al., 1999; Ballesteros et al., 2001; Shi et al., 2002) have been used to infer that activation of GPCRs is associated with movements of their TMs, particularly TM6. Mutagenesis of the residues that contact agonists and/or their modification by fluorescent probes, spin labels,

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ABBREVIATIONS: β_2AR, β_2 adrenergic receptor; EPI, epinephrine; PHE, phenylephrine; SYN, synephrine; HAL, halostachine; TM, transmembrane segment; WT, wild-type/wild type; CAM, constitutively active mutant; GPCR, G-protein-coupled receptor; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; HEK, human embryonic kidney; CGP-12177, 4-[3-[(1,1-dimethylethyl)amino]2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one.

or other sulfhydryl reagents, however, would abolish or dramatically alter their functional interactions with agonist. Thus, it is particularly difficult to determine movements of crucial contact residues, although this information is vital to the elucidation of the mechanism by which agonist interaction with these residues contributes to receptor activation and thus to agonist efficacy.

In the two-state model, receptor exists in an equilibrium between two conformations, the active, R*, and the inactive, R (Leff, 1995). Agonist binds with higher affinity to R* than to R, thereby stabilizing R* and shifting the equilibrium R↔R* to the right. Agonist efficacy is related, therefore, to the ratio between the affinity of a drug for R and R* (affinity shift) and reflects the degree of stabilization of receptor in, and its transition to, R*. The experimental measurement of efficacy, however, is highly system-dependent; in a number of heterologous expression systems, the weak partial agonist dopamine has been shown to display a high intrinsic activity at the β_2AR, with ~90% of the effect of the full agonist epinephrine (Del Carmine et al., 2002). Agonist efficacy has also been approached experimentally by measuring the ratio between the affinities of high- and low-affinity components of agonist binding (Kearn et al., 1999). However, in membranes of COS-7 or HEK 293 cells expressing high levels of wild-type (WT) β_2AR, GTP has minimal effects on agonist binding, consistent with the receptor being in excess over G-protein and therefore being predominantly in the low-affinity state (Samama et al., 1993). This makes it difficult to measure this ratio in such a heterologous expression system.

Constitutively active receptor mutants (CAM) are active in the absence of agonist (Samama et al., 1993; Rasmussen et al., 1999; Ballesteros et al., 2001). These receptors seem to more readily assume the R* state and have substantially higher affinity for agonists than does WT receptor. Although signaling by a CAM is dependent on the presence of G-protein, its higher propensity for being in R* and therefore its higher affinity for agonist is observed even in the absence of G-protein (Samama et al., 1993). Thus, a CAM provides a model for the active state of the receptor, and the ratio between the affinities (affinity shift) of agonists for WT and CAM β_2AR was shown to correlate with their efficacies, even in an expression system in which the high-affinity component of agonist binding could not be measured (Samama et al., 1993).

As discussed above, many structure-activity relationship studies, by testing the binding to WT β_2AR of an assortment of modified adrenergic ligands, have determined the chemical groups that are important for ligand function. In this study, we extend these structure-activity relationship studies by examining the binding of a series of systematically substituted phenethylamine compounds to a series of β_2ARs with different proportions in R*, including WT and CAM, to investigate structural changes in the binding site that accompany receptor activation.

Materials and Methods

Numbering of Residues. Residues are numbered according to their positions in the human β_2AR sequence. We also index residues relative to the most conserved residue in the transmembrane segment in which it is located (Ballesteros and Weinstein, 1995). By definition, the most conserved residue is assigned the position index "50" (e.g., Pro211$^{5.50}$) and therefore Val210$^{5.49}$ and Leu212$^{5.51}$. This indexing simplifies the identification of aligned residues in different GPCRs.

β_2 Plasmids and Site-Directed Mutagenesis. The DNA sequence encoding the WT human β_2 adrenergic receptor, epitope-tagged at the amino terminus with the cleavable influenza-hemagglutinin signal sequence followed by the "FLAG" epitope (Kodak IBI, New Haven, CT) and tagged with six histidines at the carboxy terminus (Kobilka, 1995), was a gift from Dr. B. Kobilka (Stanford Brain Research Institute, Palo Alto, CA). This DNA was subcloned into the bicistronic expression vector pcin4 (Rees et al., 1996), a gift from Dr. S. Rees (GlaxoSmithKline, Uxbridge, Middlesex, UK), thereby creating the vector pcin4-SFβ_2H. The CAM β_2 receptor (Samama et al., 1993) (L266S/K267R/H269K/L272A) was a gift from Dr. R.J. Lefkowitz (Department of Biochemistry, Duke University Medical Center, Durham, NC), and the fragment encoding the CAM mutation was subcloned into pcin4-SFβ_2H to create the vector, pcin4-SFCAMβ_2H. The mutation of Asp79$^{2.50}$ to Asn (D79N) was generated by the polymerase chain reaction-mediated mutagenesis using Pfu polymerase (Stratagene, La Jolla, CA). The polymerase chain reaction-generated DNA fragment containing the mutation was subcloned into the pcin4-SFβ_2H plasmid, and the mutation was confirmed by DNA sequencing.

Cell Culture and Transfection. HEK 293 cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1) containing 3.15 g/liter glucose and 10% bovine calf serum at 37°C and 5% CO_2. Cell transfection with the WT, the CAM, and the D79N β_2AR and selection for generation of cells stably expressing the receptors were performed as described previously (Javitch et al., 1997; Liapakis et al., 2000).

Membrane Preparation. Cell suspensions, prepared as described previously (Liapakis et al., 2000), were centrifuged at 1000*g* for 5 min at 4°C, and the pellets were homogenized in 1 ml of binding buffer (25 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, and 0.006% bovine serum albumin) using an OMNI 1000 Polytron homogenizer at setting 26 to 30 for 10 to 15 s at 4°C. The homogenates were centrifuged at 13,500*g* for 10 min at 4°C, and the membrane pellets (from a confluent 100-mm dish) were resuspended by homogenization as described above in 1 ml of binding buffer. The membrane suspensions were diluted (typically 1:20–1:40) in binding buffer and used for radioligand binding studies.

[^{3}H]CGP-12177 Binding. Aliquots of diluted membrane suspension (200 μl) were incubated in binding buffer with increasing concentrations of the various agonists tested (obtained from Sigma-Aldrich, St. Louis, MO and Tocris Cookson Inc., Ellisville, MO) in the presence of 0.7 nM [^{3}H]CGP-12177 (Amersham Biosciences Inc., Piscataway, NJ) in competition experiments or with varying concentrations of [^{3}H]CGP-12177 (50–1200 pM) in saturation experiments. The total volume was adjusted to 0.5 ml, and the binding experiments were performed as described previously (Javitch et al., 1997; Liapakis et al., 2000). The amount of membrane used was adjusted to insure that no ligand depletion occurred (i.e., that the specific binding was always equal to or less than 10% of the total concentration of the added radioligand). Specific [^{3}H]CGP-12177 binding was defined as total binding less nonspecific binding in the presence of 1 μM alprenolol (Sigma/RBI, Natick, MA). Data for saturation and competition binding were analyzed by nonlinear regression analysis using Prism 3.0 (GraphPad Software, San Diego, CA). IC_{50} and K_d values were obtained by fitting the data from the competition studies to a one-site competition model and from the saturation studies to a one-site binding (hyperbola) model, respectively. K_i values were determined using the equation $K_i = IC_{50}/(1 + L/K_d)$ (Cheng and Prusoff, 1973).

cAMP Accumulation Assays. HEK 293 cells stably expressing WT or D79N β_2AR were plated onto 96-well cell culture plates (pretreated with poly-L-lysine, 0.1 mg/ml) at a density of 40,000 to 60,000 cells/well. After incubation overnight at 37°C in 5% CO_2, the cells were 95 to 100% confluent. The medium was removed, and 100

μl of Opti-MEM (Invitrogen, Carlsbad, CA) with or without (control) 135 μM *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was added. The cells were incubated for 1 h at 37°C. The medium was removed, and 100 μl of assay buffer (25 mM HEPES, pH 7.4, 2 mM choline, 288 mM sucrose, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mM 3-isobutyl-1-methylxanthine) was added. After a 1-h incubation at 37°C, more assay buffer without (basal levels) or with increasing concentrations of agonists was added to a total volume of 200 μl, and the incubation was continued for 10 min at 37°C. At the end of the incubation, the assay buffer was removed. The cells were placed on ice and lysed with 3% trichloroacetic acid. Lysates were incubated on ice for 30 to 60 min and stored at −20°C. After 1 to 5 days, frozen lysates were thawed, centrifuged at 1800*g* for 10 min at 4°C, and the supernatants were neutralized with 2 N NaOH. Quantification of cAMP in the neutralized supernatants was performed using a competitive binding assay as described previously by Watts et al. (1998) and Nordstedt and Fredholm (1990), with minor modifications. Supernatants were transferred to polypropylene minitubes (20 μl/tube) containing buffer B (100 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 5 mM EDTA) with 1 to 1.1 nM [2,8-^{3}H]-adenosine 3′,5′-cyclic phosphate (Amersham Biosciences Inc.). Subsequently, cAMP-binding protein (~100 mg of crude bovine adrenal cortex extract in 500 μl of buffer B) was added to each tube. After incubation on ice for 2.5 to 3 h, the mixtures were filtered through GF/B glass fiber filters as described for radioligand binding assays. The amount of cAMP in each sample (one tenth of a well) was determined by comparison with a standard curve of known concentrations of unlabeled cAMP (0.3–100 pmol/tube). EC_{50} values were obtained by fitting the data to a one-site sigmoidal dose response model using nonlinear regression analysis.

Results

In this study, we tested the functional properties of three β_2ARs using a series of different adrenergic ligands. These ligands are phenethylamine and its derivatives that were created by adding one or more of the following functional groups: N-CH_3, β-OH, and catechol OHs (Fig. 1). Epinephrine is the fully substituted phenethylamine derivative containing all of the above functional groups.

Activation of Adenylyl Cyclase by WT β_2AR. High expression of the WT β_2AR in HEK 293 cells (B_{max} = ~100 fmol/cm^2 of culture dish; data not shown) resulted in masking of the partial agonist activity of the drugs tested in this study. Thus, the maximal stimulation of cAMP accumulation by the partial agonists phenylephrine, synephrine, dopamine, and halostachine was similar to that of the full agonist epinephrine (EPI) (data not shown). To overcome this problem, we measured agonist-stimulated cAMP accumulation after inactivating 98 to 99% of the receptors by pretreatment with the alkylating reagent EEDQ (Belleau et al., 1969). In the resulting low receptor reserve environment, we found that phenylephrine, synephrine, halostachine, and dopamine were indeed partial agonists, being 40, 24, 19, and 46% as effective as the full agonist, epinephrine, in maximally stimulating cAMP accumulation in WT receptor (Table 1 and Fig. 2). In contrast, the intrinsic activities of norepinephrine and methyldopamine were similar to that of epinephrine, sug-

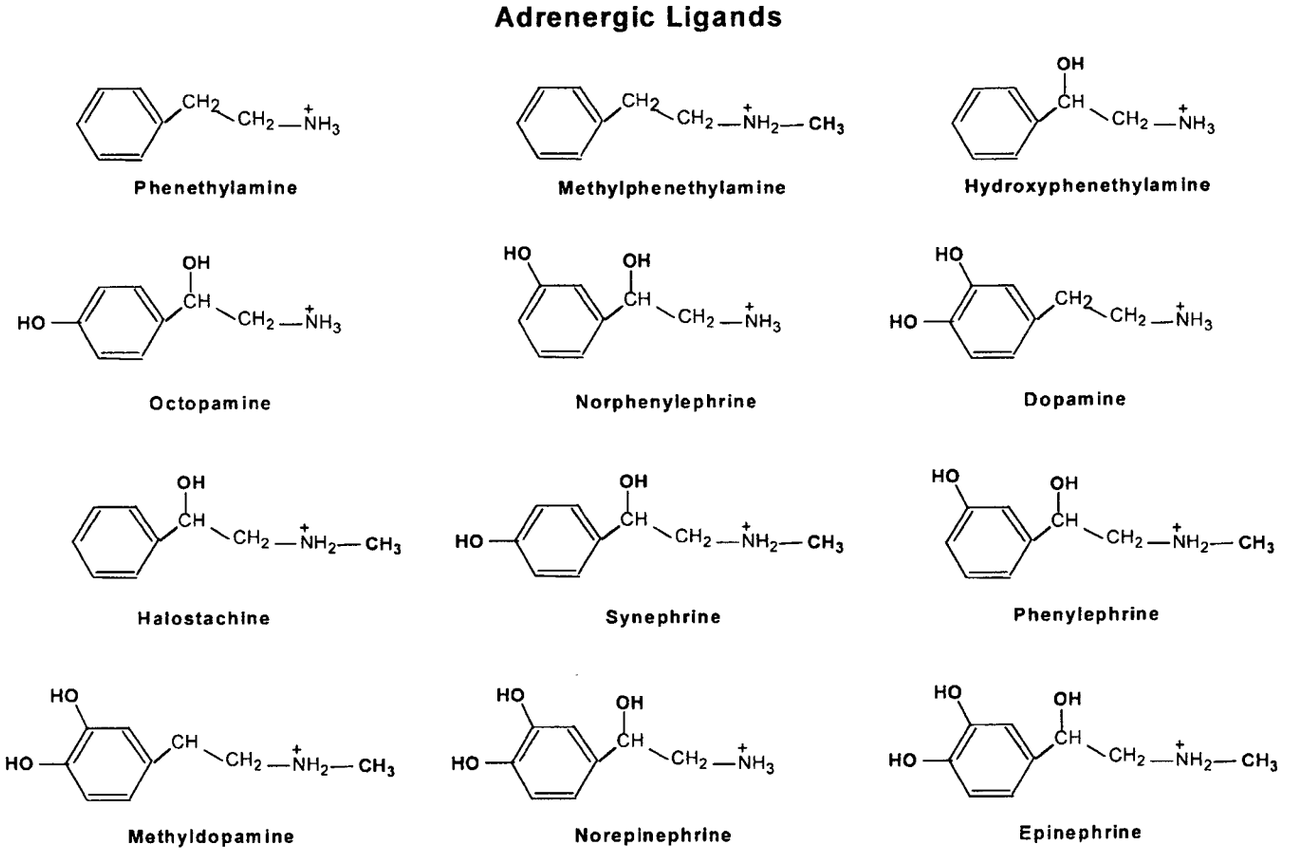

Fig. 1. Chemical structures of adrenergic ligands. The adrenergic ligands used in this study are phenethylamine and its derivatives that were created by adding to phenethylamine one or more of the following functional groups: N-CH_3, β-OH, and catechol OHs. Epinephrine is the fully substituted phenethylamine derivative containing all of the above functional groups.

gesting that these ligands, which lack the N-CH_3 or the β-OH, respectively, fully (or nearly fully) activated the β_2AR.

