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INTERAÇÃO FUNCIONAL ENTRE O RECEPTOR DO PEPTÍDEO LIBERADOR DE GASTRINA E A VIA DE SINALIZAÇÃO DO AMP CÍCLICO/PROTEÍNA QUINASE A: UM ESTUDO *IN VITRO* E *IN VIVO*

CAROLINE BRUNETTO DE FARIAS

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Orientador: Prof. Dr. Rafael Roesler

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ÍNDICE

LISTA DE ABREVIATURAS

BB: Bombesina (*bombesin)*

BRS3: Receptor preferencial para o subtipo 3 de bombesina *(bombesin-like receptor 3)*

COX2: Cicloxigenase 2 (c*yclooxygenase 2*)

DAG: Diacilglicerol (d*iacylglycerol*)

ERK: Quinase reguladora de sinal extracelular (*extracellular signal-regulated kinases)*

FAK: *Focal-adhesion kinase (*quinase de adesão focal)

GPCR: Receptores acoplados a proteína G *(G protein-coupled receptors)*

GRP: Peptídeo liberador de gastrina (*gastrin-releasing peptide)*

GRPR: Receptor preferencial ao peptídeo liberador de gastrina (*gastrin releasing peptide receptor)*

IP3: Inositol 1,4,5-trifosfato (*inositol 1,4,5-trisphosphate*)

MAPK: Proteína quinase mitógeno- ativada (m*itogen-activated protein kinases)*

NMB: Neuromedina B (*neuromedin B*)

NMBR: Receptor preferencial a neuromedina B (*neuromedin B receptor*)

NMC: Neuromedina C (*neuromedin C*)

PGE2: Prostaglandina E2 (*prostaglandin E2*)

PI3K: Fosfatidilinositol 3 quinase (*phosphoinositide 3-kinases*)

PIP2: Fosfatidilinositol (4,5)-bifosfato (*phosphatidylinositol (4,5)-bisphosphate*)

PKC: Proteína quinase C (*protein kinase C*)

PLA2: Fosfolipase A2 (*phospholipase A2*)

PLC-β: Fosfolipase C- β (*phospholipase C- β*)

ROCK: Proteína quinase serina-treonina associada à proteína Rho (*Rho-associated serine-threonine protein kinase)*

RESUMO

Muitas evidências demonstram que o peptídeo liberador de gastrina (GRP) é um fator de crescimento que afeta funções neuroendócrinas, incluindo proliferação e diferenciação celular, comportamento alimentar, formação de memória, respostas a estresses, desenvolvimento de neoplasias, desordens neurológicas e psiquiátricas. Porém, os eventos moleculares pelos quais isso ocorre ainda não são totalmente compreendidos.

No presente estudo, nós avaliamos as interações entre o receptor do peptídeo liberador de gastrina (GRPR) e a via de sinalização celular da PKA, tanto na proliferação celular de glioblastoma humano (*in vitro)* quanto na consolidação da memória no hipocampo de ratos Wistar (*in vivo*).

Mostramos que o GRP age em sinergismo com agentes que estimulam a via do cAMP/PKA, promovendo a proliferação de células de glioblastoma humano, pois o tratamento com GRP combinado com um ativador de adenilil ciclase (AC), forskolin, ou um análogo de cAMP, 8-Br-cAMP, ou um inibidor do tipo IV de fosfodiesterase, rolipram, aumentaram a proliferação das células de U-138MG, quando avaliadas pelo método de MTT. Nenhum destes compostos teve efeito sozinho. O mRNA de GRPR e a expressão protéica em U-138MG foram detectados pelas técnicas de RT-PCR e imuno-histoquímica.

No estudo *in vivo a* bombesina em baixas doses induziu um aumento na consolidação da memória. O resultado foi potencializado na combinação com um ativador do receptor de dopamina D1/D5 (D1R), além de ser prevenido quando combinado com um inibidor da via da PKA.

Os resultados sugerem que GRP e GRPR interagem com a via de sinalização cAMP/PKA tanto na estimulação da proliferação celular em linhagem de câncer humano quanto na modulação da memória no hipocampo de ratos.

Palavras-chave

Bombesina • Peptídeo liberador de gastrina • Receptor do peptídeo liberador de gastrina • cAMP • Proteína quinase A • Glioblastoma • Tumores cerebrais • Hipocampo • Consolidação de memória

ABSTRACT

Increasing evidence indicates that gastrin-releasing peptide (GRP) acts as an autocrine growth factor for brain tumors as well as been implicated in memory formation, however, underlying molecular events are poorly understood. In the present study, we examined interactions between the GRPR and cellular signaling pathways in influencing memory consolidation in the hippocampus and on proliferation of glioblastoma cell *in vitro*.

We show here that GRP acts synergistically with agents that stimulate the cAMP/PKA pathway to promote proliferation of human gliobastoma cells. Treatment with GRP combined with the adenylyl cyclase (AC) activator forskolin, the cAMP analog 8-Br-cAMP, or the phosphodiesterase type IV (PDE4) inhibitor rolipram increased proliferation of U138-MG cells in vitro measured by MTT assay. None of the compounds had an effect when given alone. GRP receptor (GRPR) mRNA and protein expression in U138-MG cells was detected by reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry. We investigated the interactions between the GRPR and the PKA pathway in male Wistar rats. BB-induced enhancement of consolidation was potentiated by co infusion of activators of the dopamine D1/D5 receptor (D1R) pathway and prevented by a PKA inhibitor.

The results suggest that GRP and the GRPR interact with the cAMP/PKA signaling pathway in stimulating a cancer cell line proliferation and in memory modulation by hippocampal.

Key words

Bombesin-like peptides • Gastrin-releasing peptide • Gastrin-releasing peptide receptor • cAMP signaling • Protein Kinase A • Glioblastoma • Brain tumor • Hippocampus • Memory consolidation

1 INTRODUÇÃO

1.1 Peptídeo Liberador de Gastrina

O peptídeo bombesina (BB) foi originalmente isolado da pele da rã *Bombina bombina* (ANASTASI *et al*., 1971). Outros três peptídeos homólogos a bombesina foram posteriormente descobertos: o peptídeo liberador de gastrina (GRP), neuromedina C (NMC) e neuromedina B (NMB) (McDONALD *et al*., 1979).

Estes peptídeos foram classificados de acordo com a região COOH-terminal (KROOG *et al*., 1995; PRESTON *et al*., 1996). O GRP e NMC têm uma leucina como penúltimo resíduo da região C terminal enquanto NMB tem um fenilalanina como penúltimo resíduo (SHIN *et a*l., 2006).

O peptídeo liberador de gastrina é o homólogo humano de bombesina (ver figura 1). O GRP possui 27 aminoácidos e é codificado por um gene que está localizado no cromossomo 18 (SCOTT *et al.,* 2004).

Bombesina

Pyr-Gln-Arg-Leu-Gly-Asn-Gln-**Trp-Ala-Val-Gly-His-Leu-Met-NH2**

GRP

Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-**Trp-Ala-Val-Gly-His-Leu-Met-NH2**

Figura 1: Seqüências de aminoácidos de bombesina e GRP. As porções C-terminais de bombesina e GRP, marcadas em negrito, são idênticas (SUNDAY *et al*., 1988).

Em humanos três subtipos de receptores que se ligam aos peptídeos da família da bombesina foram identificados: o receptor preferencial ao peptídeo liberador de gastrina (GRPR), receptor preferencial a neuromedina B (NMBR) e receptor para o subtipo 3 de bombesina (BRS3) (CASSANO *et al*., 2001).

De acordo com Patel e colaboradores (2004), o GRP se liga a GRPR com alta afinidade e a BRS3 com baixa afinidade.

O GRPR é um receptor transmembrana com 384 aminoácidos, é codificado por um gene que está localizado no cromossomo X (Xp22) e, pertencente à família dos receptores acoplados a proteína G com sete domínios transmembrana (BENYA *et al*., 2000; XIAO *et al*., 2001). (Figura 2).

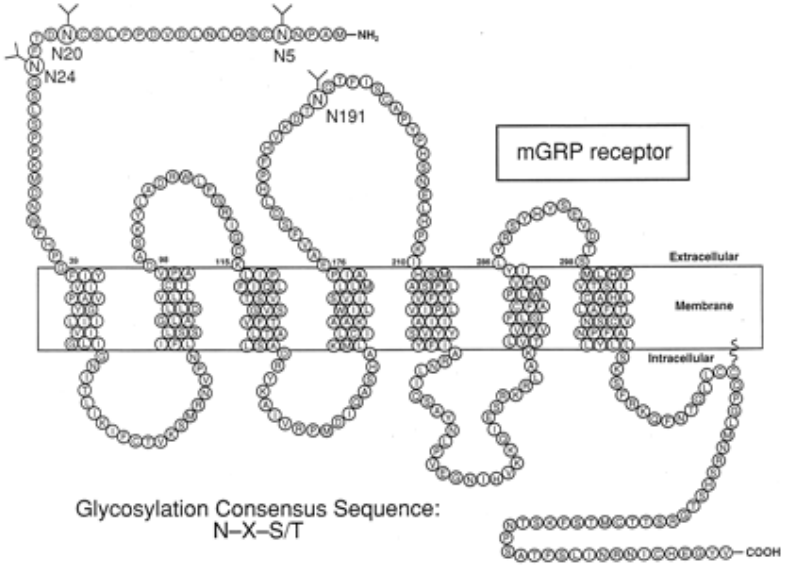

Figura 2. Estrutura molecular do GRPR (BENYA *et al*., 2000).

O peptídeo liberador de gastrina é um importante fator de crescimento autócrino e parácrino (CUTTITA *et al*., 1985; KIM *et al*., 2002), pois afeta a proliferação e diferenciação celular pela ligação ao seu receptor (SCOTT *et al*.,

2004; PATEL *et al*., 2004; MOODY & MERALI, 2004; OHKI-HAMAZAKI *et al*., 2005; PATEL *et al*., 2006; ROESLER *et al*., 2006).

O GRP tem uma atividade mitogênica potente, promovendo o crescimento do tecido normal e tumoral (FRUCHT *et al*., 1991; CASANUEVA *et al*., 1996; CASSANO *et al*., 2001; SCOTT *et al*., 2007).

Radulovic e colaboradores (1991) desenvolveram um antagonista sintético para o receptor de bombesina/GRP, [DTpi6, Leu13C-(CH2NH)Leu14]BN (RC-3095). Este peptídeo foi testado contra diferentes tipos de cânceres, demonstrando ser um potente agente antitumoral (RADULOVIC *et al*., 1991; LIEBOW *et al*., 1994; CASANUEVA *et al*., 1996; SZEPESHAZI *et al*., 1997; CHATZISTAMOU *et a*l., 2001; SCHWARTSMANN *et al*., 2004; 2005; CORNÉLIO *et al*., 2007).

1.2 O Papel Fisiológico do GRP

O peptídeo liberador de gastrina foi assim denominado por estimular a liberação da gastrina *in vivo* com atividade similar a da bombesina (PRESTON *et al*., 1996).

Em humanos, a distribuição de GRP no tecido normal é restrita ao sistema nervoso central (BATTEY & WADA, 1991), células neuroendócrinas do pulmão de fetos (SCOTT *et al*., 2004), fibras nervosas no plexo mientérico do trato gastro-intestinal (MORAN *et al.,* 1988), mucosa intestinal (CHU *et al*., 1995) e glândulas mamárias (SZEPESHAZI *et al.,* 1991; PRESTON *et al*., 1996).

GRP participa da regulação de diversos processos fisiológicos no sistema nervoso central, sistema imune, pulmonar e gastro-intestinal,

(CARROLL *et al*., 1999). Este peptídeo age como um hormônio gastro-intestinal estimulando o crescimento epitelial do intestino (DEMBIŃSK *et al.,* 1991), a liberação de gastrina através de células G antrais, a secreção exócrina pancreática e modulando a motilidade gastro-intestinal (SCOTT *et al.*, 2004).

Bombesina e GRP estão envolvidos na indução da secreção de hormônios e ácidos gástricos, secreção de muco, regulação da contração do músculo esquelético, promoção de quimiotaxia, modulação neuronal, controle da temperatura corporal (MARKI *et al*., 1981), regulação do ritmo circadiano (ALBERS *et al*., 1991), saciedade (McCOY & AVERY, 1990), certos aspectos do comportamento e formação da memória (LIEBOW *et al.,* 1994; CARROLL *et al.,* 2000, CASSANO *et al*., 2001; CASANUEVA *et al*., 1996; ROESLER *et al*., 2006).

Análises imuno-histoquímicas usando anticorpos contra BB, GRP e NMB revelaram a existência de peptídeos similares altamente conservados no cérebro e em tecidos gástricos de várias espécies animais (OHKI-HAMAZAKI *et al*., 2005).

1.2.1 GRP e Memória

Bombesina e GRP são implicados na regulação de memórias emocionais, comportamento alimentar, plasticidade sináptica e funcionamento do sistema imune (CARNEY *et al.,* 1985; FLOOD & MORLEY, 1988; McCOY & AVERY, 1990; MERALI *et al.*, 2002; ROESLER *et al.,* 2004b). Além disso, são relacionados a condicionamento de medo e respostas a estresses (CRAWLEY & MOODY, 1983; MERALI *et al*., 2002; SHUMYATSKY *et al*., 2002; YAMADA *et al*., 2002a; b).

No cérebro, o GRPR está presente nos dendritos, corpo celular dos neurônios nas regiões da amígdala lateral e hipocampo dorsal (WOLF & MOODY, 1985; ZARBIN *et al.*, 1985; BATTEY & WADA, 1991; KAMICHI *et al.,* 2005)*, O* hipotálamo também possui alta densidade de receptores GRP (MOODY *et al.,* 1988*;* BATTEY & WADA, 1991; LADENHEIM *et al.,* 1990).

Administrações sistêmicas de BB ou GRP melhoram a retenção da memória (RASHIDY- POUR & RAZVANI, 1998; SANTO-YAMADA *et al*., 2001), enquanto que infusões do antagonista de GRPR na área CA1 do hipocampo ou na amígdala basolateral, imediatamente após o treino dos animais, prejudicam a retenção da memória de curta e longa duração em teste de condicionamento aversivo (ROESLER *et al*., 2003, 2004c; VENTURELLA *et al*., 2005).

Esses resultados sugerem que receptores de GRP nas áreas cerebrais estão envolvidos na formação e consolidação da memória (ROESLER *et al*., 2006).

1.3 O Papel Patológico de GRP

Recentes evidências indicam que os receptores de GRP estão relacionados a doenças neurodegenerativas e neuropsiquiátricas, incluindo Doença de Alzheimer's (AD), autismo e ansiedade (ITO *et al*., 1994; ISHIKAWA-BRUSH *et al.*, 1997; MELLER *et al*., 2004; ROESLER *et al*., 2004a; GIBSON & HUANG, 2005; ROESLER *et al*., 2006).

Alterações na densidade de GRPR e disfunções em BB têm sido mostradas em fibroblastos e leucócitos de pacientes com AD (ITO *et al*., 1994; GIBSON AND HUANG, 2005).

Martins e colaboradores (2005) mostraram que ratos tratados com o RC-3095, apresentaram indicativos de mais altos níveis de ansiedade do que os controles.

Dantas e colaboradores (2006) demonstraram que baixas doses de RC-3095 (1 μg), prejudicam a retenção da memória em tarefas de esquiva inibitória, enquanto que altas doses (10 μg na região CA1 do hipocampo dorsal) aumentam a retenção da memória. Esses diferentes efeitos podem modular circuitos neuronais distintos, alterando os resultados comportamentais (MOUNTNEY *et al*., 2006).

Além disso, GRPR é associado a processos inflamatórios tais como o desenvolvimento de sepse, pois o seu antagonista atenua a liberação de citocinas pró-inflamatórias em modelos *in vitro* e *in vivo* (DAL-PIZZOL *et al*., 2006).

O GRP também é relatado em processos neoplásicos e parece estar relacionado à promoção de tumores (PRESTON *et al*., 1996). Alguns estudos têm mostrado que tanto o GRP quanto o GRPR são expressos de forma aberrante em muitos tipos de cânceres como em glioblastomas (WADA *et al*., 1991; WANG *et al*., 1992), próstata (SCHULZ *et al*., 2006; SUN *et al*., 2000), câncer gastro-intestinal (CARROLL *et al*., 1999; 2000; KIM *et al*., 2000; REUBI *et al*., 2002; PATEL *et al*., 2006), de pulmão, (CORJAY *et al*., 1991; MOODY *et al*., 1992; SIEGFRIED *et al*., 1997; MOODY *et al*., 2006), de mama (XIAO *et al*., 2001), de ovário (SUN *et al*., 2000), neuroblastoma (SEBESTA *et al.,* 2001) e câncer de cabeça e pescoço (PRESTON *et al*., 1996; JENSEN *et al*., 2001). E GRPR pode estar vinculado a características invasivas bem como com a progressão da doença (CARROLL *et al.,* 2000; PATEL *et al*., 2004).

Scott e colaboradores (2004) sugerem que a expressão aberrante do receptor de GRP pode ser suficiente para a estimulação autócrina e parácrina de alguns carcinóides e para dirigir a proliferação das células neoplásicas.

Em células que expressam alto número de GRPR, há uma desensitização crônica do receptor quando comparado às células que expressam pouco GRPR (MILLAR & ROZENGURT, 1990).

Bombesina, GRP e NMB atuam como um fator de crescimento tumoral (KROOG *et al*., 1995; PRESTON *et al*., 1996), promovendo a proliferação celular e aderência das células tumorais na matriz extracelular (GLOVER *et al*., 2004).

RC-3095 tem se mostrado eficaz na diminuição da progressão de lesões pré-malignas bem como no crescimento e diferenciação celular *in vitro* e *in vivo*, incluindo cânceres gástricos, colorretais, pancreáticos, de mama, de próstata e de pulmão (SZEPESHAZI *et al.,* 1991; 1992; PINSKI *et al*, 1994a; 1994b; LIEBOW *et al*., 1994; MAHMOUD *et al.,* 1991; HALMOS & SCHALLY, 1997; KOOPAN *et al*, 1998; KIARIS *et al.,* 1999; DORSAM & GUTKIND, 2007).

1.3.1 Glioblastoma Multiforme e GRP

Os tumores do sistema nervoso central (SNC) podem ser classificados de acordo com o tipo histológico, localização e grau de malignidade (PIZZO & POLACK, 1993). Muitos destes tumores são derivados de células precursoras dos astrócitos, incluindo os glioblastomas multiformes (GBM) (COTRAN, 2000).

Os tumores cerebrais são a terceira causa de morte relacionada a cânceres em adultos e a segunda em crianças (PARKER *et al.,* 1997), sendo

os glioblastomas o tipo mais comum e incurável (HOCHBERG & PRUITT, 1980).

GMB são compostos por múltiplos tipos celulares, apresentando uma heterogeneidade em termos morfológicos, agressividade, potencial de invasividade e sensibilidade à radiação (SAXENA *et al.,* 1999; BRANDES, *et al.,* 2000).

Estes tumores são altamente malignos, podendo desenvolver-se em qualquer parte do cérebro ou medula espinhal, entretanto apresentam maior incidência no lobo frontal, temporal e parietal. Os pacientes com GMB tendem a ter respostas insatisfatórias ao tratamento e, ao contrário dos outros tumores do SNC, ocorre recidiva da doença na grande maioria dos casos (FINLAY *et al.,* 1995; WISOFF *et al.*, 1998). Por isso, a sobrevida é baixa, variando de 12 a 15 meses após o diagnóstico (CHANG *et al.*, 1995).

A terapia convencional para este tipo de tumor compreende neurocirurgia, radioterapia local e quimioterapia, porém o tratamento clássico não tem produzido cura nos pacientes e geralmente apenas prolonga, mas não drasticamente, as taxas de sobrevida, (SZEPESHAZI *et al.*, 1991; SCHULTZ *et al*., 2005; TAKHAR *et al*., 2004). Por isso, novas modalidades de tratamento são necessárias urgentemente.