Agonist Binding to WT and CAM β_2AR. The affinities of the various agonist derivatives for WT and CAM were determined from competition experiments of [^{3}H]CGP-12177 performed under equilibrium conditions in membranes from HEK 293 cells stably expressing the receptors (Table 1 and Fig. 3, A and B). Phenethylamine, which contains the protonated amine and the aromatic ring, had equal low affinities for the WT and CAM, resulting in an affinity shift of ~1 (defined as the ratio between their apparent binding affinities for the WT and the CAM) (Table 1). In contrast, epinephrine, which contains the catechol OHs, the β-OH, and the N-CH_3 in addition to the protonated amine and the aromatic ring, had an affinity 400- and 16,000-fold higher than phenethylamine in WT and CAM, respectively, thereby resulting in an affinity shift of ~54 (Table 1).

The maximal ability of the agonists tested to stimulate cAMP accumulation in HEK cells stably expressing WT was highly correlated with their affinity shift (r^2 = 0.87, p < 0.005). Thus, the full agonists epinephrine, methyldopamine, and norepinephrine had the highest affinity shifts (54, 68, and 78, respectively), whereas the partial agonists synephrine, phenylephrine, halostachine, and dopamine had affinity shifts of 18, 17, 7, and 10, respectively (Table 1 and Fig. 4).

To investigate the interrelationship between the effects of the various functional groups, we expressed the changes in affinity resulting from the addition of each group to the set of substituted phenethylamine compounds. Addition to phenethylamine of either the catechol OHs (dopamine), the β-OH (hydroxyphenethylamine), or the N-CH_3 (methylphenethylamine) had no significant effect on affinity for the WT receptor (Table 1 and Fig. 5). Addition of any one of these functional groups to a compound bearing one other group increased their affinities for WT only 4- to 10-fold [considering the catechol OHs as one group and, thus, including adding the N-CH_3 to hydroxyphenethylamine or dopamine (Fig. 5), adding the β-OH to methylphenethylamine or dopamine (Fig. 5), or adding the catechol OHs to methylphenethylamine or hydroxyphenethylamine (Fig. 5)]. In marked contrast, addition of the N-CH_3 to norepinephrine (Fig. 5), the β-OH to methyldopamine (Fig. 5), or the catechol OHs to halostachine (Fig. 5) (in each case, creating epinephrine) resulted in a synergistic increase of their affinities for WT of 60- to 120-fold.

Although addition of the catechol OHs to halostachine produced a 120-fold increase in affinity for WT, addition to halostachine of the *m*-OH alone (thus making phenylephrine) increased the affinity for WT by only 3.5-fold, whereas addition of the *p*-OH alone (thus making synephrine) had no effect (Table 1). Thus, there was also a dramatic synergistic effect of the simultaneous presence of the catechol OHs, even starting with the presence of the β-OH and the N-CH_3. These results are in agreement with a previous study in which the synergism of the catechol OHs on binding affinity was abolished when either the catechol OHs or the -OH group on the side chain at positions $203^{5.42}$, $204^{5.43}$, or $207^{5.46}$ were removed (Liapakis et al., 2000). These results suggest that the synergistic effect of the catechol OHs on the affinity is associated with their interaction with all three OHs at positions $Ser203^{5.42}$, $Ser204^{5.43}$, and $Ser207^{5.46}$ in β_2AR.

The synergistic effect of multiple functional groups on affinity was different in the CAM. In contrast to WT, in which the presence of two other functional groups was necessary to enable the full effect of adding the third group, in CAM, a large synergistic effect was achieved by combining the catechol OHs (again considered as one group) with either the N-CH_3 or the β-OH. Thus, addition of the N-CH_3 to dopamine to make methyldopamine (Fig. 5), β-OH to dopamine to make norepinephrine (Fig. 5), or catechol OHs to methylphenethylamine or hydroxyphenethylamine to make methyldopamine or norepinephrine, respectively (Fig. 5), produced nearly the same increase in affinity as adding these groups to the appropriate compounds to make epinephrine. Despite this enhanced synergism in the CAM, the simultaneous presence of the catechol OHs was still necessary to produce this effect, just as it was necessary in WT. Although addition of the catechol OHs to halostachine produced a 980-fold increase in

TABLE 1

Agonist binding to wild-type and CAM β_2AR and stimulation of cAMP accumulation by wild-type β_2AR

Competition binding assays were performed on HEK 293 membranes stably expressing wild-type or CAM β_2AR, and cAMP accumulation studies were performed in intact HEK 293 cells stably expressing the wild-type β_2AR as described under *Materials and Methods*. The K_i values were determined according to the method of Cheng and Prusoff using the IC_{50} values from the competition binding isotherms (Cheng and Prusoff, 1973). The affinity shift is the ratio between the K_i values for WT and CAM β_2AR. K_i/K_{iPE} is the ratio between the K_i values for phenethylamine and each drug. The potencies (EC_{50} values) of agonists to stimulate cAMP accumulation were determined by nonlinear regression analysis. Values are presented as mean ± S.E. from three to eight independent experiments.

	[^{3}H]CGP-12177 Binding					cAMP Accumulation	
	Wild Type		CAM				
	$-\log K_i$ ± S.E.	K_i/K_{iPE}	$-\log K_i$ ± S.E.	K_i/K_{iPE}	Affinity Shift	$-\log EC_{50}$ ± S.E.	% MaximalStimulation
Epinephrine	7.16 ± 0.08	407	8.89 ± 0.13	16218	54	7.00 ± 0.11	100
Phenylephrine	5.62 ± 0.08	12	6.85 ± 0.10	148	17	5.65 ± 0.09	40 ± 5
Synephrine	5.00 ± 0.07	2.8	6.25 ± 0.18	37	18	4.98 ± 0.09	24 ± 3
Halostachine	5.08 ± 0.06	3.4	5.90 ± 0.09	17	6.6	4.73 ± 0.14	19 ± 4
Norepinephrine	5.40 ± 0.11	7.1	7.29 ± 0.07	407	78	5.61 ± 0.07	101 ± 10
Dopamine	4.35 ± 0.07	0.6	5.33 ± 0.05	4.5	10	4.50 ± 0.10	46 ± 7
Methyldopamine	5.37 ± 0.04	6.6	7.20 ± 0.09	331	68	5.40 ± 0.11	88 ± 10
Norphenylephrine	4.64 ± 0.04	1.2	5.35 ± 0.08	4.7	5.1	N.D.	N.D.
Octopamine	4.81 ± 0.16	1.8	5.09 ± 0.17	2.6	1.9	N.D.	N.D.
Hydroxyphenethylamine	4.53 ± 0.05	1.0	4.88 ± 0.04	1.6	2.2	N.D.	N.D.
Methylphenethylamine	4.18 ± 0.07	0.4	4.54 ± 0.02	0.7	2.3	N.D.	N.D.
Phenethylamine	4.55 ± 0.38	1.0	4.68 ± 0.42	1.0	1.3	N.D.	N.D.

N.D., not determined.

affinity for CAM (Fig. 5), addition to halostachine of the *m*-OH alone (thus making phenylephrine) increased the affinity for CAM by only 9-fold, whereas addition of the *p*-OH alone (thus making synephrine) increased the affinity for CAM by only 2-fold (Table 1).

cAMP Accumulation and Ligand Binding Studies on D79N β_2AR. The highly conserved $Asp^{2.50}$ in TM2 has been shown to be important for activation in β_2AR (Chung et al., 1988) as well as other GPCRs. Consistent with these reports, mutation of this residue to asparagine, creating $D79^{2.50}N$, dramatically decreased the potency and maximal activity of epinephrine to stimulate cAMP accumulation by 200- and 5-fold, respectively (Fig. 6A).

To test whether the impaired ability of $D79^{2.50}N$ β_2AR to reach the active conformation affected the binding properties of the receptor, we determined the affinities of various agonist derivatives for the D79N mutant in competition experiments with [^{3}H]CGP-12177 (Fig. 6B). It is interesting that the aforementioned synergism of the various functional groups in epinephrine on the binding affinity for WT and CAM was dramatically smaller in D79N: addition of the N-CH_3 to norepinephrine, the β-OH to methyldopamine, and the catechol OHs to halostachine, thereby making epinephrine, produced only a 7- to 13-fold increase in affinity for the $D79^{2.50}N$ mutant (Fig. 7). Thus, despite the addition of the functional groups to a compound containing two groups, these values were similar to those seen in WT for adding the functional groups to a compound bearing only one group, consistent with impaired synergism and with an impaired ability of $D79^{2.50}N$ to reach the active conformation. We refer to such a mutant as a "constitutively inactive" mutant to make the point that relative to WT, its isomerization is shifted toward the inactive state, whereas a CAM is shifted toward the active state.

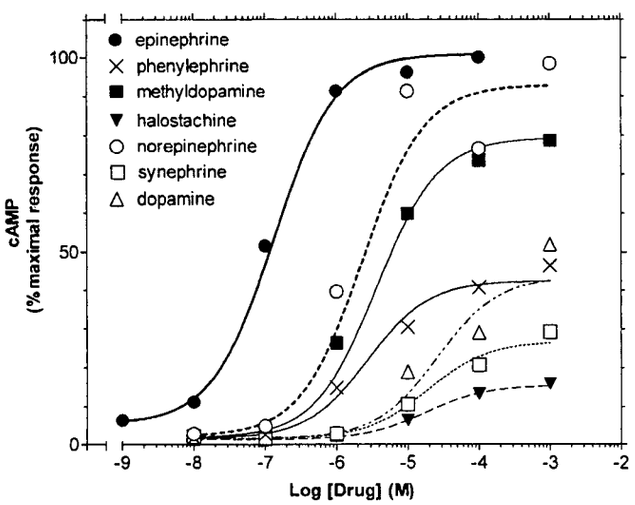

Fig. 2. Agonist-induced stimulation of cAMP accumulation by wild-type β_2AR. Stimulation of cAMP accumulation by the indicated concentrations of the series of agonists was performed in intact HEK 293 cells stably expressing the wild-type β_2AR after treatment with EEDQ, as described under *Materials and Methods*. The data were fit to a one-site sigmoidal dose-response model by nonlinear regression, and the logEC_{50} values were calculated and shown in Table 1. Maximal cAMP accumulation is presented as the percentage of the maximal stimulation obtained with 1 mM epinephrine. The values shown in each curve are from a representative experiment performed four to eight times with similar results.

Discussion

In contrast to the two-state model (Leff, 1995) in which a receptor isomerizes between an inactive state, R, and an active state, R*, many studies have suggested that β_2AR (Ghanouni et al., 2001; Swaminath et al., 2003) and other GPCRs (Noda et al., 1996; Tachibanaki et al., 1997) exist not only in R* and R but in intermediate conformations, R's, as well. Thus, under the equilibrium conditions of our binding studies, β_2AR may exist in a distribution of states, R↔R's↔R*. Based on the model of Weiss et al. (1996), if a receptor exists in an equilibrium between more than two

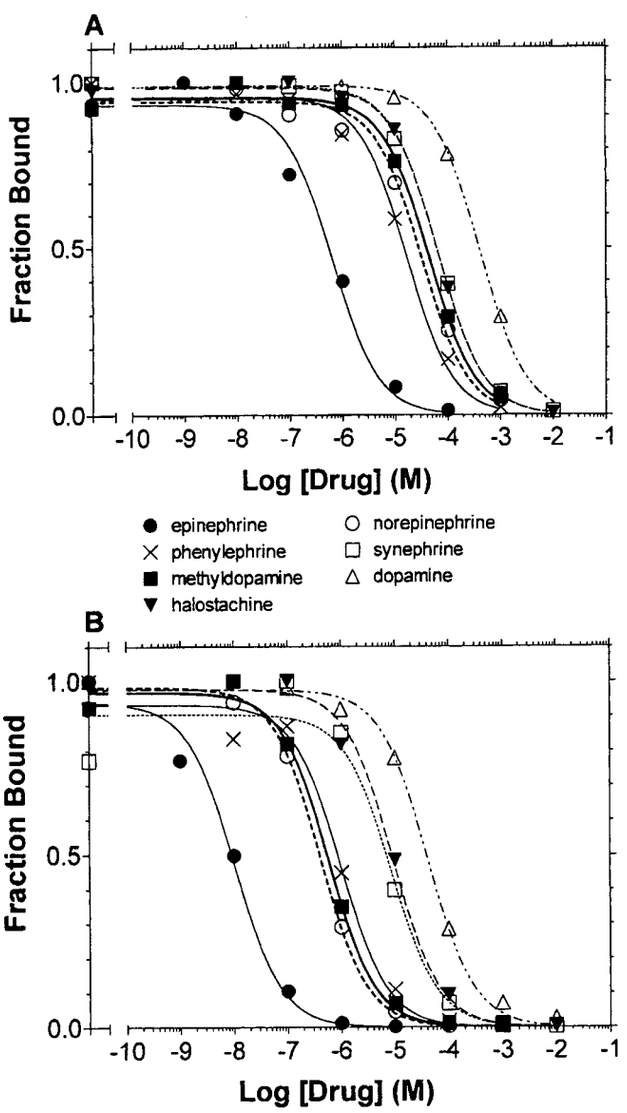

Fig. 3. Competition binding isotherms of adrenergic agonists to wild-type and CAM β_2AR. Competition of [^{3}H]CGP-12177–specific binding of the series of agonists was performed, as described under *Materials and Methods*, on membranes from HEK 293 cells stably expressing wild type (A) or CAM β_2AR (B). The data were fit to a one-site competition model by nonlinear regression, and the logIC_{50} values were calculated. The logK_i values determined from the logIC_{50} values are shown in Table 1. The values shown in each curve are from a representative experiment performed three to eight times with similar results. Of the 12 agonists screened, only those tested for stimulation of cAMP accumulation are shown above.

conformational states, the apparent agonist affinity will represent an average value derived from 1) agonist affinities for each state and 2) the relative proportion at equilibrium of each of the unbound receptor states. Apparent agonist affinity for β_2AR should, therefore, represent an average value derived by the affinities for R, R's, and R* and by the distribution of the unbound receptor among these states. Therefore, a rightward shift of R↔R's↔R* equilibrium, such as that seen after constitutive activation of β_2AR in a CAM, would increase the proportion of receptors in R* and thus the agonist apparent affinity, thereby resulting in an affinity shift compared with WT. In contrast, in a constitutively inactive mutant, such as D79$^{2.50}$N, this equilibrium is shifted leftward, presumably reflecting a decrease in the proportion of receptors in R* and therefore a decreased agonist apparent affinity. Indeed, the apparent affinity of epinephrine for CAM was ~54-fold higher than that for WT, whereas the affinity of epinephrine for D79$^{2.50}$N was ~70-fold lower than that for WT, consistent with the notion that CAM isomerizes toward R* to a much larger degree than WT but that WT isomerizes toward R* to a greater extent than D79$^{2.50}$N. This interpretation is consistent with our previous observation that in a rhodopsin homology model, the distance between Asp$^{2.50}$ and known agonist contact residues is too great for Asp$^{2.50}$ to be a direct agonist contact (Shi and Javitch, 2002) and that the effect of the mutation of Asp$^{2.50}$ on agonist binding in β_2AR and in other GPCRs is an indirect effect mediated through a change in the distribution of conformational states.

Because R* couples to and activates G-proteins, agonist efficacy must also be related to the degree of receptor transition toward R* and thus to the affinity shift. We found, as was reported previously for a different set of ligands (Samama et al., 1993), that the ratio (affinity shift) between the apparent binding affinities of the agonists tested for CAM and WT was correlated with their maximal abilities to stimulate cAMP accumulation in WT receptor (intrinsic activity). The differential propensities of WT, CAM, and D79$^{2.50}$N β_2AR to isomerize toward R* make these receptors excellent tools to investigate, as discussed below, the structural changes in the binding site that accompany receptor activation as well as to discriminate between different possible conformational states of receptor.

The presence of the catechol OHs and either the β-OH or the N-CH_3 was absolutely required for full activation of the receptor and for full affinity shift. Thus, despite having much lower affinities than epinephrine, norepinephrine and methyldopamine were full, or nearly full, agonists and had large affinity shifts. These results suggest that the interactions of β_2AR with the catechol OHs, the β-OH, and the N-CH_3 of epinephrine are optimized in R*, thus stabilizing R* and leading to receptor activation.

Mutagenesis and molecular modeling studies have shown that the contact residues for the catechol OHs are Ser203$^{5.42}$, Ser204$^{5.43}$, and Ser207$^{5.46}$ in TM5 (Strader et al., 1989; Liapakis et al., 2000), whereas the contact residues for the protonated amine and the β-OH are Asp113$^{3.32}$ in TM3 (Strader et al., 1988) and Asn293$^{6.55}$ in TM6 (Wieland et al., 1996), respectively. The contact site for the N-CH_3 is less clear but may be in TM7 (Gouldson et al., 1997). Thus, the role of epinephrine is to stabilize an activation-associated altered conformational relationship between TM3, TM5, TM6, and possibly TM7. Activation-associated conformational changes, consisting of translations and/or rotations of

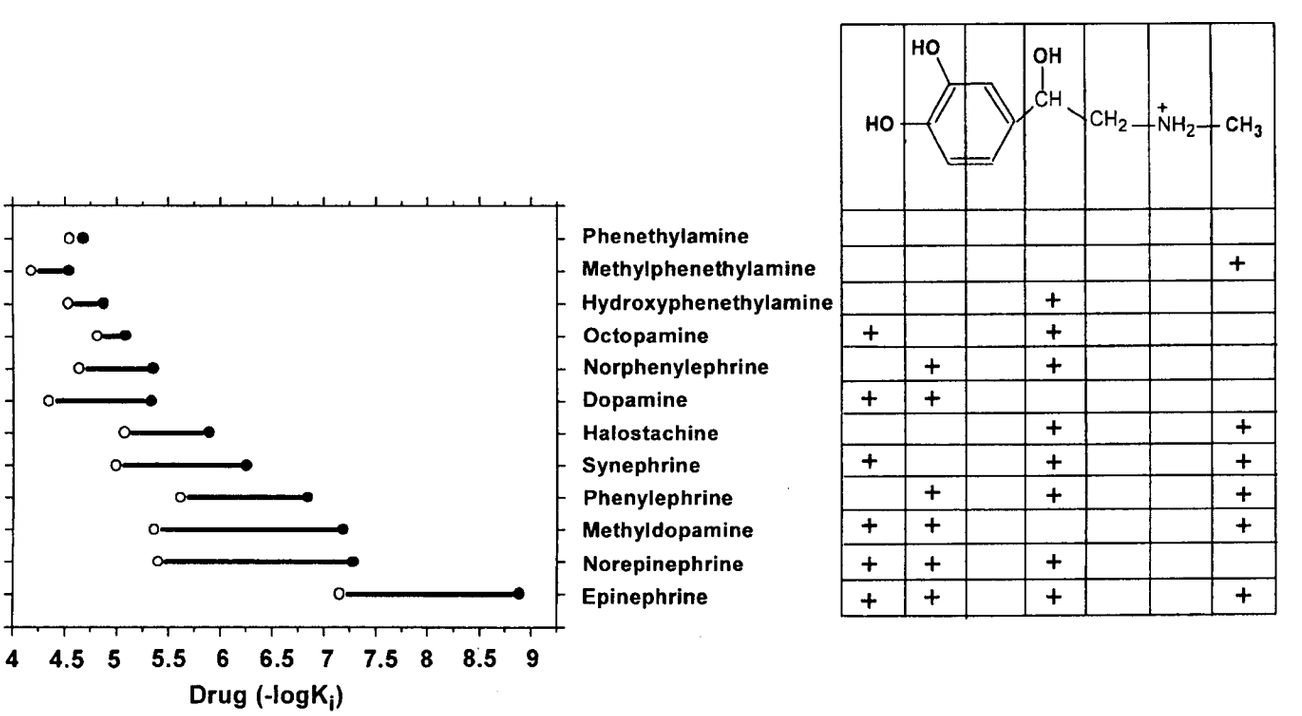

Fig. 4. Chemical structure of agonists and their affinities for wild-type and CAM β_2AR. The affinities of agonists for CAM and wild-type β_2AR are represented as ● and ○, respectively; the connecting line represents the size of the affinity shift. Epinephrine has four functional groups that were substituted in these experiments: two catechol OHs, a N-CH_3 group, and a β-OH group. +, the presence of the specified chemical groups for each phenethylamine derivative.

TM3, TM5, and TM6, have been proposed based on biophysical and biochemical studies in adrenergic and rhodopsin receptors (Farahbakhsh et al., 1993; Farrens et al., 1996; Gether et al., 1997; Javitch et al., 1997; Marjamaki et al., 1999; Shi et al., 2002).

To elucidate further details of the interrelationship of the conformational changes of these various contact residues, we analyzed systematically the energetic contributions of adding each of the functional groups to various substituted phenethylamine derivatives. In WT, the effect of adding each of the groups was 60- to 120-fold greater when added to the otherwise fully substituted compounds norepinephrine, methyldopamine, and halostachine (thus making epinephrine) compared with adding them to phenethylamine itself (Fig. 5). The observed synergistic effect of multiple functional groups on the resulting affinity is associated with the ability of receptor to isomerize toward R*, because the $D79^{2.50}N$ mutation, which dramatically impaired activation, resulted in a quite substantial loss of synergism (Fig. 7). In addition, this synergistic effect suggests that an initial interaction between two or more contacts may promote an intermediate conformation of β_2AR, R′, that allows more optimal contact with the remaining functional group. In agreement with our data, spectroscopy studies on β_2AR have suggested the presence between R and R* of an intermediate conformation, R′, induced by an initial agonist binding (Ghanouni et al., 2001; Swaminath et al., 2003). The existence of intermediate conformations has also been proposed for multiple GPCRs, including rhodopsin (Tachibanaki et al., 1997), AT_1-angiotensin (Noda et al., 1996), and M_1-muscarinic (Ward et al., 1999).

The pattern of these effects was very different in CAM than in WT in that combination of the catechol OHs with only either the β-OH or the N-CH_3 (thus making norepinephrine or methyldopamine, respectively) was sufficient to produce a synergistic effect on the resulting affinity as large as that observed by adding each of the functional groups to the otherwise fully substituted compounds norepinephrine, methyldopamine, and halostachine (thus making epinephrine) (Fig. 5). This synergistic affinity gain from the addition of only two functional groups to produce norepinephrine or methyldopamine is consistent with the CAM mutation enriching the receptor in R′ and/or R*, resulting in synergism

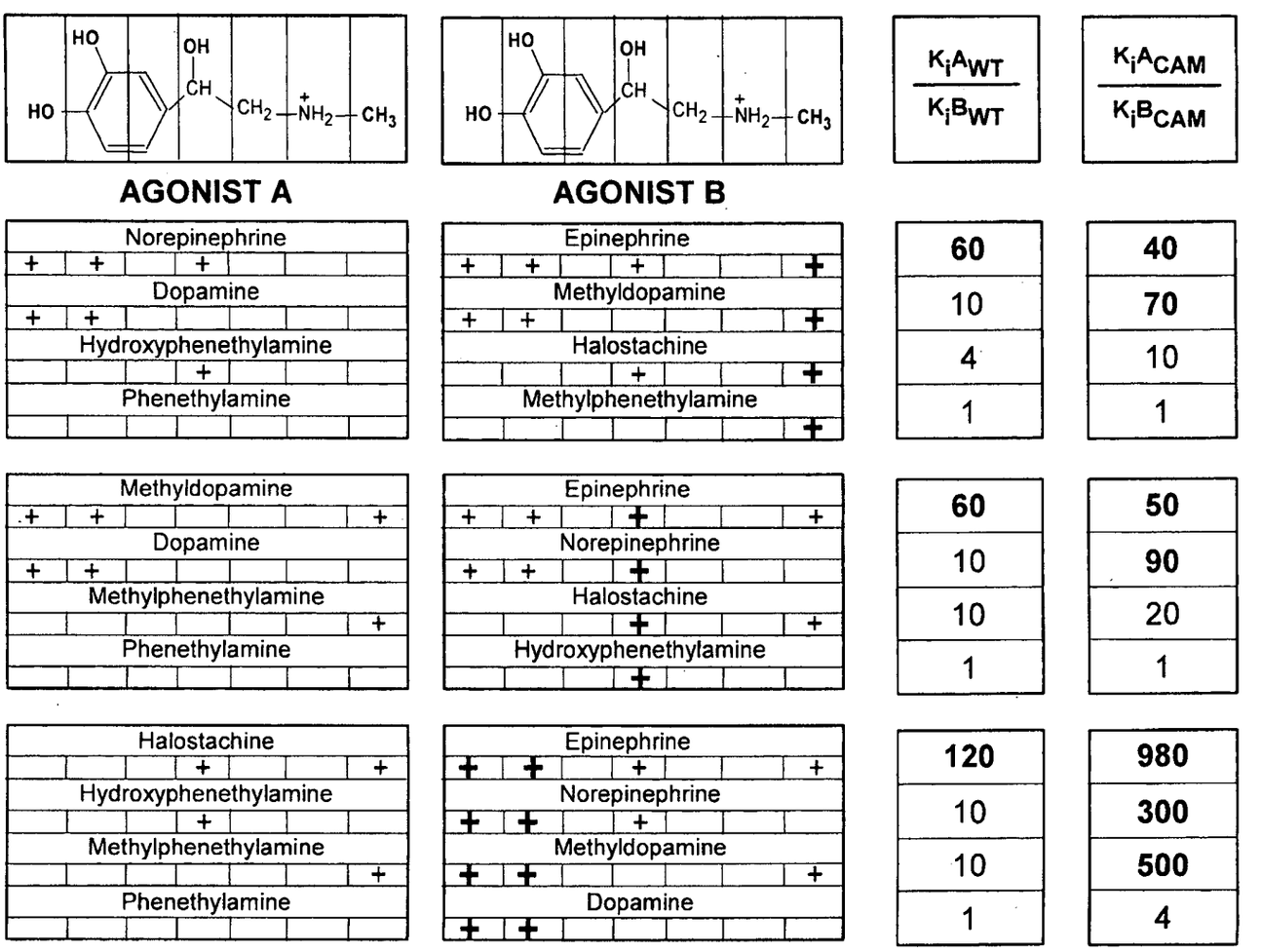

Fig. 5. Affinity change after the addition of N-CH_3, β-OH, or catechol OHs into various phenethylamine derivatives. Epinephrine has four functional groups that were substituted in these experiments: two catechol OHs, a N-CH_3 group, and a β-OH group. +, the presence of the specified chemical groups for each phenethylamine derivative; the individual group added in each case to agonist A to make agonist B is shown by a bold +. The addition of these functional groups to agonist A changed its affinity for WT and CAM β_2AR by K_iA_{WT}/K_iB_{WT}- and K_iA_{CAM}/K_iB_{CAM}-fold, respectively. K_iA_{WT}, K_iA_{CAM}, K_iB_{WT} and K_iB_{CAM} are the affinities of agonists A and B for WT and CAM β_2AR, shown in Table 1. The K_iA_{WT}/K_iB_{WT} and K_iA_{CAM}/K_iB_{CAM}A values in bold represent a large synergistic effect on the resulting affinity after addition of various functional groups to agonist A.