Muitas linhagens celulares derivadas de glioblastomas humanos, como em U-87MG, U-373MG, U-118, D-247MG, U-251MG, D-245MG, D-54MG, A-172MG, D-270MG, U-1242MG, SJ-S6 e SJ-G2 expressam receptores funcionais de BB e GRP (MOODY *et al*., 1989; SHARIF *et al.*, 1997), indicando que antagonistas destes receptores podem ter um valor clínico no tratamento desses tumores. E, de fato, RC-3095 inibe o crescimento celular das linhagens

de GMB, U-87MG e U373MG, após a estimulação com GRP (PINSKI *et al*., 1994b). Além disso, em modelos de enxerto não autólogo, a proliferação celular também é inibida com o uso de RC-3095 (KIARIS *et al.,* 1999). Glover e colaboradores (2004) mostraram que GRP e GRPR atuam na diferenciação de células tumorais em camundongos *knockout.*

A interação entre GRP e outros mitógenos potenciais pode ocorrer em nível de membrana celular pela modulação de receptores GRP (PRESTON *et al.*, 1996). Além disso, estudos dirigidos por Patel e colaboradores (2006) sugerem que múltiplos fatores de crescimento devem atuar em conjunto para a progressão das neoplasias.

Nos últimos anos, muitos avanços têm permitido um melhor entendimento da biologia tumoral, viabilizando assim a identificação de novos alvos terapêuticos e consecutivamente o desenvolvimento de novas drogas antitumorais com mecanismos de ação específicos (FERNANDO & HURWITZ, 2003; SCHALLY *et al.*, 2004).

1.4 Vias de Sinalização Celular Envolvidas com GRPR

Os receptores de GRP se ligam a uma proteína Gαq, ativando fosfolipase C-β (PLC-β) que cliva o fosfatidilinositol bifosfato (PIP2), resultando na produção de diacilglicerol (DAG) e inositol 1,4,5-trifosfato (IP3), elevando os níveis de cálcio intracelular e deste modo ativando cascatas de proteínas quinases (GILADI *et al*., 1993; SHUMYATSKY *et al*., 2002; ROESLER *et al.*, 2003; MOODY & MERALI, 2004; ROESLER *et al*., 2004a; 2004b; 2006; PATEL *et al*., 2004; OHKI-HAMAZAKI *et al*., 2005) (Figura 3).

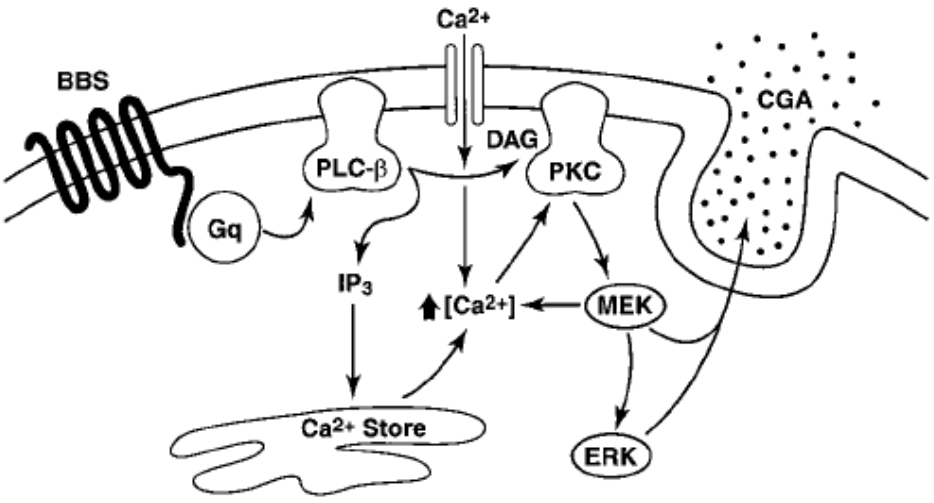

Figura 3. Modelo de via de sinalização de receptores de GRPR (HELLMICH *et al*., 1999).

Entre as vias de sinalização celular acionadas pelos receptores de GRP já foram caracterizadas a da proteína quinase ativada por mitógeno (mitogen-activated protein kinase, MAPK), proteína quinase C (protein kinase C, PKC), quinase de adesão focal (focal adhesion kinase, FAK), e proteína quinase regulada por sinais extracelulares (extracellular signal-regulated protein kinase, ERK) (APRIKIAN *et al*., 1997; HELLMICH *et al*., 1999; KIM *et al.,* 2000; QU *et al*., 2002; XIAO *et al.,* 2003; CHEN & KROOG, 2004; SCHWARTSMANN *et al.,* 2005; STANGELBERGER *et al*., 2005; THOMAS *et al*., 2005).

Os receptores de GRP, além de promoverem crescimento e proliferação também estão envolvidos na migração celular e angiogênese, pois estimulam a pequena GTPase Rho (LEFRANC *et al.,* 2002), que tem um papel central na migração celular através do estímulo da proteína ROCK (MARINISSEN & GUTKIND, 2001), e ativam a fosfolipase A2 (PLA2) e cicloxigenase 2 (COX2),

aumentando a produção de prostaglandina E2 (PGE2) (ROZENGURT *et al*., 2002) (Figura 4).

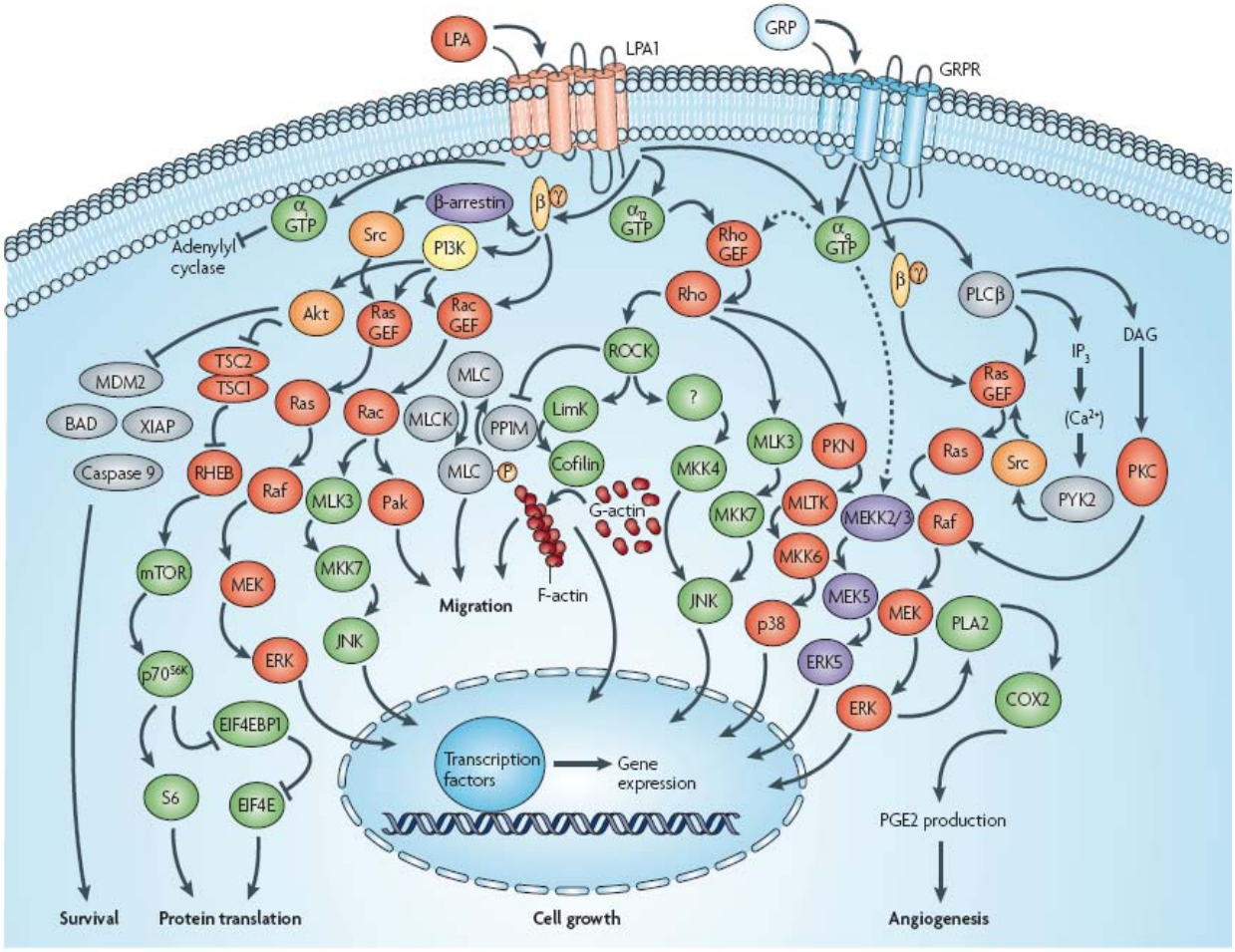

Figura 4. Vias de sinalização ativadas pelo GRPR (DORSAM & GUTKIND, 2007).

Alguns estudos demonstraram que os níveis intracelulares de cAMP são importantes na regulação do crescimento e diferenciação celulares (CHO-CHUNG & CLAIR, 1993).

Em 1977, Furman e Shulman compararam os níveis de cAMP e avaliaram a atividade de adenilil ciclase no cérebro normal e em gliomas, mostrando existir uma correlação inversa entre o grau de malignidade e os níveis de cAMP em gliomas malignos.

A interação entre GRPR e a sinalização de cAMP/PKA é controversa. Segundo Chan & Wong (2005) GRPR ativa diretamente PKC, mas não PKA. Outros autores indicam que a inibição de cAMP/PKA não afeta respostas celulares mediadas por GRP (KIM *et al.,* 2000; QU *et al*., 2002). Todavia, Qin e colaboradores descrevem que a inibição da proliferação produzida pelo bloqueio de GRPR está associado à diminuição dos níveis de cAMP em células de adenocarcinoma pancreático. Entretanto, os mecanismos de sinalização celular de cAMP/PKA envolvidos nas ações geradas por GRP e GRPRs ainda não estão totalmente esclarecidos tanto em processos fisiológicos quanto nos patológicos.