with fewer functional groups in our equilibrium binding assays. This enrichment in R′ and/or R* also results in the increased basal activity of CAM.

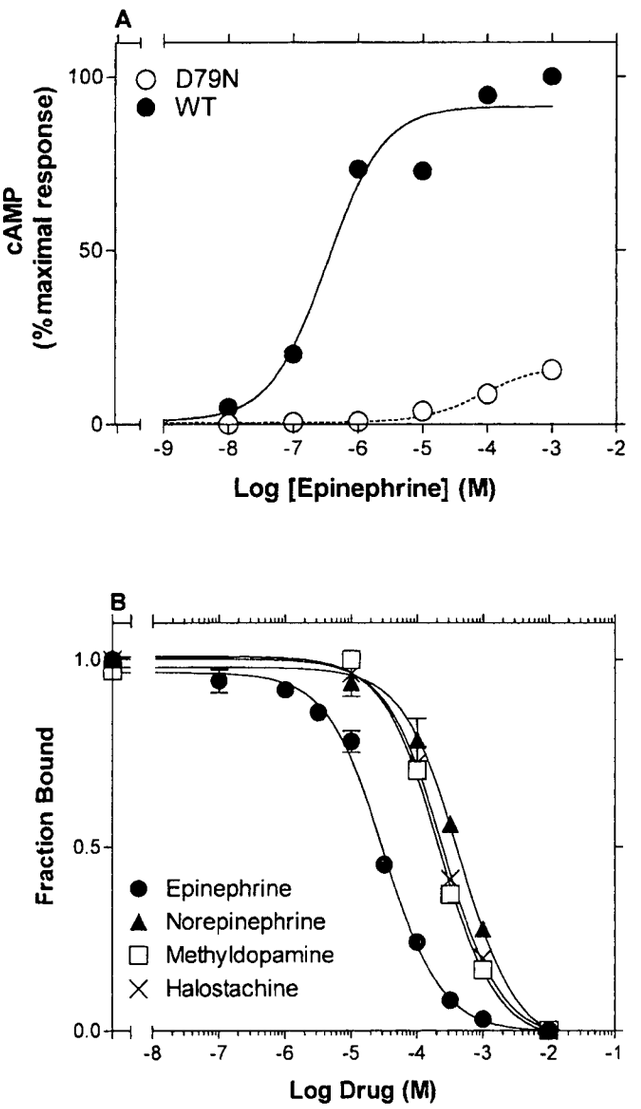

Fig. 6. Epinephrine-induced stimulation of cAMP accumulation by wild-type and $D79^{2.50}N$ β_2AR and ligand binding to $D79^{2.50}N$ β_2AR. A, stimulation of cAMP accumulation by the indicated concentrations of EPI was performed, as described under *Materials and Methods* and after treatment with EEDQ, in intact HEK 293 cells stably expressing WT and $D79^{2.50}N$ β_2AR. The data from three experiments were fit to a one-site sigmoidal dose-response model by nonlinear regression, and the $-\log EC_{50}$ values (mean ± S.E.) were 4.79 ± 0.36 for $D79^{2.50}N$ and 7.08 ± 0.33 for WT β_2AR. Maximal cAMP accumulation obtained by stimulation of $D79^{2.50}N$ with 1 mM EPI is presented as the percentage of the maximal cAMP accumulation obtained by stimulation of WT with 1 mM EPI. The values shown in each curve are from a representative experiment performed three times with similar results. B, competition of [^{3}H]CGP-12177–specific binding by various ligands was performed, as described under *Materials and Methods*, on membranes from HEK 293 cells stably expressing $D79^{2.50}N$ β_2AR. The data were fit to a one-site competition model by nonlinear regression, and the $\log IC_{50}$ values were calculated. The $-\log K_i$ values (mean ± S.E.) determined from the $\log IC_{50}$ values, according to the method of Cheng and Prusoff (1973), were 5.32 ± 0.05 for epinephrine, 4.21 ± 0.06 for norepinephrine, 4.46 ± 0.07 for methyldopamine, and 4.32 ± 0.14 for halostachine. The values shown in each curve are from a representative experiment performed three to four times with similar results.

Because stabilization of a fully active state by agonists requires the presence of either the β-OH or the N-CH_3, which interact with residues in TM6 and possibly in TM7, respectively, in combination with the catechol OHs and the protonated amine, which interact with TM5 and TM3 residues, respectively, it is possible that in R′ and/or R*, TM6 and TM7 are conformationally linked and rearrange in concert such that either the interaction of the β-OH with TM6 or the possible interaction of the N-CH_3 with TM7 is sufficient for full efficacy. This is consistent with a molecular model of 5-hydroxytryptamine$_{2A}$ receptor in which TM6 and TM7 have been suggested to interact with each other and an activation-associated movement of TM7 affects the motion of TM6 (Luo et al., 1994). In addition, it is possible that the active species of β_2AR stabilized by the two functional groups of methyldopamine and norepinephrine might represent different receptor conformations than that stabilized by the three functional groups of epinephrine, despite the fact that all of these active states are able to activate maximal cAMP accumulation. The presence of different active conformations of GPCRs that are stabilized by different agonists has been proposed previously (Perez et al., 1996; Seifert et al., 1999; Strange, 1999; Ghanouni et al., 2001).

Stabilization of fully active species requires the presence of both catechol OHs, since removal from epinephrine of either *p*-OH or *m*-OH resulted in partial activation and loss of synergism regardless of the presence of both β-OH and N-CH_3. These results are in agreement with previous studies (Strader et al., 1989; Liapakis et al., 2000) and suggest that the interactions between the catechol OHs and the cluster of serines in TM5 play a critical role in the proper positioning of the catechol ring in the binding site crevice and probably the proper positioning of the side chains and/or orientation of the TM5 to achieve the fully active state.

In the dopamine D2 receptor, a cluster of aromatic residues consisting of $Phe382^{6.44}$, $Trp386^{6.48}$, $Phe389^{6.51}$, $Phe390^{6.52}$, $His393^{6.55}$ in TM6, $Tyr416^{7.43}$ in TM7, and $Phe198^{5.47}$ in TM5 plays an important role in ligand binding and receptor activation (Javitch et al., 1998). Most of these residues are conserved in β_2AR, and in this receptor, activation has been inferred to be associated with coordinated rotamer changes of $Trp286^{6.48}$ and $Phe290^{6.52}$ in the aromatic cluster along with $Cys285^{6.47}$ (Shi et al., 2002). This rotamer "toggle switch" may modulate the movement of TM6 about the kink formed by $Pro^{6.50}$, which is associated with the movement of the cytoplasmic end of TM6 relative to that of TM3, during receptor activation. Based on this model, an agonist could promote the active state by stabilizing $Phe^{6.52}$ in the *t* rotamer conformation, thus allowing $Trp^{6.48}$ to adopt the *t* conformation, but only in the context of the complete set of interactions described above.

Acknowledgments

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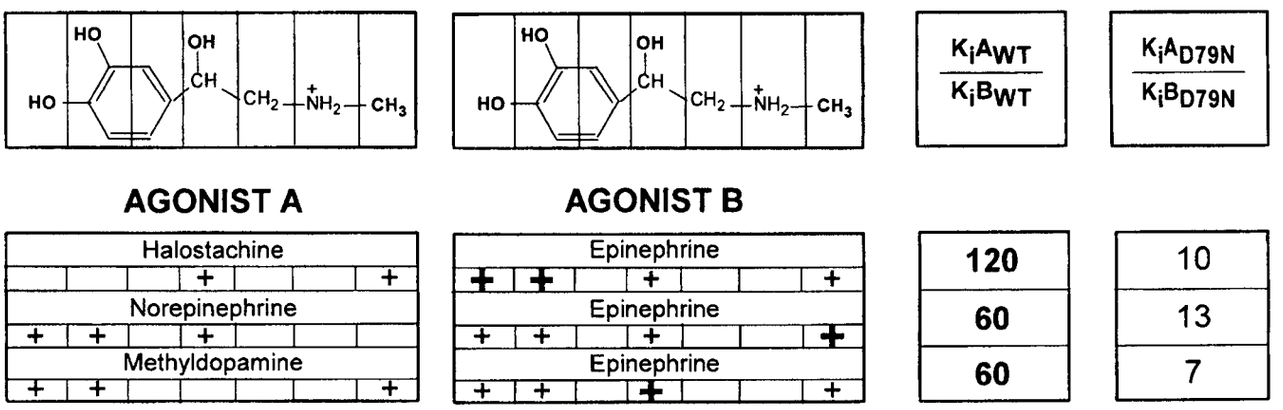

Fig. 7. Affinity change after the addition of the catechol OHs, a β-OH, or a N-CH_3 into various phenethylamine derivatives. Epinephrine has four functional groups that were substituted in these experiments: two catechol OHs, a N-CH_3 group, and a β-OH group. +, the presence of the specified chemical groups for each phenethylamine derivative; the individual group added in each case to agonist A to make agonist B is shown by a bold +. The addition of these functional groups to agonist A changed its affinity for the WT and $D79^{2.50}N$ β_2AR by K_iA_{WT}/K_iB_{WT}- and K_iA_{D79N}/K_iB_{D79N}-fold, respectively. K_iA_{WT}, K_iA_{D79N}, K_iB_{WT}, and K_iB_{D79N} are the affinities of agonists A and B for WT and $D79^{2.50}N$ β_2AR, shown in Table 1 and Fig. 5B. The K_iA_{WT}/K_iB_{WT} and K_iA_{D79N}/K_iB_{D79N} values in bold represent a large synergistic effect on the resulting affinity after addition of various functional groups to agonist A.

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Molecular Modelling Study of the Lidocaine, Procaine, and Their Metabolites

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The AM1 quantum-chemical method has been used to determine stable structures and proton affinities of local anaesthetics lidocaine, procaine, and their main metabolites. The reaction enthalpies for the deethylation and hydroxylation of the lidocaine and the hydrolysis of procaine were computed. The lipophilic properties of these compounds were also investigated. The observed relative activities of lidocaine and its active metabolites correlated with both the calculated proton affinity and lipophilicity of these compounds.