2 OBJETIVOS

Este trabalho objetiva avaliar a interação entre o GRPR e a via de AMPc/PKA envolvidos na proliferação celular de glioblastoma humano *in vitro* e no processo de consolidação de memória no hipocampo dorsal, *in vivo.*

Visando ordenar os assuntos abordados, o trabalho está apresentado na forma de dois artigos.

O Capítulo 1 trata do estudo sobre a relação da via de sinalização cAMP/PKA e GRP na proliferação celular de glioblastoma humano *in vitro.* Para isso, avaliamos os efeitos da estimulação do receptor GRP sozinho ou combinado a ativadores envolvidos com a via cAMP/PKA: forskolin, um ativador de adenilil ciclase (AC); 8-Br-cAMP, um análogo de cAMP ou; rolipram, um inibidor do tipo IV de fosfodiesterase, na linhagem de glioblastoma humano, U-138MG. Ainda, confirmamos a expressão do receptor funcional nesta linhagem.

O Capítulo 2 apresenta os experimentos relacionados ao envolvimento de bombesina com a via de sinalização cAMP/PKA na consolidação da memória no hipocampo de ratos Wistar. Para isso, utilizamos bombesina sozinha ou em adição a: forskolin, 8-Br-cAMP, RC-3095, SKF 38393 (um agonista do receptor de dopamina D1/D5 (D1R) ou Rp-cAMP (um inibidor da via da PKA). Os resultados referentes a esta dissertação estão apresentados na Figura 4 do artigo 2. Os dados mostrados nas demais figuras foram ou poderão ser apresentados em outras teses e dissertações.

3 CAPÍTULO 1

Synergistic Stimulation of Proliferation of U138-MG Glioblastoma Cells by Gastrin-Releasing Peptide in Combination with Agents that Enhance cAMP Signaling

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Laboratory Investigation

Stimulation of Proliferation of U138-MG Glioblastoma Cells by Gastrin-Releasing Peptide in Combination with Agents that Enhance cAMP Signaling

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Running head: GRP interacts with cAMP signaling in glioma cells

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Key Words

Bombesin-like peptides • Gastrin-releasing peptide • Gastrin-releasing peptide receptor • cAMP signaling • Glioblastoma • Brain tumor

Abstract

Increasing evidence indicates that gastrin-releasing peptide (GRP) acts as an autocrine growth factor for brain tumors. However, it remains unclear whether the cAMP/protein kinase A (PKA) signaling pathway plays a role in mediating the mitogenic effects of GRP. We show here that GRP combined with agents that stimulate the cAMP/PKA pathway promotes proliferation of human gliobastoma cells. Treatment with GRP combined with the adenylyl cyclase (AC) activator forskolin, the cAMP analog 8-Br-cAMP, or the phosphodiesterase type IV (PDE4) inhibitor rolipram increased proliferation of U138-MG cells in vitro measured by MTT assay. None of the compounds had an effect when given alone. GRP receptor (GRPR) mRNA and protein expression in U138-MG cells were detected by reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry. The results suggest that GRP and the GRPR interact with the cAMP/PKA signaling pathway in stimulating cancer cell proliferation.

Introduction

Gastrin-releasing peptide (GRP), a mammalian bombesin (BB)-like peptide, has emerged as a major autocrine growth factor involved in the development of a variety of human cancers, and aberrant expression of both GRP and its receptor (GRPR, also known as BB2 receptor) has been reported in many types of tumors (reviewed in [1, 2]). GRPR expression has been characterized in human glioma cells [3, 4], and GRPR activation by its agonists BB or GRP stimulates mitogenesis in several glioma cell lines [5-7].

Signal transduction mechanisms downstream of GRPR activation mediating the mitogenic effects of GRP in cancer cells are proposed to include the protein kinase C (PKC), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK), and phosphatidylinositol 3-kinase (PI3K) signaling pathways [8-16]. However, the involvement of the cAMP/protein kinase A (PKA) pathway remains controversial. The GRPR is coupled to the G_q family of G proteins, which directly activates the PKC but not the PKA pathway [17]. In addition, previous studies have indicated that inhibition of the cAMP/PKA pathway does not affect GRP-elicited cellular responses [11, 12]. However, Qin et al. [18] have described that inhibition of proliferation produced by GRPR blockade is associated with a decrease of cAMP levels in human pancreatic adenocarcinoma cells. Moreover, it remains unclear whether cAMP signaling inhibits or stimulates glioma growth. Evidence indicates that stimulators of the cAMP/PKA pathway inhibit proliferation and induce apoptosis in A-172 and C6 glioma cell lines [19-21]. In order to address these issues, in the present study

we have evaluated the effects of GRP alone or combined with stimulators of the cAMP/PKA pathway on proliferation of U138-MG human glioblastoma cells. In addition, GRPR mRNA and protein expression in U138-MG cells was assessed by reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry.

Materials and Methods

Cell culture and treatments

All experimental protocols were approved by the institutional Research Ethics Committee. The U138-MG cell line was obtained from American Type Culture Collection (Rockville, Maryland, USA). The cells were plated into 96 multiwell plates (TPP) at a density of 3 X 10^3 cell per well in sextuple, and grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Carlsbad, USA) containing 2% (w/v) H-glutamine and 15% (v/v) fetal bovine serum (FBS; Sorali, Campo Grande, Brazil). After 24 h, the cells were preincubated for 30 min with vehicle (2% dimethyl sulfoxide), the adenylyl cyclase activator forskolin (10 μM; Fluka, St. Gallen, Switzerland), the cAMP analog 8-Br-cAMP (10 μM; Sigma-Aldrich), or the phosphodiesterase type IV (PDE4) inhibitor rolipram (10 μM; Sigma-Aldrich). GRP (0.1 μM; Sigma-Aldrich, St Louis, USA) or an equivalent volume of saline (NaCl 0.9 %) was then added to the plates and cell viability was measured after 48 h. Concentrations of GRP, forskolin, 8-Br-cAMP, and rolipram were based on previous studies [5, 19, 20].

Cells were kept at a temperature of 37 °C, a minimum relative humidity of 95% and an atmosphere of 5% CO_2 in air.

MTT assay

Cell viability was measured by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich), which measures the mitochondria activity, 48 h after the treatment. The cells were washed with Hank's Balanced Salt Solution (HBSS; Invitrogen, São Paulo, Brazil) and 90µl of DMEM plus 10µl of MTT 5mg/mL solution was added to each well then were incubated for 4h at 37° C. The plate was left at room temperature until be completely dry. Dimethyl sulfoxide (DMSO) was added and the absorbance was read in 492 nm in a multiplate reader.

RT-PCR analysis of GRPR mRNA expression

Total RNA was extracted from U-138MG cells using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions, and RNA concentration was determined spectrophotometrically. The cDNA species were synthesized with Moloney murine leukemia virus (MMLV) RT (Promega, Madison, WT). The GRPR primers were designed according to the corresponding Gene Bank sequence (access NM_005314): upstream 5'-GTCAAGTCCATGCGAAACG-3' and downstream 5'-

GGGTGTCTGTCTTCACACT-3', which yield a 190-bp RT-PCR product. The presence of amplifiable mRNA was confirmed by performing RT-PCR for β-actin transcripts using the following primers, upstream GAGACCTTCAACACCCCAG and downstream GTG GTG GTG AAG CTG TAG C, which yield a 210-bp RT-PCR product. The PCR reaction was performed in a total volume of 20μL using a concentration of 0.04mM dNTPs, 0.2U Taq polymerase in the supplied reaction buffer, 0.3mM $MgCl_2$, and 10ρmol of each primer. The amplification consisted of 1 minute at 95°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension of primers at 72°C for 45 s and followed by a final extension at 72°C for 10 minutes. The products were electrophoresed through 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet illumination.

Immunohistochemistry

Expression of the GRPR protein was accessed by immunohistochemistry. The primary antibody used was a rabbit polyclonal antibody against GRPR (OPA1-15619, Affinity Bioreagents, Golden, CO, USA), corresponding to the second extracellular loop of human GRPR.

U138-MG cells were seeded in flasks of 25 cm^3. The cells grown until confluency and were then detached with a trypsin/EDTA solution. After centrifugation, the cell pellet was resuspended in 3 ml formol and embedded into paraffin wax.

Four-μm-thick sections were mounted on organosilane-coated slides and dried overnight at 37°C. Sections were deparaffinized in stove, rehydrated in

graded alcohols, and washed with distilled water. The procedure to antigenic recuperation was performed in the microwave, the inactivation of the endogenous peroxidase through immersion in hydrogen peroxide and blocking cross-reaction with normal serum. The primary antibody diluted in solution (1:50) was incubated for 12 hours, at 4°C, followed by an application of the complex biotin streptavidin-biotin-peroxidase (LSAB, Dako) and the revelation with diaminobenzidine tetrahidroclore (Kit DAB, Dako). Cells were lightly counterstained with hematoxylin-eosin as a control.

Statistics

Data are shown as mean±SEM number of cells. Differences between mean values were evaluated by one-way analysis of variance (ANOVA) followed by multiple comparisons using the least significant difference test.

Results

GRP in combination with stimulators of the cAMP/PKA pathway increases proliferation of U138-MG glioblastoma cells

Proliferation of U138-MG cells measured by MTT assay was significantly enhanced by GRP combined with forskolin, 8-Br-cAMP, or rolipram (all ps < 0.01 compared to control cells). None of the compounds significantly affected cell proliferation when given alone (fig. 1).

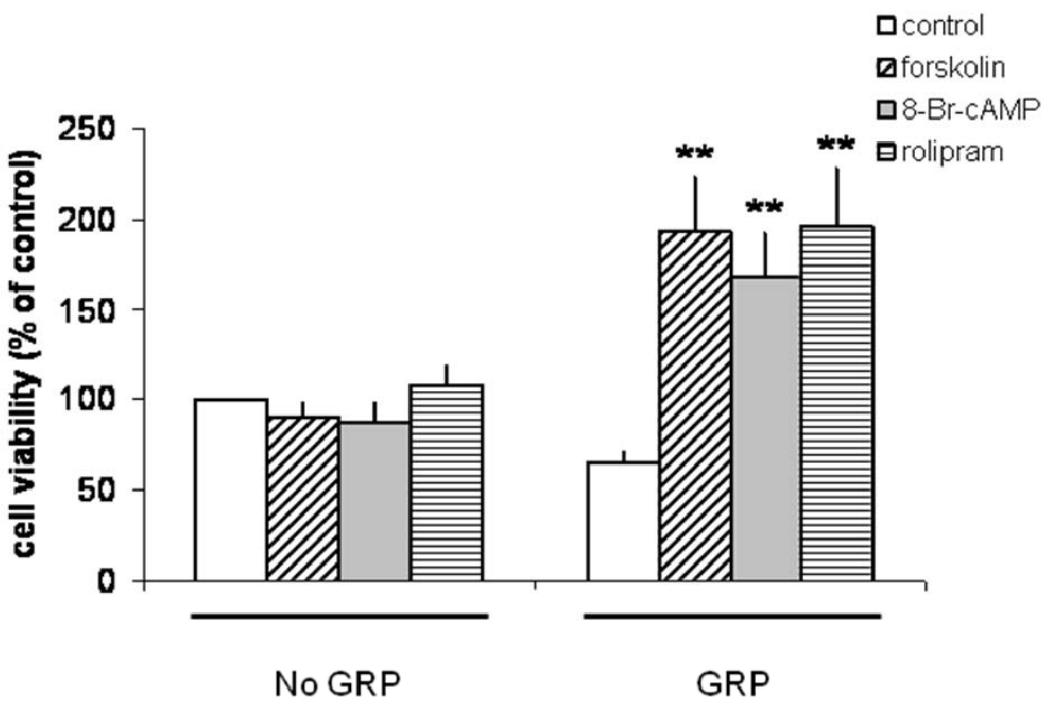

Fig. 1. Gastrin-releasing peptide (GRP) combined with stimulators of the cAMP/PKA signaling pathway enhance proliferation of U138-MG human glioblastoma cells in vitro. Cells were treated with GRP (0,1 μM), the adenylyl cyclase activator forskolin (10 μM), the cAMP analog 8-Br-cAMP (10 μM), the phosphodiesterase type IV (PDE4) inhibitor rolipram (10 μM), or GRP combined with forskolin, 8-Br-cAMP or rolipram. Cell viability was measured using MTT assay 48 h after treatment as described in Materials and Methods. Data represent the mean±S.E.M of 3 different experiments performed in sextuple wells each. The mean value for control cells was taken as 100%; ** $p < 0.01$ compared to control cells.

RT-PCR analysis of GRPR mRNA expression in U138-MG glioblastoma cells

RT-PCR analyses demonstrated that U138-MG cells express mRNA for GRPR. A transcript size of 190 bp, representing a fragment of the GRPR, was identified in the cells (fig. 2).

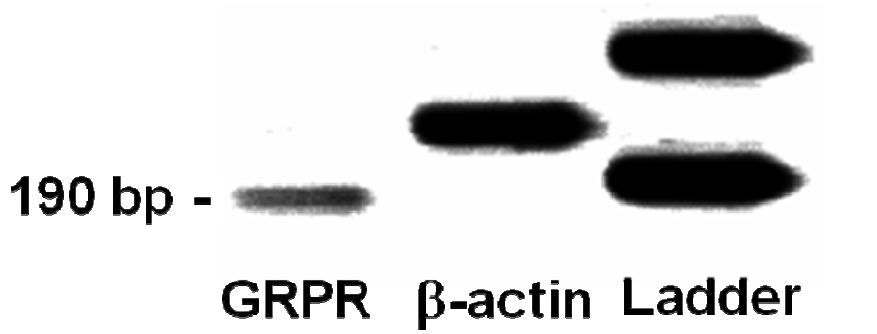

Fig. 2. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of gastrin-releasing peptide receptor (GRPR) mRNA expression in U138-MG human glioblastoma cells. RNA was extracted from U138-MG cells and RT-PCR analysis was performed as described in Materials and Methods. A transcript size of 190 bp representing a fragment of the GRPR was identified.

Immunohistochemical detection of GRPR in U138-MG glioblastoma cells

Immunohistochemical analysis using a synthetic rabbit polyclonal antibody against GRPR corresponding to the second extracellular loop of human GRPR confirmed that U138-MG cells express GRPR (fig. 3).

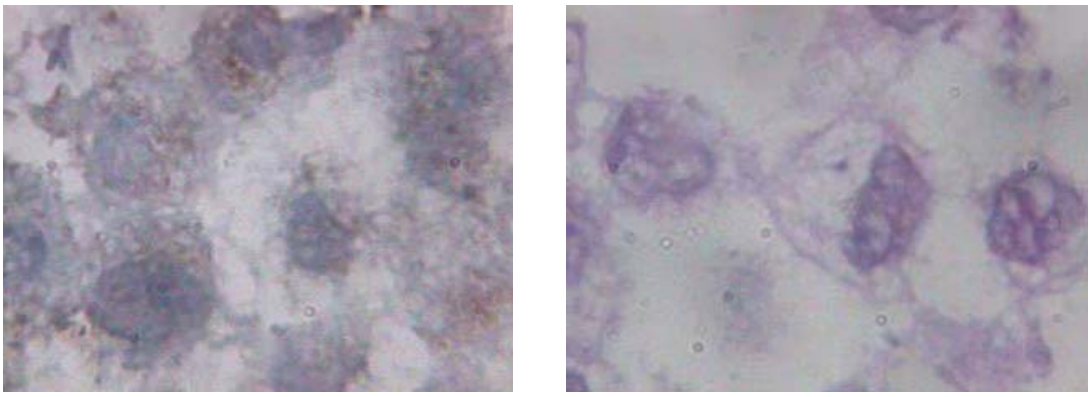

Fig. 3. Immunohistochemical analysis of gastrin-releasing peptide receptor (GRPR) expression in U138-MG human glioblastoma cells. Sections were incubated with anti-GRPR antibody, sequentially treated with biotinylated anti-rabbit IgG and streptavidin-biotin-peroxidase solution, and then developed with diaminobenzidine as chromogen (*brown/*left). Cell nuclei were lightly counterstained with hematoxilin-eosin as a control (*blue/*right).

Discussion

Previous studies have not described interactions between drugs acting at the GRPR and modulators of cAMP signaling in glioma cells, and have not examined GRPR expression and function in the U138-MG glioblastoma cell line. Our results show that GRP combined with three different activators of the cAMP/PKA signaling pathway stimulated proliferation of U138-MG cells in vitro. None of the compounds had an effect when given alone at the concentrations used in this study. In addition, we report that GRPR mRNA and protein are expressed in the U138-MG cell line. To our knowledge, these findings are the

first to suggest that the agents that stimulate GRPR and the cAMP/PKA pathway might act in synergistic or additive combination to promote glioma cell proliferation.

G-protein coupled receptors and protein kinase signaling pathways have been proposed as targets for the development of anticancer therapies (for recent reviews, see [22, 23]. The GRPR has increasingly featured as a therapeutic target in cancer [1, 2], and GRPR antagonists have been investigated as experimental anticancer drugs for the treatment of brain tumors for over 12 years [5, 24]. Thus, several studies have aimed at characterizing the intracellular molecular mechanisms mediating GRPR-triggered actions in cancer cells [11, 14, 16].

Most previous studies examining the signal transduction mechanisms underlying the effects of GRPR activation have suggested that the cAMP/PKA pathway is not involved in GRPR-triggered cellular responses [11, 12]. In addition, the role of cAMP signaling in proliferation of glioma cells remains unclear, and evidence suggests that activation of the cAMP/PKA pathway inhibits glioma cell proliferation. For instance, up-regulation of the cAMP/PKA pathway by activators including forskolin, 8-Br-cAMP, and rolipram has been shown to decrease proliferation and lead to apoptosis in the malignant glioma cell line A-172 [19, 20]. Moreover, stimulators of cAMP signaling inhibit, whereas PKA inhibition enhances, proliferation of rat C6 glioma cells [21]. In the light of this evidence, it has been proposed that stimulation of the cAMP/PKA pathway could be a therapeutic strategy for the treatment of brain tumors [19-21]. Our present report is in contrast to those findings and suggests that at least under some conditions (namely, co-activation with GRPR), stimulation of the

cAMP/PKA cascade might promote rather than inhibit proliferation of glioblastoma cells. Our finding is consistent with data indicating that inhibition of tumor growth by GRPR antagonism is related to reduced cAMP production in SW-1990 human pancreatic adenocarcinoma [18], as well as with our previous in vivo study showing that neuronal GRPR interacts with the cAMP/PKA pathway in regulating brain function in rats [25]. Future experiments should examine whether PKA inhibitors can inhibit proliferation and/or prevent stimulatory effects of GRP agonists in glioma cells.

The mechanisms mediating the effects of GRP combined with cAMP signaling stimulators might involve activation of the MAPK/ERK pathway. GRPR is coupled to the G_q family of G proteins, which directly activates the PKC but not the cAMP PKA pathway [9, 17]. Stimulation of the PKC pathway by GRPR can in turn activate MAPK [8, 9, 13, 17, 26], and the PKA and MAPK pathways can interact to promote cell proliferation (reviewed in [27]). One possibility is that a GRPR-triggered increase in $[Ca^{2+}]$ leads to stimulation of Ca^{2+}-responsive adenylyl cyclase (AC), thus further enhancing the raise in cAMP levels induced by stimulators of the cAMP/PKA pathway and leading to increased activation of the MAPK/ERK pathway. Chan and Wong [17] have shown that a rise in $[Ca^{2+}]$ elicited by GRPR stimulation leads to increased AC activity and cAMP levels in COS-7 cells, and MAPK/ERK activity is synergistically enhanced by Ca^{2+} and activators of the cAMP/PKA pathway in neurons [28].

It should be noted that treatment with GRP alone at the concentration used in this study failed to increase proliferation of U138-MG cells. If anything, it induced a decrease of about 35% in cell viability, although this effect did not reach statistical significance (Fig. 1; $p = 0.16$ compared to control cells).