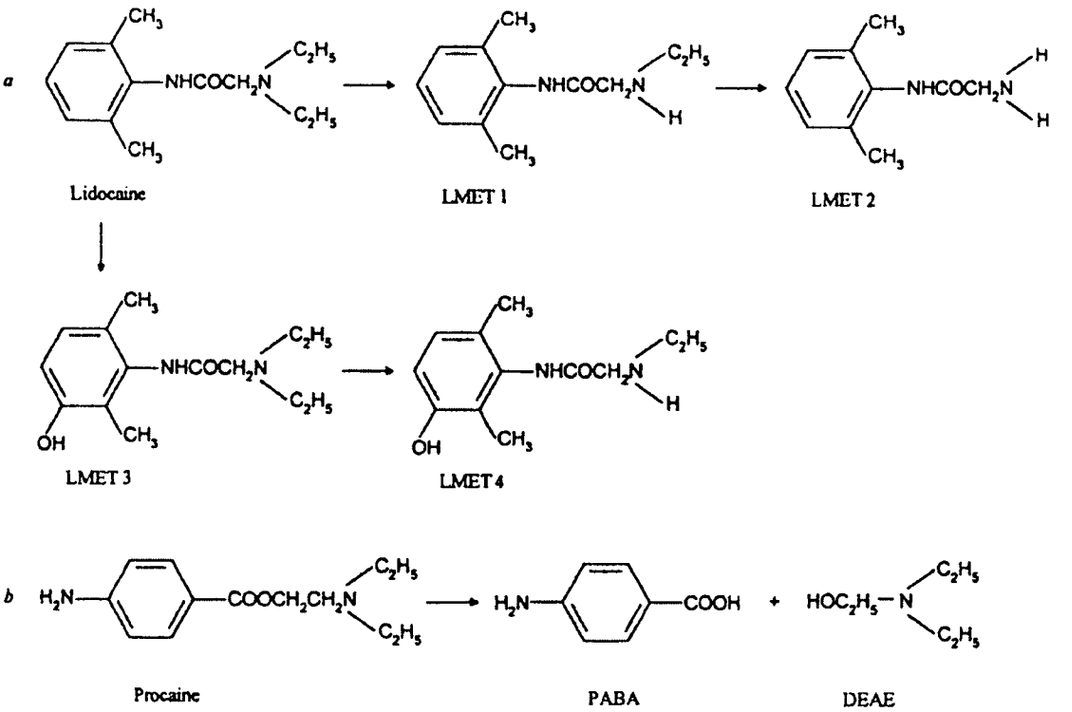
Procaine and lidocaine are well known clinically used local anaesthetics (LA) [1]. Lidocaine also exhibits considerable antiarrhythmic activity [2]. These compounds, like many other neuroactive drugs, must penetrate into or across the neuronal plasmalemma to be pharmacologically active [2]. In order to explain the molecular mode of actions of these drugs, the effect of procaine and lidocaine on the cell membranes was intensively experimentally investigated [3—6]. Based on the premise that the knowledge of a detailed three-dimensional structure of these drugs must be important for the explanation of the structure—activity relationships, some authors devoted their work to the investigation of the molecular structure of procaine [7—10] and lidocaine [11—15].

The use of these drugs may be complicated by the presence of active metabolites [16, 17]. The metabolic fate of the procaine and lidocaine has been extensively studied [16—19] and principal metabolites were determined [16, 18—21] (Scheme 1). It is assumed that procaine metabolites interact with neuronal voltage-gated Na^+ channels in a manner similar to that of procaine and other local anaesthetics [16]. Similarly, it is shown [17] that also metabolites of the lidocaine-type antiarrhythmics can bind to this class drug receptor associated with the sodium channel. Considering the chemical structures of LA's there are several loci on the channel where LA is likely to bind. However, none of these have been definitely identified using the techniques of molecular biology. The absence of three-dimensional structural data for transmembrane receptors presents a challenge to the application of molecular modelling methods to gain insights into the recognition and binding process. The results of theoretical modelling [22—29] of interactions of associative sites of LA's with polar groups (as carboxylate, phosphate, amine, amide) of membranes have been used to identify molecular determinants of recognition and binding process. The effect of medium on the equilibrium geometry and interaction energy of the LA—carboxylate complexes was investigated [29]. Effect of hydration on stable conformations of LA has also been studied [30].

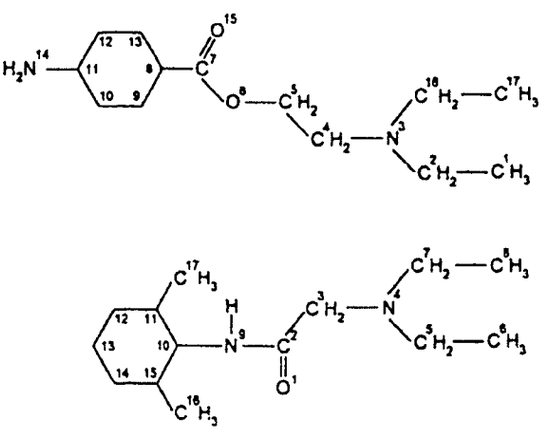
Based on these results we applied the methods of theoretical chemistry to a group of two most frequently used LA's (procaine, lidocaine) and their six metabolites. Of particular interest were physicochemical properties of these species (structural characteristics, proton affinity, reaction enthalpy, partition coefficient) and how these parameters correlate with available experimental (physicochemical and biological) data. The calculations reported here also represent the first molecular modelling study of procaine and lidocaine metabolites.

THEORETICAL METHODS

The geometries of procaine and its metabolites *para*-aminobenzoic acid (PABA) and diethylaminoethanol (DEAE), lidocaine and its metabolites ω-diethylamino-2,6-dimethylacetanilide (LMET1), ω-amino-2,6-dimethylacetanilide (LMET2), 3-hydroxy-ω-diethylamino-2,6-dimethylacetanilide (LMET3), and 3-hydroxy-ω-ethylamino-2,6-dimethylacetanilide (LMET4) (Scheme 1) were fully energy-optimized using the quantum-chemical MO-SCF AM1 method [31]. The molecular graphic and molecular modelling studies of the AM1 optimized structures were carried out by means of the MOLGEN 3.0 program [32]. For the calculation of partition coefficients the MGP program [33] was used. The labeling of the atoms of procaine and lidocaine is shown in Scheme 2.



Scheme 1. Metabolic pathways for lidocaine (*a*) and procaine (*b*).



Scheme 2. Numbering of atoms in procaine and lidocaine.

The semiempirical quantum-chemical AM1 method allows the calculation of the standard ($T = 298$ K) formation enthalpies [34], $\Delta H^0_{f,298}$. The proton affinity of base PA(B) can be computed by the equation

$$\mathrm{PA(B)} = \Delta H^0_{f,T}(\mathrm{H^+, g}) + \Delta H^0_{f,T}(\mathrm{B, g}) + \Delta H^0_{f,T}(\mathrm{BH^+, g}) \quad (1)$$

$\Delta H^0_{f,T}$ represents the heat of formation of the species stated between parenthesis. For $\Delta H^0_{f,298}(\mathrm{H^+, g})$ the experimental value 1537.1 kJ mol^{-1} is taken [35]. All quantum-chemical calculations were performed using the AMPAC program [36].

RESULTS AND DISCUSSION

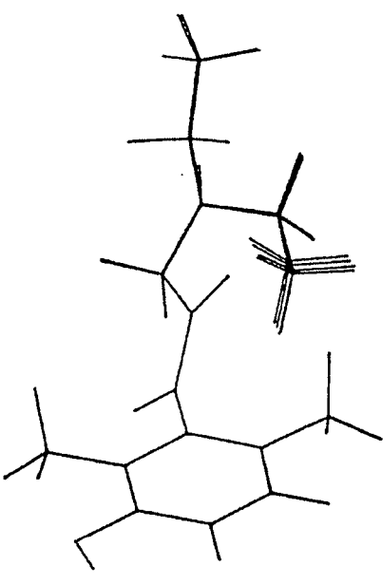
Geometry

The quantum-chemical calculations of conformational energy maps [7, 8, 13, 30] reveal the presence of several stable conformations in basic and protonized LA's. Without taking into account any environment, the most stable conformations correspond to bent structures stabilized by intramolecular hydrogen bonds. Among the several binding possibilities of these drugs to the receptor the strongest interaction is that of ion-pair type [22—29]. The actual, biologically active conformation may be better represented by structures observed in the ion pairs of some LA's. Therefore, the thermodynamically stable X-ray structures of lidocaine [37] and procaine [38] hydrochlorides were considered as starting geometries for the AM1 energy optimizations of drugs in this study.

The structures of lidocaine and its four metabolites were fully optimized by the AM1 method. Selected AM1 geometric values for the lidocaine and its metabolites are listed in Table 1. As it is seen from this table the deethylation and/or hydroxylation practically does not change the overall mutual arrangement of the hydrophobic (aromatic) part and hydrophilic (amine) group of the drug. The torsion angle Ψ defining the orientation of the aromatic and amide groups, is practically the same in all compounds studied and its value is close to those found in different crystal environments (67.9—76.9°, Refs. [37, 39, 40]). The amide group is in all compounds investigated nonplanar, but the deviation from planarity is small (8—10°,

Table 1. Selected AM1 Optimized Parameters for Lidocaine and its Metabolites

	Lidocaine		LMET1	LMET2	LMET3	LMET4
	AM1	X-Ray [39]				
			Bond lengths r/pm			
O-1—C-2	124.5	122.2	124.5	124.5	124.4	124.5
C-2—C-3	154.3	151.4	154.2	154.3	154.2	154.3
C-3—N-4	144.3	146.7	143.8	143.5	144.2	143.8
C-2—N-9	138.9	134.3	138.7	138.6	139.1	138.9
N-9—C-10	141.5	143.2	141.4	141.5	141.4	141.4
			Bond angles Φ/°			
O-1—C-2—N-9	122.5	125.1	122.9	123.3	122.5	123.0
C-3—C-2—N-9	115.7	113.4	116.1	116.4	115.6	116.0
C-2—C-3—N-4	116.9	114.5	115.7	115.3	117.0	115.7
C-2—N-9—C-10	124.6	122.3	124.9	124.8	124.7	124.8
			Torsion angles Ψ/°			
C-15—C-10—N-9—C-2	66.0	67.9	65.5	66.1	65.8	65.7
C-10—N-9—C-2—O-1	351.7	353.8	351.1	350.5	351.3	351.0
N-9—C-2—C-3—N-4	157.2	149.7	158.5	159.1	158.5	159.2
C-2—C-3—N-4—C-5(H-5)	67.3	79.0	57.8	60.1	67.7	54.8



Scheme 3. The superposition of the AM1 optimized conformers of lidocaine and its four metabolites studied.

Table 2. Selected AM1 Optimized Parameters for Procaine

	Procaine	
	AM1	X-Ray [38]
	Bond lengths r/pm	
C-8—C-7	146.4	146.8
C-7—O-15	123.6	121.6
C-7—O-6	137.3	136.1
O-6—C-5	143.5	143.9
C-5—C-4	153.3	150.2
C-4—N-3	144.7	150.8
	Bond angles Φ/°	
C-8—C-7—O-6	114.2	112.2
C-8—C-7—O-15	128.2	127.6
C-7—O-6—C-5	116.4	116.4
O-6—C-5—C-4	106.9	109.2
C-5—C-4—N-3	118.4	116.3
C-4—N-3—C-2	112.9	112.0
	Torsion angles Ψ/°	
C-9—C-8—C-7—O-15	176.2	185.9
C-8—C-7—O-6—C-5	178.4	179.4
C-7—O-6—C-5—C-4	176.3	172.7
O-6—C-5—C-4—N-3	71.0	70.3
C-5—C-4—N-3—C-2	61.3	60.0

Table 1). A little bit lower deviation from planarity of the amide group (about 5—6°) was also observed in the crystals of lidocaine salts [37, 39, 40]. The N-9—C-2—C-3—N-4 fragment adopts the nonplanar *trans* conformation in both lidocaine and its metabolites with very close torsion angles (Table 1). The very low deviations of the structures of metabolites from the optimized lidocaine conformer are also manifested in Scheme 3. This scheme was prepared by means of the molecular graphics program MOLGEN 3.0 and represents the superposition of the lidocaine metabolites studied with respect to the lidocaine. The superposition was carried out with respect to the atoms C-10, C-11, and N-9, respectively. A goodness of fit was expressed in terms of Root Mean Square (RMS) value (equal to 0.001). The major features of the geometry predicted by AM1 for the lidocaine are in agreement with those of the previous X-ray study [39] (Table 1).

The selected bond lengths, bond angles, and torsion angles, together with the experimental X-ray values [38] of the procaine hydrochloride are given in Table 2. In general, the values obtained with the AM1 method and experimental data are very similar. However, several differences are seen. The length C-4—N-1 was computed considerably shorter and the length

Table 3. AM1 Calculated A, B, and C Interatomic Distances (in pm), Proton Affinities, and Relative Activity (RA) of the Compounds Studied

Compound	A($C_{arom}\cdots O$)		B($N\cdots O$)		C($C_{arom}\cdots N$)		PA/(kJ mol^{-1})	RA[b]
	B	BH^+	B	BH^+	B	BH^+		
Lidocaine	287	288	290	282 297[a]	504	499 485[a]	916.7	100
LMET1	288	290	285	270	499	497	900.8	85
LMET2	288	291	282	267	498	495	879.0	26
LMET3	288	290	285	270	499	497	915.5	
LMET4	287	289	290	288	501	500	900.0	
Procaine	243	244	493	492 497[a]	523	525 521[a]	935.2	
DEAE							933.1	

a) X-Ray data; *b*) Relative activity in man with respect to the lidocaine (Refs. [44, 45]).

C-5—C-4 longer than the experimental values. As regards bond angles, the largest difference between theory and experiment (2.3°) was observed for the O-6—C-5—C-4 angle. As follows from the comparison of the torsion angles (Table 2) the stereochemistry of the C—CO—C—C—N connecting chain of procaine is in the optimized structure close to those found in the crystal of procaine hydrochloride [38]. The carboxylic group of procaine is calculated to be practically coplanar with the aromatic ring. The C-8—C-7—O-6—C-5 and C-7—O-6—C-5—C-4 fragments exist predominantly in the planar arrangement. On the other hand, the O-6—C-5—C-4—N-3 and C-5—C-4—N-3—C-2 groups are in stable *gauche* conformations.