Previous evidence has indicated that GRP only moderately (i.e., by about 15%) stimulates proliferation of the U373-MG and U87-MG [5]. In addition, the GRPR agonist bombesin alone might fail to stimulate cancer cell proliferation in cell lines that are sensitive to the inhibitory effects of GRPR antagonists [29]. It is possible that the poor stimulation of proliferation by exogenous GRP and bombesin is related to the production of endogenous GRP by the cells [5, 29]. In pilot studies using different GRP doses ranging from 0.01 and 1.0 μM, we failed to observe any significant stimulatory effect of GRP alone on stimulation of U138-MG cells (data not shown). However, further experiments using other doses of GRP are required to verify whether GRP alone can stimulate proliferation of U138-MG cells and whether these cells produce endogenous GRP.

In summary, the present study shows that the GRPR is expressed in U138-MG human glioblastoma cells; indicates that the GRPR interacts with the cAMP/PKA signaling pathway in promoting glioma cell proliferation; and suggests that activation of the cAMP/PKA pathway might play a stimulatory rather than inhibitory role in proliferation of glioma cells. Additional experiments are required to further examine the functional interactions between the GRPR and cAMP signaling in different glioma cell lines and investigate the possible role of the cAMP/PKA pathway as a target for treating malignant gliomas.

Acknowledgements

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301578/2006-0 to R.R.; the South American Office for Anticancer Drug Development (SOAD; Porto Alegre, Brazil); and the Children's Cancer Institute (ICI-RS; Porto Alegre, Brazil). Authors thank Maína Bonetti for technical assistance.

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4 CAPÍTULO 2

Molecular Mechanisms Mediating Gastrin-Releasing Peptide Receptor Modulation of Memory Consolidation in the Hippocampus

Situação: Publicado

Revista: Neuropharmacology 51 (2006) 350 - 357

Os resultados referentes a esta dissertação estão apresentados na Figura 4 do Capítulo 2. Os dados mostrados nas demais figuras foram ou poderão ser apresentados em outras teses e dissertações.

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Molecular mechanisms mediating gastrin-releasing peptide receptor modulation of memory consolidation in the hippocampus

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Abstract

Although the gastrin-releasing peptide-preferring bombesin receptor (GRPR) has been implicated in memory formation, the underlying molecular events are poorly understood. In the present study, we examined interactions between the GRPR and cellular signaling pathways in influencing memory consolidation in the hippocampus. Male Wistar rats received bilateral infusions of bombesin (BB) into the dorsal hippocampus immediately after inhibitory avoidance (IA) training. Intermediate doses of BB enhanced, whereas a higher dose impaired, 24-h IA memory retention. The BB-induced memory enhancement was prevented by pretraining infusions of a GRPR antagonist or inhibitors of protein kinase C (PKC), mitogen-activated protein kinase (MAPK) kinase and protein kinase A (PKA), but not by a neuromedin B receptor (NMBR) antagonist. We next further investigated the interactions between the GRPR and the PKA pathway. BB-induced enhancement of consolidation was potentiated by coinfusion of activators of the dopamine D1/D5 receptor (D1R)/cAMP/PKA pathway and prevented by a PKA inhibitor. We conclude that memory modulation by hippocampal GRPRs is mediated by the PKC, MAPK, and PKA pathways. Furthermore, pretraining infusion of BB prevented beta-amyloid peptide (25–35)-induced memory impairment, supporting the view that the GRPR is a target for the development of cognitive enhancers for dementia.

Keywords: Bombesin-like peptides; Gastrin-releasing peptide receptor; Beta-amyloid peptide; Cellular signaling; Hippocampus; Memory consolidation

1. Introduction

When memory is formed, new information acquired by the nervous system is at first labile and becomes subsequently stable through a process of consolidation involving long-lasting synaptic modifications. Extensive pharmacological and genetic evidence indicates that consolidation of long-term

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memory for spatial and contextual tasks in rats involves a number of neurotransmitter receptors and intracellular signal transduction pathways, as well as protein synthesis and gene expression in the CA1 area of the dorsal hippocampus (for reviews see Izquierdo and Medina, 1997; Alberini, 1999; McGaugh, 2000; Silva, 2003; Tonegawa et al., 2003).

The gastrin-releasing peptide-preferring receptor (GRPR, also known as BB2 receptor) has recently emerged as a system importantly involved in regulating memory formation (Williams and McGaugh, 1994; Rashidy-Pour and Razvani, 1998; Shumyatsky et al., 2002; Roesler et al., 2003; Santo-Yamada et al., 2003; Roesler et al., 2004b,c; Martins et al., 2005; Venturella et al., 2005). The GRPR is a member of the bombesin (BB)-like peptide receptor subfamily of G-protein coupled receptors, and is expressed in the cell surfaces of several mammalian tissues. Other subtypes of mammalian BB receptors include the neuromedin B receptor (NMBR). Within the brain, the GRPR occurs on dendrites and cell bodies of neurons in regions including the dorsal hippocampus and lateral amygdala (Wolf and Moody, 1985; Zarbin et al., 1985; Battey and Wada, 1991; Kamichi et al., 2005). GRPR activation by the amphibian peptide BB or its mammalian counterpart gastrin-releasing peptide (GRP) affects a range of cellular and neuroendocrine functions, including cell proliferation and differentiation, cancer growth, feeding behavior, and stress responses (for recent reviews, see Moody and Merali, 2004; Ohki-Hamazaki et al., 2005; Roesler et al., 2006). Recent evidence has also implicated the GRPR in neurodegenerative and neuropsychiatric disorders including Alzheimer's disease (AD), autism, and anxiety (Ito et al., 1994; Ishikawa-Brush et al., 1997; Meller et al., 2004; Roesler et al., 2004a; Gibson and Huang, 2005; for a review, see Roesler et al., 2006).

Findings from early pharmacological studies have indicated that systemic administration of GRPR agonists can improve memory retention in rodent models (Flood and Morley, 1988; Rashidy-Pour and Razvani, 1998). In addition, previous studies from our laboratory have indicated that infusions of a GRPR antagonist into either the CA1 hippocampal area or the basolateral amygdala impair consolidation of memory for aversive conditioning (Roesler et al., 2003, 2004c; Venturella et al., 2005). Furthermore, GRPR-deficient mice show altered fear conditioning and synaptic plasticity in the amygdala (Shumyatsky et al., 2002). Together, these findings indicate that GRPRs in brain areas including the dorsal hippocampus and amygdala are involved in memory formation.

The signal transduction mechanisms underlying the actions of the GRPR in the brain are poorly understood. Studies using cancer and neuroendocrine cell lines have indicated that cellular responses to GRPR activation require the protein kinase C (PKC) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) signaling pathways (Hellmich et al., 1999; Kim et al., 2000; Qu et al., 2002; Xiao et al., 2003; Chen and Kroog, 2004; Schwartsmann et al., 2005; Stangelberger et al., 2005; Thomas et al., 2005). The involvement of the cAMP/protein kinase A (PKA) pathway in mediating GRPR actions remains to be clarified (Kim et al., 2000; Qu et al., 2002). Previous studies have not examined the intracellular signaling mechanisms mediating the modulatory effects of GRPR activation on memory. In the present study, we used previously established behavioral training and hippocampal infusion procedures to investigate interactions between the GRPR and the PKC, MAPK, and PKA signaling pathways in memory consolidation in the dorsal hippocampus. We have also examined whether GRPR activation in the hippocampus alters memory impairment induced by intrahippocampal infusion of beta-amyloid peptide (Abeta) (25–35).

2. Methods

2.1. Animals

Adult male Wistar rats (220–315 g at time of surgery) from the State Health Research Foundation (FEPPS-RS) were housed five to a cage in a temperature-controlled colony room with food and water available ad libitum, and maintained on a 12-h light/dark cycle (lights on at 07:00 h). Behavioral procedures were conducted during the light phase of the cycle between 10:00 and 17:00 h. All experimental procedures were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals (NIH publication No. 80-23 revised 1996). All efforts were made to minimize the number of animals and their suffering.

2.2. Surgery

Animals were implanted under thionembutal anesthesia (30 /kg, i.p.) with bilateral 9.0-mm, 23-gauge guide cannulae aimed 1.0 mm above the CA1 area of the dorsal hippocampus as described in previous studies (Bevilaqua et al., 1997; Walz et al., 2000; Roesler et al., 2003; Quevedo et al., 2004; Venturella et al., 2005). Coordinates (anteroposterior, −4.3 mm from bregma, mediolateral, ±3.0 mm from bregma, ventral, −1.4 mm from dura) were obtained from the atlas of Paxinos and Watson (1997). Animals were allowed to recover at least 7 days after surgery.

2.3. Behavioral training

We used the single-trial step-down inhibitory avoidance (IA) conditioning as an established model of aversively motivated, hippocampus-dependent memory (Izquierdo and Medina, 1997; Taubenfeld et al., 1999; McGaugh, 2000). In IA training, animals learn to associate a location in the training apparatus with an aversive stimulus (footshock). Consolidation of long-term memory for IA in rats has been previously shown to depend on activation of a number of neurotransmitter receptors and protein kinase pathways as well as protein synthesis and gene expression in the dorsal hippocampus (Bevilaqua et al., 1997; Izquierdo and Medina, 1997; Quevedo et al., 1999; Taubenfeld et al., 1999; McGaugh, 2000; Walz et al., 2000; Quevedo et al., 2004).

The IA behavioral training and retention test procedures were described in previous reports (Bevilaqua et al., 1997; Quevedo et al., 1999, 2004; Walz et al., 2000; Roesler et al., 2003; Roesler et al., 2004b,c; Venturella et al., 2005). The IA apparatus was a 50 × 25 × 25-cm acrylic box (Albarsch, Porto Alegre, Brazil) whose floor consisted of parallel caliber stainless steel bars (1 mm diameter) spaced 1 cm apart. A 7-cm wide, 2.5-cm high platform was placed on the floor of the box against the left wall. On the training trial, rats were placed on the platform and their latency to step down on the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, rats received a 0.4-mA, 2.0-s footshock and were removed from the apparatus immediately after the footshock. A retention test trial was carried out 24 h after training. The retention test trial was procedurally identical to training, except that no footshock was presented. Step-down latencies on the retention test trial (maximum 180 s) were used as a measure of IA retention.

2.4. Drugs and infusion procedures

Intrahippocampal infusion procedures have been described in previous reports (Bevilaqua et al., 1997; Walz et al., 2000; Quevedo et al., 1999, 2004; Roesler et al., 2003; Venturella et al., 2005). At the time of infusion, a 30-gauge infusion needle was fitted into the guide cannula. The tip of the infusion needle protruded 1.0 mm beyond the guide cannula and was aimed at the CA1 area of the dorsal hippocampus. Drugs were infused during a 30-s period. After the infusion of drug or vehicle, the infusion needle was left in place for an additional minute to allow diffusion of the drug away from the needle tip.

For the first experiment, BB (0.002, 0.01, 0.05, or 0.25 μg in 0.5 μl; Sigma, St. Louis, MO, USA) was dissolved in saline (SAL, 0.9% NaCl) and infused bilaterally into the hippocampus immediately after IA training. Control animals were given a 0.5-μl bilateral infusion of SAL. For the second experiment, the GRPR antagonist [D-Tpi^{6}, Leu^{13} psi(CH_2NH)-Leu^{14}] bombesin (6–14) (RC-3095, 0.2 μg in 0.5 μl; Zentaris GmbH, Frankfurt, Germany), the NMBR antagonist BIM 23127 (0.1 μg in 0.5 μl; Sigma), the PKC inhibitor Gö 7874 (0.5 pg in 0.5 μl; Calbiochem, San Diego, USA), the MAPK kinase inhibitor PD098059 (5.0 ng in 0.5 μl; Calbiochem), the PKA inhibitor Rp-cAMPs (0.02 μg in 0.5 μl; Sigma), or vehicle (VEH, 2% dimethylsulfoxide (DMSO) in SAL; 0.5 μl) were infused bilaterally into the hippocampus 10 min prior to IA training, and BB (0.01 μg in 0.5 μl) or SAL were infused immediately after training. For the third experiment, RC-3095 (0.2 μg in 0.5 μl), the dopamine D1/D5 receptor (D1R) agonist SKF 38393 (7.5 μg in 0.5 μl; Sigma), the adenylyl cyclase (AC) stimulator forskolin (0.5 μg in 0.5 μl; Sigma), the cAMP analog 8-Br-cAMP (1.25 μg in 0.5 μl; Sigma), or the PKA inhibitor Rp-cAMPS (0.02 μg in 0.5 μl; Sigma), were infused alone or in combination with BB (0.01 μg in 0.5 μl) immediately after IA training. All drugs were dissolved in VEH. Control animals were given a bilateral 0.5-μl infusion of VEH. For the fourth experiment, BB (0.002 μg in 0.5 μl) or SAL (0.5 μl) were bilaterally infused into the hippocampus 10 min prior to IA training, and distilled water (DW, 0.5 μl) or Abeta (25–35) (0.02 μg in 0.5 μl; Sigma) were infused immediately after training. Previous experiments from our laboratory have indicated that a single intrahippocampal infusion of Abeta (25–35) at 0.02 μg induces IA memory impairment without causing significant neuronal death in the CA1 area (Luft et al., unpublished results). For all experiments, drug doses were chosen on the basis of previous studies (Bevilaqua et al., 1997; Vianna et al., 2000a,b; Walz et al., 2000; Freir et al., 2001; Roesler et al., 2003; Costello and Herron, 2004; Quevedo et al., 2004; Tsushima and Mori, 2005) and pilot experiments. Although higher doses of Gö 7874 and Rp-cAMPS have been shown to impair memory retention when infused immediately posttraining into the hippocampus in previous studies from our group (Vianna et al., 2000a,b; Quevedo et al., 2004), lower doses of those drugs were used in the present study which did not affect IA memory. Drug solutions were freshly prepared before each experiment.

2.5. Histology

Twenty-four to 48 h after behavioral testing, the animals were killed by decapitation and their brains were removed, stored in 5% formalin for at least 72 h and verified for infusion site placements as follows: 0.5 μl of a 4% methylene blue solution was infused as described above and the extension of the dye was taken as indicative of diffusion of the drugs previously given to each rat (Bevilaqua et al., 1997; Walz et al., 2000; Quevedo et al., 1999, 2004; Roesler et al., 2003; Venturella et al., 2005). Only data from animals with correct infusion sites (314 rats) were included in the final analysis (Fig. 1).

2.6. Statistics

Data are mean ± SEM retention test latencies to step-down (s). Comparisons of training and retention test step-down latencies among groups were performed using Kruskal–Wallis analysis of variance followed by Mann–Whitney *U*-tests, two-tailed, when necessary (Bevilaqua et al., 1997; Walz et al., 2000; Quevedo et al., 1999, 2004; Roesler et al., 2003; Venturella et al., 2005). In all comparisons, $P < 0.05$ was considered to indicate statistical significance.

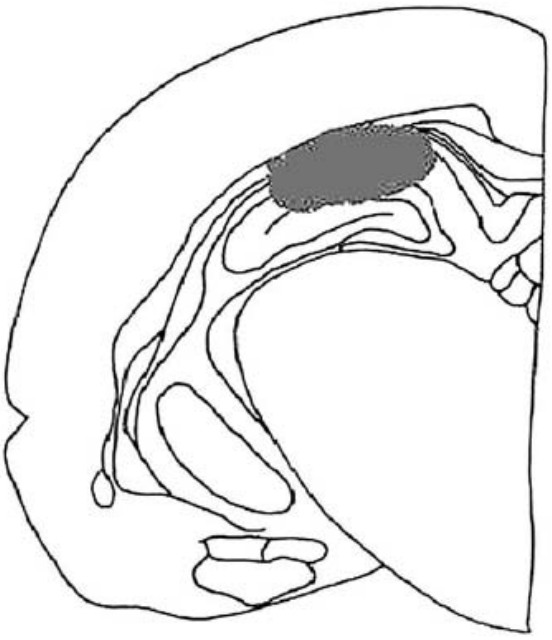

Fig. 1. Drawing of the plane A −4.3 mm of the atlas of Paxinos and Watson (1997) showing the area (hatched) where the infusion sites considered to be correct were placed.

3. Results

3.1. Bombesin modulation of memory consolidation in the hippocampus

The first experiment examined the effects of posttraining intrahippocampal infusions of BB on IA memory retention. BB at the doses of 0.01 and 0.05 μg induced a significant

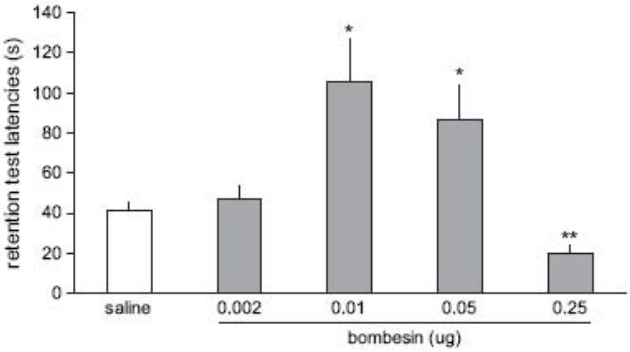

Fig. 2. Bombesin (BB)-induced modulation of memory consolidation in the hippocampus. Data are mean ± SEM 24-h retention step-down latencies (s) of rats given bilateral 0.5 μl-infusions of BB (0.002, 0.01, 0.05, or 0.25 μg) or saline (SAL) into the dorsal hippocampus immediately after inhibitory avoidance (IA) training ($n = 7–12$ animals per group). *$P < 0.05$ and ** $P < 0.01$ compared to the SAL-treated group.

enhancement of IA memory retention (both Ps <0.05 compared to the SAL-treated group), whereas BB at 0.25 μg impaired retention ($P < 0.01$ compared to the SAL-treated group) (Fig. 2). There was no significant difference among groups in training trial latencies ($P = 0.50$, overall mean ± SEM training trial step-down latency (s) was 9.14 ± 0.51). The results indicate that low and high doses of BB induce opposite effects on IA memory consolidation in the dorsal hippocampus.

3.2. Bombesin-induced enhancement of memory consolidation in the hippocampus depends on GRPRs, PKC, MAPK, and PKA

The second experiment examined the mechanisms underlying the effect of a memory-enhancing dose of BB into the hippocampus. Results are shown in Fig. 3. Posttraining infusion of BB at 0.01 μg induced significant IA retention enhancement ($P < 0.01$ compared to the control group treated with SAL and VEH). Pretraining infusions of the GRPR antagonist RC-3095, the PKC inhibitor Gö 7874, the MAPK kinase inhibitor PD 098059, or the PKA inhibitor Rp-cAMPS did not affect retention, but prevented the retention enhancement induced by posttraining BB. Animals treated with a pretraining infusion of the NMBR antagonist BIM 23127 and a posttraining infusion of BB showed a significantly higher retention than the control group treated with SAL and VEH ($P < 0.05$). There was no significant difference among groups in training trial latencies ($P = 0.27$, overall mean ± SEM training trial step-down latency (s) was 8.88 ± 0.51). The results suggest that BB-induced enhancement of memory retention in the hippocampus depends on GRPRs, PKC, MAPK and PKA, but not NMBRs.

3.3. Bombesin-induced enhancement of memory consolidation in the hippocampus is potentiated by stimulators of the D1R/cAMP/PKA pathway and prevented by a PKA inhibitor

The finding that PKA inhibition prevented BB-induced memory enhancement was somewhat unexpected because previous studies in cancer cells have indicated that PKA inhibitors do not affect the cellular effects of GRPR activation (Kim et al., 2000; Qu et al., 2002). We thus decided to further evaluate the interactions between the GRPR and the PKA pathway in the hippocampus. The third experiment examined the effects of a memory-enhancing dose of BB coinfused with stimulators of the D1R/cAMP/PKA pathway or a PKA inhibitor after IA training. Results are shown in Fig. 4. The group treated with BB alone showed a significant enhancement of IA retention ($P < 0.01$ compared with the VEH group). Coinfusion with RC-3095 prevented the BB-induced retention enhancement, indicating that the BB effect was mediated by GRPR activation. The D1R receptor agonist SKF 38393, the AC activator forskolin, and the cAMP analog 8-Br-cAMP did not affect retention when infused alone, but potentiated the memory-enhancing effect of BB. The groups treated with BB combined with SKF 38393, forskolin, or 8-Br-cAMP showed significantly higher retention latencies than the group treated with BB alone (all Ps <0.05). Infusion of an otherwise ineffective dose of the PKA inhibitor Rp-cAMPS prevented the memory-enhancing effect of BB. There was no significant difference among groups in training trial latencies ($P = 0.49$, overall mean ± SEM training trial step-down latency (s) was 7.77 ± 0.48). The results suggest that the enhancing effect of BB on IA memory retention in the hippocampus requires PKA and is potentiated by stimulation of the D1R/cAMP/PKA pathway.