For more quantitative description of the structural differences of both drugs we also determined the intermolecular distances A($C\cdots O$), B($N\cdots O$), and C($C\cdots N$) connecting the nonbonding nitrogen, oxygen, and aromatic carbon (C-8, C-10) atoms, respectively, specifying the separation of the two polar groups of drugs able to interact with the corresponding binding sites of the membrane [24—28] (Table 3). The analysis of these separations among the common functional groups of LA's and their metabolites reveals that they are within certain narrow intervals. However, the interatomic separations between amine and oxygen atoms are very different for lidocaine (282 pm) and procaine (492 pm). This suggests that the binding sites for these two drugs should be different. The calculated analogous distances for metabolites of the lidocaine are very close to those found for parent drug (Table 3). Thus the common pharmacophoric patterns of lidocaine and its metabolites indicate that these species may bind to the same receptor. *Sheldon et al.* [17] have recently shown experimentally that antiarrhythmics of the lidocaine type and their metabolites bind to the same antiarrhythmic receptor. This is not surprising given that our fully optimized structures of lidocaine metabolites are very similar to that of their parent drug (Table 3, Scheme 3).

Proton Affinity and Reaction Enthalpy

At physiological pH, procaine, lidocaine, and their metabolites can occur in positively charged or uncharged forms. The protonation site is the amino group. Table 3 summarizes the AM1 calculated proton affinities (PA) of compounds studied. For evaluation of proton affinities the fully geometry-optimized structures of base and corresponding cation were used. The highest values of PA were computed for the tertial amines and with the decreasing alkylation the PA also decreases (Table 3). The same dependence was observed experimentally for vapour phase protonation of methyl-substituted ammonia [41]. The experimental gas-phase PA's for methylamine, dimethylamine, and trimethylamine (896.2 kJ mol^{-1}, 923.0 kJ mol^{-1}, and 944.4 kJ mol^{-1}) (Ref. [41]) are in general agreement with corresponding AM1 calculated PA's (Table 3) for lidocaine and procaine derivatives investigated. The computed proton affinities correlate with the relative activities of lidocaine and its active metabolites (Table 3). A good correlation ($R = 0.971$) was found between the relative activity and proton affinity of these three agents. The very close PA computed for the procaine and its simpler metabolite diethylaminoethanol (Table 3) shows that the side aromatic parts of the molecule do not significantly influence the basicity of the hydrophilic amino group of drug. The practically equal basicity of procaine and its metabolite diethylaminoethanol (DEAE) is probably responsible for the fact that DEAE, after intravenous injection, shows many actions of procaine [16]. Thus it is possible that DEAE interacts with neuronal Na^+ channels in a manner similar to that of procaine.

The reaction enthalpies for the deethylation and hydroxylation of the lidocaine and the hydrolysis of the procaine are shown in Table 4. Although these calculations do not take into account the entropy changes and effect of medium, it was hoped that in comparison with the same reaction for the hydrolysis and/or hydroxylation of the compounds studied these factors

Table 4. Reaction Enthalpies for the Reactions of the Lidocaine and Procaine

No.	Reaction	$\Delta H^0_{f,298}$ (kJ mol^{-1})
1	Lidocaine + H_2O ⟶ LMET1 + C_2H_5OH	−15.9
2	LMET1 + H_2O ⟶ LMET2 + C_2H_5OH	−1.3
3	Lidocaine + H_2O ⟶ LMET3 + H_2	46.9
4	LMET3 + H_2O ⟶ LMET4 + C_2H_5OH	−16.3
5	LMET1 + H_2O ⟶ LMET4 + H_2	46.5
6	Procaine + H_2O ⟶ PABA + DEAE	−18.4

Table 5. Calculated and Experimental Partition Coefficients, log *P*

Compound	log *P*	
	Calculated	Experimental [2]
Lidocaine	2.41	2.56
LMET1	1.69	
LMET2	0.84	
LMET3	2.02	
LMET4	1.30	
Procaine	1.73	2.0
PABA	0.79	
DEAE	0.40	

would at least partially cancel. For the deethylation (reactions *1*, *2*, and *4*) of the lidocaine and its metabolites the exothermic reactions with small absolute values of enthalpies are characteristic. The deethylation of the tertiary amine is energetically more favourable in comparison with the same reaction of the secondary amine (Table 4). The larger, but positive reaction enthalpies were found for the hydroxylation reactions *3* and *5* (Table 4). The computed enthalpies for deethylation and hydroxylation of lidocaine qualitatively correlate with the *in vivo* experiments. These experiments [21] have shown that the capacity of lidocaine 3-hydroxylation is small and saturable at low substrate concentrations while *N*-deethylation is not saturable. The hydrolysis of the procaine is exothermic with the calculated enthalpy of −18.4 kJ mol^{-1}. Another question arises as to whether there should be a barrier to the formation of reaction products. The activation energies for the reactions *1*—*6* (Table 4) are not known. Their magnitude may have (together with the catalytic influence of substrate) some influence on gas phase stability.

Hydrophobic Properties

Hydrophobicity of local anaesthetic is one of decisive physicochemical parameters which is responsible for its ability to penetrate a hydrophobic domain of the membrane. A clear correlation between potency and hydrophobicity is observed [42]. Because the base of local anaesthetic is more membrane-permeant, it accounts for most of the bulk passage of drug through membrane hydrophobic barriers [2]. Hydrophobicity of a drug is usually measured as *P*, the partition coefficient of the molecule in the water—octanol system. Table 5 contains the calculated log *P* of compounds under study using the MGP program. Log *P* was computed from the hydrophobic atomic parameters defined by *Crippen et al.* [43] and is in a very good agreement with the available experimental partition coefficients for lidocaine and procaine. The metabolites were calculated more hydrophilic than their parent drugs. Similarly, the nonactive hydroxylated metabolites of the lidocaine (LMET3 and LMET4) are more hydrophilic than the corresponding active compounds (lidocaine and LMET1). In man, LMET1 and LMET2 have 85 % (Ref. [44]) and 26 % (Ref. [45]) of the activity of lidocaine. Using regression analysis with calculated log *P* as the independent variable a very good correlation with the relative activity (RA) of lidocaine (100 %), LMET1 (85 %), and LMET2 (26 %) was obtained

$$\mathrm{RA} = -8.374 + 47.798 \pm 13.855 \log P \qquad (2)$$
$$R = 0.9602; \quad \mathrm{SE} = 15.443; \quad F = 11.832$$

As it is well known [2] the hydrophobicity of local anaesthetic significantly contributes to the potency of the drug, because it increases its accessibility to some functional sites in the membrane. The presence of the polar hydroxyl group on the aromatic part of the metabolites LMET3 and LMET4 considerably decreases the lipophilicity of this moiety of the compound which should prevent their penetration through the hydrophobic part of the membrane to the right place and create the effective interaction with the receptor in order to produce measurable pharmacological response.

CONCLUSION

This theoretical study was set out to determine physicochemical information about lidocaine and procaine metabolites and their parent compounds for which a relatively small amount of experimental structural data exists, considering their pharmacological importance. Using the molecular modelling methods the following conclusions can be drawn.

1. The structural diversity of lidocaine and procaine resulting from the molecular modelling studies indicates that these drugs could bind to different receptors. The deethylation and/or ring-hydroxylation of the lidocaine does not change the conformation of the side chain containing the —N(H)—C(O)—C—N< atoms and reveals the common pharmacophoric patterns of lidocaine and its metabolites.

2. The deethylation of lidocaine and the hydrolysis of procaine are in vapour state exothermic reactions. On the contrary, the hydroxylation of lidocaine is found to be highly endothermic.

3. A different computed partition coefficient, log P found for active and inactive metabolites of lidocaine should explain their different biological behaviour.

The data obtained in this work will be useful for further investigations of the mechanism of action of local anaesthetics on the molecular level.

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Translated by M. Remko

WHAT IS CLAIMED:

1. Use of an activatable matrix-degrading enzyme (AMDE) for formulation of a medicament for sub-epidermal administration for temporally degrading a component of the extracellular matrix (ECM) for treating a disease or condition of the ECM, wherein:

the AMDE is formulated in an amount sufficient, when exposed to an activator, to degrade a component of the ECM associated with the disease or condition;

the AMDE is a matrix-degrading enzyme that is inactive in the ECM following sub-epidermal administration in the absence of the activator; and

the AMDE is active for a limited time in the ECM following sub-epidermal administration upon exposure to an activating condition provided by the activator.

2. The use of claim 1, wherein the medicament is for single dosage administration, and contains an amount of AMDE that is or is about 10 μg to 100 mg, 50 μg to 75 mg, 100 μg to 50 mg, 250 μg to 25 mg, 500 μg to 10 mg, 1 mg to 5 mg or 2 mg to 4 mg.

3. The use of claim 1, wherein the enzyme is substantially inactive at neutral pH.

4. The use of claim 1 that is formulated for subcutaneous administration, intramuscular administration, intralesional administration or intradermal administration.

5. The use of claim 1, wherein the activator is formulated in the same composition as the AMDE or in a separate composition.

6. The use of claim 1, wherein the AMDE is selected from among a cathepsin, a calpain, and a heparanase.

7. The use of claim 6, wherein the AMDE is a cathepsin and the cathepsin is a cysteine protease or aspartic protease.

8. The use of claim 7, wherein the cathepsin is selected from among cathepsin S, cathepsin K, cathepsin L, cathepsin B, cathepsin C, cathepsin H, cathepsin F, cathepsin O, cathepsin R, cathepsin V, cathepsin W, cathepsin D, and cathepsin E.

9. The use of claim 8, wherein the cathepsin is cathepsin L.

10. The use of claim 7, wherein the cathepsin has a sequence of amino acids selected from any of SEQ ID NOS: 57, 60, 1,65, 180, 68,71,74, 77, 183, 186, 189, 80, 195, 90, 93 and 96, or is an allelic, or species variant or other variant of any of SEQ ID NOS: 57, 60, 1, 65, 180, 68,71,74,77, 183, 186, 189, 80, 195, 90, 93 and 96.

11. The use of claim 1, wherein the activating condition is selected from among pH, ionic strength, temperature and metal ions.

12. The use of claim 11, wherein the AMDE requires more than one activating condition for activity.

13. The use of claim 11, wherein the activating condition is pH.

14. The use of claim 13, wherein the activating condition is an acidic pH.

15. The use of claim 14, wherein:

the AMDE is inactive at neutral pH;

the activator provides an acidic pH activating condition to activate the enzyme; and

the AMDE is active at pH 5.5.

16. Use of a cathepsin L for formulation of a medicament for sub-epidermal administration under the outerlayer of the skin for treatment of a collagen-mediated disease or condition, wherein:

the collagen-mediated disease or condition is associated with accumulated fibrous tissue rich in collagen; and

the cathepsin L is formulated in an acidic buffer providing an acidic pH activating condition for administration, whereby the cathepsin L is active for a limited time following sub-epidermal administration to degrade the collagen component of the ECM in the fibrous of the skin.

17. The use of claim 14 or claim 16, wherein the pH is an acidic pH that is or is about in a range of 3 to 6.5, or 3.5 to 6, or 3 to 5.5, or 3 to 4.5, or 4 to 6, or 4 to 5.5, or 4.5 to 5, or 5 to 6.

18. The use of claim 17, wherein the pH is about or is 3, 3.5,4,4.5, 5, 5.5,6 or 6.5.

19. The use of claim 14, wherein the activator provides the acidic pH activating condition as a buffered solution at the pH.

20. The use of claim 16 or claim 19, wherein the buffer contains an acid selected from among 2-(N-morpholino)ethanesulfonic acid (MES), acetic acid, citric acid, citric phosphate and histidine.

21. The use of claim 16 or claim 19, wherein the ionic strength of the buffer is selected such that the AMDE is active for a predetermined time at neutral pH.

22. The use of claim 21, wherein the predetermined time is or is about 1 minute, 2 minutes, 3 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 1 hour, 2 hours, 3 hours or 4 hours.

23. The use of claim 15, wherein the AMDE is a lysosomal enzyme.

24. The use of claim 15, wherein the AMDE is a cathepsin or a heparanase.

25. The use of claim 24, wherein the AMDE is a cathepsin and the cathepsin is a cysteine protease or aspartic protease.

26. The use of claim 25, wherein the cathepsin is selected from among cathepsin B, cathepsin F, cathepsin H, cathepsin K, cathepsin L, cathepsin V and cathepsin D.

27. The use of claim 26, wherein the cathepsin has a sequence of amino acids selected from any of SEQ ID NOS: 65, 71, 183,68,60, 1, 189 and 90, or is an allelic, or species variant or other variant of any of SEQ ID NOS: 65,71, 183,68,60, 1, 189 and 90.

28. The use of claim 26, wherein the cathepsin is cathepsin L.

29. The use of claim 16 or claim 28, wherein the cathepsin L enzyme is a single chain form.

30. The use of claim 16 or claim 28, wherein the cathepsin L enzyme is a two chain form.

31. The use of claim 16 or claim 28, wherein the cathepsin L enzyme comprises:

a heavy chain having the sequence of amino acids set forth as amino acids 1-175 of SEQ ID NO:1 or a sequence that exhibits at least 90% sequence identity to the sequence of amino acids 1-175; and

a light chain having the sequence of amino acids set forth as amino acids 179-220 of SEQ ID NO:1 or a sequence that exhibits at least 90% sequence identity to the sequence of amino acids 179-220.

32. The use of claim 16 or claim 28, wherein the cathepsin L has the sequence of amino acids set forth in SEQ ID NO: 1 or is an allelic, species or other variant thereof.

33. The use of claim 1, wherein the AMDE is a mature protein that is a single chain or two chain form.

34. The use of claim 11, wherein:

the AMDE is modified to be temperature sensitive, whereby the AMDE is active at a temperature les than 37°C; the activator provides an activating condition that is a temperature less than 37°C;

the AMDE is inactive at 37°C.

35. The use of claim 11, wherein the activating condition is temperature and the temperature is or is about 20 °C, 21 °C, 22 °C, 23 °C, 24 °C, 25 °C, 26 °C, 27 °C, 28 °C, 29 °C or 30 °C.

36. The use of claim 34, wherein the AMDE is selected from among a pancreatic elastase, an elastase-2A, an elastase-2B, a neutrophil elastase, a proteinase-3, an endogenous vascular elastase, a cathepsin G, a mast cell chymase, a mast cell tryptase, a plasmin, a thrombin, a granzyme B, a cathepsin S, a cathepsin K, a cathepsin L, a cathepsin B, a cathepsin C, a cathepsin H, a cathepsin F, a cathepsin O, a cathepsin R, a cathepsin V, a cathepsin W, a calpain 1, a calpain 2, a legumain, a cathepsin Z, a cathepsin D, a cathepsin E, an MMP-1, an MMP-8, an MMP-13, and MMP-18, an MMP-2, an MMP-9, an MMP-3, an MMP-10, an MMP-11, an MMP-7, an MMP-26, an MMP-12, an MMP-14, an MMP-15, an MMP-16, and MMP-17, an MMP-19, an MMP-20, an ADAMTS-1, an ADAMTS-2, an ADAMTS-3, an ADAMTS-4, an ADAMTS-5, an ADAMTS-14 and a heparanse or allelic or species variants or other variants thereof.

37. The use of claim 34, wherein the activator is a cold buffer.

38. The use of claim 34, wherein the activator is provided in the same composition as the AMDE or in a separate composition.

39. The use of claim 11, wherein the activating condition is a metal ion and the metal ion is Ca2+.

40. The use of claim 1, wherein the one or more ECM components is selected from among a collagen, an elastin, a fibronectin or a proteoglycan.

41. The use of claim 16 or claim 40, wherein the ECM component is collagen and the collagen is selected from among type I, type II, type III or type IV collagen.

42. The use of claim 16 or claim 40, wherein the collagen is type I collagen.

43. The use of claim 1, wherein the disease or condition of the ECM is a collagen-mediated disease or condition.

44. The use of claim 16 or claim 43, wherein the collagen-mediated disease or condition is selected from among cellulite, Dupuytren's disease, Peyronie's disease, Ledderhose fibrosis, stiff joints, existing scars, scleroderma, lymphedema and collagenous colitis.

45. The use of claim 44, wherein the collagen-mediated disease or condition is stiff joints that is frozen shoulder.

46. The use of claim 44, wherein the collagen-mediated disease or condition is existing scars that is selected from among surgical adhesions, keloids, hypertrophic scars and depressed scars.

47. A pharmaceutical composition, comprising an activatable matrix-degrading enzyme (AMDE) formulated for sub-epidermal administration for use in temporally degrading a component of the extracellular matrix (ECM) for treating a disease or condition of the ECM,, wherein:

the AMDE is formulated in an amount sufficient, when exposed to an activator, to degrade a component of the ECM associated with the disease or condition;

the AMDE is a matrix-degrading enzyme that is inactive in the ECM following sub-epidermal administration in the absence of the activator; and

the AMDE is active for a limited time in the ECM following sub-epidermal administration upon exposure to an activating condition provided by the activator.

48. The composition of claim 47, wherein the composition is for single dosage administration, and contains an amount of AMDE that is or is about 10 μg to 100 mg, 50 μg to 75 mg, 100 μg to 50 mg, 250 μg to 25 mg, 500 μg to 10 mg, 1 mg to 5 mg or 2 mg to 4 mg.

49. The composition of claim 47, wherein the enzyme is substantially inactive at neutral pH.

50. The composition of claim 47 that is formulated for subcutaneous administration, intramuscular administration, intralesional administration or intradermal administration.

51. The composition of claim 47, wherein the activator is formulated in the same composition as the AMDE or in a separate composition.

52. The composition of claim 47, wherein the AMDE is selected from among a cathepsin, a calpain, and a heparanase.

53. The composition of claim 52, wherein the AMDE is a cathepsin and the cathepsin is a cysteine protease or aspartic protease.

54. The composition of claim 53, wherein the cathepsin is selected from among cathepsin S, cathepsin K, cathepsin L, cathepsin B, cathepsin C, cathepsin H, cathepsin F, cathepsin O, cathepsin R, cathepsin V, cathepsin W, cathepsin D, and cathepsin E.

55. The composition of claim 54, wherein the cathepsin is cathepsin L.

56. The composition of claim 53, wherein the cathepsin has a sequence of amino acids selected from any of SEQ ID NOS: 57,60, 1,65, 180,68,71,74,77, 183, 186, 189, 80, 195, 90, 93 and 96, or is an allelic, or species variant or other variant of any of SEQ ID NOS: 57, 60, 1, 65, 180, 68, 71,74,77, 183, 186, 189, 80, 195, 90, 93 and 96.

57. The composition of claim 47, wherein the activating condition is selected from among pH, ionic strength, temperature and metal ions.

58. The composition of claim 57, wherein the AMDE requires more than one activating condition for activity.

59. The composition of claim 57, wherein the activating condition is pH.

60. The composition of claim 59, wherein the activating condition is an acidic pH.

61. The pharmaceutical composition of claim 60, wherein:

the AMDE is inactive at neutral pH;

the activator provides an acidic pH activating condition to activate the enzyme; and

the AMDE is active at pH 5.5.

62. A pharmaceutical composition, comprising cathepsin L formulated for sub-epidermal administration under the outerlayer of the skin for treatment of a collagen-mediated disease or condition, wherein:

the collagen-mediated disease or condition is associated with accumulated fibrous tissue rich in collagen; and

the cathepsin L is formulated in an acidic buffer providing an acidic pH activating condition for administration, whereby the cathepsin L is active for a limited time following sub-epidermal administration to degrade the collagen component of the ECM in the fibrous of the skin.

63. The composition of claim 60 or claim 62, wherein the pH is an acidic pH that is or is about in a range of 3 to 6.5, or 3.5 to 6, or 3 to 5.5, or 3 to 4.5, or 4 to 6, or 4 to 5.5, or 4.5 to 5, or 5 to 6.

64. The composition of claim 63, wherein the pH is about or is 3, 3.5, 4, 4.5, 5, 5.5, 6 or 6.5.

65. The composition of claim 60, wherein the activator provides the acidic pH activating condition as a buffered solution at the pH.

66. The composition of claim 62 or claim 65, wherein the buffer contains an acid selected from among 2-(N-morpholino)ethanesulfonic acid (MES), acetic acid, citric acid, citric phosphate and histidine.

67. The composition of claim 62 or claim 65, wherein the ionic strength of the buffer is selected such that the AMDE is active for a predetermined time at neutral pH.

68. The composition of claim 67, wherein the predetermined time is or is about 1 minute, 2 minutes, 3 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 1 hour, 2 hours, 3 hours or 4 hours.

69. The composition of claim 61, wherein the AMDE is a lysosomal enzyme.

70. The composition of claim 61, wherein the AMDE is a cathepsin or a heparanase.

71. The composition of claim 70, wherein the AMDE is a cathepsin and the cathepsin is a cysteine protease or aspartic protease.

72. The composition of claim 71, wherein the cathepsin is selected from among cathepsin B, cathepsin F, cathepsin H, cathepsin K, cathepsin L, cathepsin V and cathepsin D.

73. The composition of claim 72, wherein the cathepsin has a sequence of amino acids selected from any of SEQ ID NOS: 65, 71, 183, 68, 60, 1, 189 and 90, or is an allelic, or species variant or other variant of any of SEQ ID NOS: 65, 71, 183, 68, 60, 1, 189 and 90.

74. The composition of claim 72, wherein the cathepsin is cathepsin L.

75. The composition of claim 47, wherein the AMDE is a mature protein that is a single chain or two chain form.

76. The composition of claim 62 or claim 74, wherein the cathepsin L enzyme comprises:

a heavy chain having the sequence of amino acids set forth as amino acids 1-175 of SEQ ID NO:1 or a sequence that exhibits at least 90% sequence identity to the sequence of amino acids 1-175; and

a light chain having the sequence of amino acids set forth as amino acids 179-220 of SEQ ID NO:1 or a sequence that exhibits at least 90% sequence identity to the sequence of amino acids 179-220.

77. The pharmaceutical composition of claim 62 or claim 74, wherein the cathepsin L has the sequence of amino acids set forth in SEQ ID NO: 1 or is an allelic, species or other variant thereof.

78. The pharmaceutical composition of claim 57, wherein:

the AMDE is modified to be temperature sensitive, whereby the AMDE is active at a temperature less than 37°C;

the activator provides an activating condition that is a temperature less than 37°C ; and

the AMDE is inactive at 37°C.

79. The pharmaceutical composition of claim 78, wherein the activating condition is temperature and the temperature is or is about 20° C, 21° C, 22° C, 23° C, 24° C, 25° C, 26° C, 27° C, 28° C, 29° C or 30° C.

80. The pharmaceutical composition of claim 78, wherein the AMDE is selected from among a pancreatic elastase, an elastase-2A, an elastase-2B, a neutrophil elastase, a proteinase-3, an endogenous vascular elastase, a cathepsin G, a mast cell chymase, a mast cell tryptase, a plasmin, a thrombin, a granzyme B, a cathepsin S, a cathepsin K, a cathepsin L, a cathepsin B, a cathepsin C, a cathepsin H, a cathepsin F, a cathepsin O, a cathepsin R, a cathepsin V, a cathepsin W, a calpain 1, a calpain 2, a legumain, a cathepsin Z, a cathepsin D, a cathepsin E, an MMP-1, an MMP-8, an MMP-13, and MMP-18, an MMP-2, an MMP-9, an MMP-3, an MMP-10, an MMP-11, an MMP-7, an MMP-26, an MMP-12, an MMP-14, an MMP-15, an MMP-16, and MMP-17, an MMP-19, an MMP-20, an ADAMTS-1, an ADAMTS-2, an ADAMTS-3, an ADAMTS-4, an ADAMTS-5, an ADAMTS- 14 and a heparanse or allelic or species variants or other variants thereof.

81. The pharmaceutical composition of claim 78, wherein the activator is a cold buffer.

82. The pharmaceutical composition of claim 78, wherein the activator is provided in the same composition as the AMDE or in a separate composition.

83. The composition of claim 57, wherein the activating condition is a metal ion and the metal ion is Ca2+.

84. The pharmaceutical composition of claim 47, wherein the one or more ECM components is selected from among a collagen, an elastin, a fibronectin or a proteoglycan.

85. The pharmaceutical composition of claim 84, wherein the ECM component is collagen and the collagen is selected from among type I, type II, type III or type IV collagen.

86. The pharmaceutical composition of claim 47, wherein the disease or condition of the ECM is a collagen-mediated disease or condition.

87. The pharmaceutical composition of claim 62 or claim 86, wherein the collagen-mediated disease or condition is selected from among cellulite, Dupuytren's disease, Peyronie's disease, Ledderhose fibrosis, stiff joints, existing scars, scleroderma, lymphedema and collagenous colitis.

88. The pharmaceutical composition of claim 87, wherein the collagen-mediated disease or condition is stiff joints that is frozen shoulder.

89. The pharmaceutical composition of claim 87, wherein the collagen-mediated disease or condition is existing scars that is selected from among surgical adhesions, keloids, hypertrophic scars and depressed scars.

90. A combination, comprising:

an activatable matrix-degrading enzyme (AMDE), wherein the AMDE is a matrix-degrading enzyme that is inactive in the ECM following sub-epidermal administration in the absence of an activator;

an activator that provides an activating condition for the AMDE such that the AMDE is active when exposed to the activator; and

one or more other agents selected from among other biologics, small molecule compounds, dispersing agents, anesthetics and vasoconstrictors.

91. The combination of claim 90, wherein the activator provides an activating condition selected from among pH, metal ions, temperature and ionic strength.

92. The combination of claim 90, wherein the AMDE is selected from among a pancreatic elastase, an elastase-2A, an elastase-2B, a neutrophil elastase, a proteinase-3, an endogenous vascular elastase, a cathepsin G, a mast cell chymase, a mast cell tryptase, a plasmin, a thrombin, a granzyrne B, a cathepsin S, a cathepsin K, a cathepsin L, a cathepsin B, a cathepsin C, a cathepsin H, a cathepsin F, a cathepsin O, a cathepsin R, a cathepsin V, a cathepsin W, a calpain 1, a calpain 2, a legumain, a cathepsin Z, a cathepsin D, a cathepsin E, an MMP-I, an MMP-8, an MMP-13, and MMP-18, an MMP-2, an MMP-9, an MMP-3, an MMP-10, an MMP-1 I, an MMP-7, an MMP-26, an MMP-12, an MMP-14, an MMP-15, an MMP-16, and MMP-17, an MMP-19, an MMP-20, an ADAMTS-1, an ADAMTS-2, an ADAMTS-3, an ADAMTS-4, an ADAMTS-5, an ADAMTS- 14 and a heparanse or allelic or species variants or other variants thereof.

93. The combination of claim 90, wherein the AMDE in the combination is for single dosage administration, and contains an amount of AMDE that is about 10 μg to 100

mg, 50 μg to 75 mg, 100 μg to 50 mg, 250 μg to 25 mg, 500 μg to 10 mg, 1 mg to 5 mg, or 2 mg to 4 mg.

94. The combination of claim 90, wherein the other agent is a dispersing agent that is a hyaluronidase.

95. The combination of claim 94, wherein the hyaluronidase is glycosylated.

96. The combination of claim 94, wherein the hyaluronidase is a PH20, or a truncated form thereof.

97. The combination of claim 96, wherein the PH20 is selected from an ovine, bovine or human PH20.

98. The combination of claim 94, wherein the hyaluronidase is soluble.

99. The combination of claim 98, wherein the hyaluronidase has the sequence of amino acids set forth in SEQ ID NOS:226-231, or is an allelic or species variant or other variant thereof.

100. The combination of claim 94, wherein the hyaluronidase is a soluble hyaluronidase provided as a composition designated rHuPH20.

101. The combination of claim 94, wherein the hyaluronidase in the combination is for single dosage administration, and contains an amount of hyaluronidase that is at or about 10 Units to 500,000 Units, 100 Units to 100,000 Units, 500 Units to 50,000 Units, 1000 Units to 10,000 Units, 5000 Units to 7500 Units, 5000 Units to 50,000 Units, or 1000 Units to 10,000 Units.

102. The combination of claim 90, wherein the other agent is an anesthetic that is a lidocaine.

103. The combination of claim 102, wherein the lidocaine in the combination is for single dosage administration and contains an amount of lidocaine that is at or about 10 mg to 1000 mg, 100 mg to 500 mg, 200 mg to 400 mg, 20 mg to 60 mg, or 30 mg to 50 mg.

104. The combination of claim 90, wherein the other agent is a vasoconstrictor that is an alpha adrenergic receptor agonist.

105. The combination of claim 104, wherein the alpha adrenergic receptor agonist is selected from among levonordefrin, epinephrine and norepinephrine.

106. The combination of claim 104, wherein the alpha adrenergic receptor agonist is epinephrine in an amount to effect vasoconstriction.

107. The combination of claim 106, wherein the epinephrine in the combination is for single dosage administration, and contains an amount of epinephrine that is at or about 10 μg to 5 mg, 50 μg to 1 mg, 50 μg to 500 μg, 50 μg to 250 μg, 100 μg to 500 μg, 200 μg to 400 μg, 1 mg to 5 mg or 2 mg to 4 mg.

108. The combination of claim 90 for use in treating a disease or condition of the ECM.

109. The combination of claim 108, wherein the AMDE is cathepsin L and the disease or condition of the ECM is cellulite.

110. A pharmaceutical composition, comprising an amount of an cathepsin L for treatment of an ECM-mediated disease or condition, wherein:

the composition is formulated for single dosage administration; and

the composition is formulated in an acid buffer that has a pH that activates the cathepsin L.

111. The composition of claim 110, wherein the amount of cathepsin L in the composition is or is about 10 μg to 100 mg, 50 μg to 75 mg, 100 μg to 50 mg, 250 μg to 25 mg, 500 μg to 10 mg, 1 mg to 5 mg, or 2 mg to 4 mg.

112. The composition of claim 110, further comprising one or more of lidocaine, epinephrine and a hyaluronidase.

113. A combination, comprising cathepsin L and a hyaluronidase.

114. The combination of claim 113, wherein the hyaluronidase is a composition designated rHuPH20.

115. The combination of claim 113, wherein the cathepsin L and the hyaluronidase are in separate compositions or are in a single composition.

116. The combination of claim 113, further comprising an acidic buffer that activates the cathepsin L.

117. The combination of claim 113, wherein the cathepsin L and the hyaluronidase are in a single composition formulated as a pharmaceutical composition.

118. A kit, comprising the combination of claim 90 or claim 113, and optionally instructions for use.

119. A container, comprising two compartments, wherein:

a first compartment contains a therapeutically effective amount of an activatable matrix-degrading enzyme (AMDE), wherein:

the AMDE is a matrix-degrading enzyme that is inactive in the ECM following sub-epidermal administration in the absence of an activator; and

the amount is for single dosage or a plurality of dosages and a single dosage is effective for treatment of a disease or condition of the ECM when exposed to an activator; and

a second compartment contains an activator, wherein the activator provides an activating condition required for activity of the enzyme .

120. The container of claim 119, wherein the enzyme is substantially active at pH 5.5.

121. The container of claims 119, wherein the enzyme is substantially inactive at neutral pH.

122. The container of claim 119, further comprising a mixing compartment.

123. The container of claim 119 that is a tube or bottle.

124. The container of claim 119 that is a syringe.

125. The container of claim 124, wherein the syringe further compris a needle for injection.

126. The container of claim 119, wherein:

the activator provides an activating condition selected from among pH, metal ion or ionic strength; and

the activator is provided in a liquid solution or suspension.

127. The container of claim 126, wherein the liquid solution is a buffer.

128. The container of claim 126, wherein the activating condition is pH that is acidic.

129. The container of claim 128, wherein the pH is or is about in a range of 3 to 6.5, or 3.5 to 6, or 3 to 5.5, or 3 to 4.5, or 4 to 6, or 4 to 5.5, or 4.5 to 5, or 5 to 6.

130. The container of claim 128,wherein the pH is about or is 3, 3.5, 4, 4.5, 5, 5.5, 6 or 6.5.

131. The container of claim 127, wherein the activator is an acidic buffered solution.

132. The container of claim 131, wherein the buffer contains an acid selected from among 2-(N-morpholino)ethanesulfonic acid (MES), acetic acid, citric acid, citric phosphate and histidine.

133. The container of claim 132, wherein the ionic strength of the buffer is selected such that the AMDE is active for a predetermined time upon administration.

134. The container of claim 126, wherein the activating condition is a metal ion and the metal ion is Ca^{2+}.

135. The container of claim 119, wherein the AMDE is selected from among a pancreatic elastase, an elastase-2A, an elastase-2B, a neutrophil elastase, a proteinase-3, an endogenous vascular elastase, a cathepsin G, a mast cell chymase, a mast cell tryptase, a plasmin, a thrombin, a granzyme B, a cathepsin S, a cathepsin K, a cathepsin L, a cathepsin B, a cathepsin C, a cathepsin H, a cathepsin F, a cathepsin O, a cathepsin R, a cathepsin V, a cathepsin W, a calpain 1, a calpain 2, a legumain, a cathepsin Z, a cathepsin D, a cathepsin E, an MMP-1, an MMP-8, an MMP-13, and MMP-18, an MMP-2, an MMP-9, an MMP-3, an MMP-10, an MMP-11, an MMP-7, an MMP-26, an MMP-12, an MMP-14, an MMP-15, an MMP-16, and MMP-17, an MMP-19, an MMP-20, an ADAMTS-1, an ADAMTS-2, an ADAMTS-3, an ADAMTS-4, an ADAMTS-5, an ADAMTS- 14 and a heparanse or allelic or species variants or other variants thereof.

136. The container of claim 119, wherein the AMDE is selected from among a cathepsin, calpain and a heparanase.

137. The container of claim 119, wherein the AMDE is a lysosomal enzyme.

138. The container of claim 119, wherein the AMDE is a mature protein that is a single chain or two chain form.

139. The container of claim 136, wherein the AMDE is a cathepsin and the cathepsin is a cysteine protease or aspartic protease.

140. The container of claim 139, wherein the cathepsin is selected from among cathepsin S, cathepsin K, cathepsin L, cathepsin B, cathepsin C, cathepsin H, cathepsin F, cathepsin O, cathepsin R, cathepsin V, cathepsin W, cathepsin D, and cathepsin E.

141. The container of claim 140, wherein the cathepsin has a sequence of amino acids selected from any of SEQ ID NOS: 57,60, 1,65, 180, 68,71, 74, 77, 183, 186, 189, 80, 195, 90, 93 and 96, or is an allelic, or species variant or other variant of any of SEQ ID NOS: 57, 60, 1, 65, 180, 68, 71, 74,77, 183, 186, 189, 80, 195, 90,93 and 96.

142. The container of claim 119, wherein the AMDE and activator are provided in an amount for multiple dosage administrations.

143. The container of claim 119, wherein the AMDE and activator are provided in a single unit dosage.

144. The container of claim 143, wherein the AMDE in the container is or is about 10 μg to 100 mg, 50 μg to 75 mg, 100 μg to 50 mg, 250 μg to 25 mg, 500 μg to 10 mg, 1 mg to 5 mg, or 2 mg to 4 mg.

145. The container of claim 119, wherein the total volume of liquid in the container is or is about 1 -100 ml, 1 -50 ml, 10- 50 ml, 10-30 ml, 1-20 ml, or 1-1 0 ml.

146. The container of claim 119, wherein the AMDE is in solution or suspension.

147. The container of claim 119, wherein the AMDE is lyophilized.

148. A container, comprising two compartments, wherein:

a first compartment contains a therapeutically effective amount of cathepsin L, wherein the amount is effective for treatment of a disease or condition of the extracellular matrix (ECM); and

a second compartment contains an acidic buffer that is one that activates the cathepsin L.

149. The container of claim 148, wherein the pH of the acidic buffer has a range that is or is about 4.5 to 6.

150. The container of claim 148, wherein the pH of the acidic buffer is or is about 4.5, 5, 5.5 or 6

151. The container of claim 148, wherein the buffer contains an acid selected from among 2-(N-morpholino)ethanesulfonic acid (MES), acetic acid, citric acid, citric phosphate and histidine.

152. The container of claim 151, wherein the ionic strength of the buffer is selected such that the AMDE is active for a predetermined time upon administration.

153. The container of claim 148, wherein the cathepsin L has a sequence of amino acids set forth in SEQ ID NO: 1, or is an allelic, or species variant or other variant of SEQ ID NO: 1.

154. The container of claim 148, wherein the cathepsin L is lyophilized.

155. The container of claim 148, wherein the cathepsin L in the container is for single dosage administration, and contains an amount of cathepsin L that is or is about 10 μg to 100 mg, 50 μg to 75 mg, 100 μg to 50 mg, 250 μg to 25 mg, 500 μg to 10 mg, 1 mg to 5mg, or 2 mg to 4 mg.

156. The container of claim 148, wherein the total volume in the container is or is about 1-100 ml, 1-50 ml, 10-50 ml, 10-30 ml, 1-20 ml, or 1-10 ml.

157. The container of claim 148, wherein the disease or condition of the ECM is a collagen-mediated disease or condition selected from among cellulite, Dupuytren's disease, Peyronie's disease, Ledderhose fibrosis, stiff joints, existing scars, scleroderma, lymphedema and collagenous colitis

158. The container of claim 148, wherein the disease or condition of the ECM is cellulite.

159. A combination, comprising:

the container of claim 148 ; and

one or more additional containers containing another pharmacologically effective agent selected from among biologics, small molecule compounds, dispersing agents, anesthetics and vasoconstrictors and combinations thereof.

160. The combination of claim 159, wherein the pharmaceutically effective agent is a dispersing agent that is a hyaluronidase.

161. The combination of claim 160, wherein the hyaluronidase is PH20.

162. The combination of claim 160, wherein the hyaluronidase is soluble.

163. The combination of claim 162, wherein the hyaluronidase has a sequence of amino acids set forth in SEQ ID NOS:226-23 1, or is an allelic or species variant or other variant thereof.

164. The combination of claim 160, wherein the hyaluronidase is glycosylated.

165. The combination of claim 160, wherein the hyaluronidase in the container is for single dosage administration, and contains an amount of hyaluronidase that is at or about 10 Units to 500,000 Units, 100 Units to 100,000 Units, 500 Units to 50,000 Units, 1000 Units to 10,000 Units, 5000 Units to 7500 Units, 5000 Units to 50,000 Units, or 1000 Units to 10,000 Units.

166. The combination of claim 159, wherein the pharmaceutically effective agent is an anesthetic that is lidocaine and the lidocaine in the container is for single dosage administration, and contains an amount of lidocaine that is at or about 10 mg to 1000 mg, 100 mg to 500 mg, 200 mg to 400 mg, 20 mg to 60 mg, or 30 mg to 50 mg.

167. The combination of claim 159, wherein the pharmaceutically effective agent is a vasoconstrictor that is an alpha adrenergic receptor agonist.

168. The combination of claim 167, wherein the alpha adrenergic receptor agonist is selected from among levonordefrin, epinephrine and norepinephne.

169. The combination of claim 168, wherein the alpha adrenergic receptor agonist is epinephrine in an amount to effect vasoconstriction.

170. The combination of claim 169, wherein the epinephne in the container is for single dosage administration, and the amount or epinephrine is at or about 10 μg to 5 mg, 50 μg to 1 mg, 50 μg to 500 μg, 50 μg to 250 μg, 100 μg to 500 μg, 200 μg to 400 μg, 1 mg to 5 mg or 2 mg to 4 mg.

171. The combination of claim 159, wherein the pharmacologically effective agents are provided in separate containers.

172. The combination of claim 159, wherein:

the additional container comprises at least two pharmacologically effective agents; and

the agents are provided as separate compositions separated from each other.

173. The combination of claim 159, wherein the pharmacologically effective agents are provided in the same container in the same composition.

174. The combination of claim 159, wherein the additional container contains a hyaluronidase, lidocaine and epinephrine.

175. The combination of claim 159, wherein the additional container(s) is a syringe.

176. The combination of claim 159, wherein the total volume in the additional container(s) is or is about 1-100 ml, 1-50 ml, 10-50 ml, 10-30 ml, 1-20 ml, or 1-10 ml.

177. A kit, comprising the container of claim 119, a device for administration and, optionally instructions for administration.

178. A kit, comprising the combination of claim 159, a device for administration and, optionally instructions for administration.