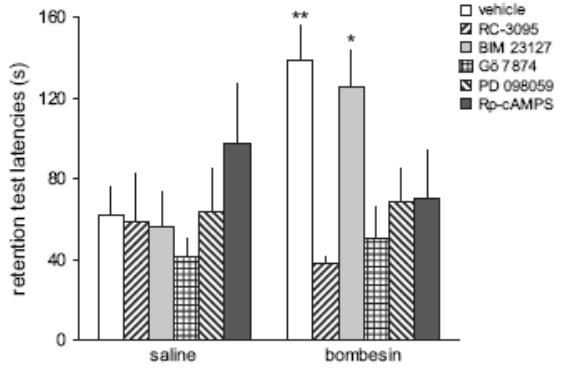

Fig. 3. Bombesin (BB)-induced enhancement of memory consolidation in the hippocampus requires gastrin-releasing peptide receptors (GRPR), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and protein kinase A (PKA). Data are mean ± SEM 24-h retention step-down latencies (s) of rats given bilateral 0.5 μl-infusions of the GRPR antagonist RC-3095 (0.2 μg), the neuromedin B receptor (NMBR) antagonist BIM 23127 (0.1 μg), the PKC inhibitor Gö 7874 (0.5 pg), the MAPK kinase inhibitor PD098059 (5.0 ng), the PKA inhibitor Rp-cAMPs (0.02 μg), or vehicle (VEH, 2% dimethylsulfoxide (DMSO) in saline (SAL)) 10 min before inhibitory avoidance (IA) training, and BB (0.01 μg in 0.5 μl) or SAL immediately after training ($n = 7$–13 animals per group). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the group treated with VEH and SAL.

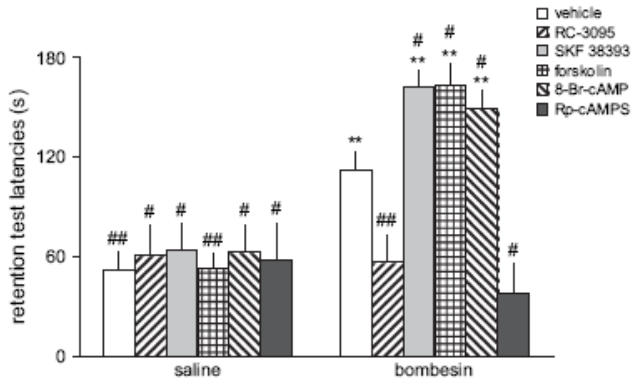

Fig. 4. Bombesin (BB)-induced enhancement of memory consolidation in the hippocampus is potentiated by activators of the dopamine D1/D5 receptor (D1R)/cAMP/protein kinase A (PKA) pathway and prevented by PKA inhibition. Data are mean ± SEM 24-h retention step-down latencies (s) of rats given bilateral 0.5 μl-infusions of the gastrin-releasing peptide receptor (GRPR) antagonist RC-3095 (0.2 μg), the D1R agonist SKF 38393 (7.5 μg), the adenylyl cyclase (AC) stimulator forskolin (0.5 μg), the cAMP analog 8-Br-cAMP (1.25 μg), the PKA inhibitor Rp-cAMPS (0.02 μg), or vehicle (VEH, 2% dimethylsulfoxide (DMSO) in saline (SAL)), alone or combined with BB (0.01 μg), immediately after inhibitory avoidance (IA) training ($n = 7$–12 animals per group). $^{**}P < 0.01$ compared to the VEH-treated group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to the group treated with BB in VEH.

3.4. Bombesin prevents beta-amyloid peptide (25–35)-induced impairment of memory consolidation in the hippocampus

Previous studies have indicated that BB-like peptides and the GRPR might be involved in the pathogenesis of AD (Ito et al., 1994; Gibson and Huang, 2005; Roesler et al., 2006). In addition, systemic administration of GRP has been shown to improve memory deficits in the scopolamine-induced amnesia model in mice (Santo-Yamada et al., 2001). Application of Abeta (25–35) to the CA1 hippocampal area in vivo and in vitro has been used as a model to investigate the impairment of synaptic plasticity associated with AD (Saleshando and O'Connor, 2000; Freir et al., 2001; Costello and Herron, 2004). Intrahippocampal and intracerebroventricular infusions of Abeta (25–35) in rats have also been used as models of cognitive impairment associated with AD (Chen et al., 1996; Stepanichev et al., 2005). The fourth experiment examined whether BB could prevent IA memory deficit induced by a single posttraining administration of Abeta (25–35) into the hippocampus. Results are shown in Fig. 5. Posttraining intrahippocampal infusion of Abeta (25–35) induced a significant impairment of IA retention ($P < 0.01$ compared to the control group given SAL and DW). Pretraining infusion of an otherwise ineffective dose of BB prevented the Abeta (25–35)-induced retention impairment. There was no significant difference among groups in training trial latencies ($P = 0.16$, overall mean ± SEM training trial step-down latency (s) was 8.20 ± 0.78). The result indicates that GRPR agonists can prevent memory impairments elicited by Abeta (25–35) in the hippocampus.

4. Discussion

The present experiments used IA behavioral training and hippocampal infusions to examine the cellular signaling mechanisms mediating the effects of BB on memory consolidation in the hippocampus. Our results can be summarized as follows: (1) lower doses of BB enhance, whereas a higher dose of BB impair consolidation of IA memory when infused posttraining into the CA1 hippocampal area; (2) the BB-induced memory enhancement in the hippocampus requires GRPRs, PKC, MAPK, and PKA, but not NMBRs; (3) the memory-enhancing effect of BB in the hippocampus is potentiated by stimulators of the D1R/cAMP/PKA pathway and prevented by PKA inhibition; and (4) BB prevents the impairment of memory consolidation induced by administration of Abeta (25–35) into the hippocampus. The use of posttraining infusions of BB rules out the possibility that the effects were due to drug-induced alterations in attentional, motivational, motor, or sensory-perceptual mechanisms at training.

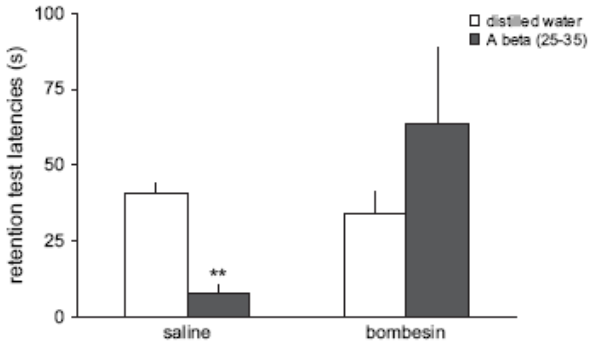

Fig. 5. Bombesin (BB) prevents beta-amyloid peptide (Abeta) (25–35)-induced impairment of memory consolidation in the hippocampus. Data are mean ± SEM 24-h retention step-down latencies (s) of rats given bilateral 0.5 μl-infusions of BB (0.002 μg) or saline (SAL) 10 min before inhibitory avoidance (IA) training, and Abeta (25–35) or distilled water (DW) immediately after training ($n = 8$–14 animals per group). **$P < 0.01$ compared to the group treated with SAL and DW.

The mammalian counterpart of BB, GRP, has been proposed to be co-released with glutamate from glutamatergic neurons, and act by binding to GRPRs on postsynaptic sites (Lee et al., 1999; Shumyatsky et al., 2002). The GRPR is expressed in neurons throughout the mammalian central nervous system, including the CA1 area of the dorsal hippocampus (Kamichi et al., 2005). Previous studies have shown that systemic administration of the GRPR agonists BB and GRP induce memory enhancement in rats and mice (Flood and Morley, 1988; Rashidy-Pour and Razvani, 1998; Santo-Yamada et al., 2001). Conversely, GRPR antagonists induce memory impairment when given systemically (Santo-Yamada et al., 2003; Roesler et al., 2004b; Martins et al., 2005) or into brain areas including the dorsal hippocampus and basolateral amygdala (Roesler et al., 2003, 2004c; Venturella et al., 2005). These findings suggest that GRPR activation plays a stimulatory role in memory formation. However, other studies have proposed that the GRPR is located predominantly on inhibitory interneurons releasing gamma-aminobutyric acid (GABA), and GRPR activation would lead to an increase in GABAergic transmission, which would in turn inhibit synaptic plasticity and memory (Lee et al., 1999; Shumyatsky et al., 2002). Consistent with this view, BB induces depolarization of inhibitory interneurons in hippocampal slices (Lee et al., 1999) and GRPR-deficient mice show enhanced fear-motivated conditioning and synaptic plasticity in the amygdala, but normal hippocampal memory (Shumyatsky et al., 2002). Thus, the role of the GRPR in hippocampal function and memory formation remains controversial. The results of the present study clearly indicate that the GRPR in the dorsal hippocampus modulates memory consolidation of an emotionally motivated, hippocampus-dependent task, and that BB at lower doses induces memory enhancement through stimulation of GRPRs in the dorsal hippocampus. How could one reconcile the present findings, together with those from other studies indicating that GRPR activation stimulates synaptic plasticity and memory, with studies suggesting that the GRPR acts as an inhibitory system? One possibility is that the GRPR is expressed on both inhibitory GABAergic neurons and excitatory glutamatergic neurons, as well as on neurons releasing other neurotransmitters such as serotonin and dopamine. Although to our knowledge there is no direct evidence for the expression of GRPRs on excitatory neurons, the recent finding

by Kamichi et al. (2005) that in the lateral amygdala only a subpopulation of cells expressing GRPRs are GABAergic neurons is consistent with the possibility that GRPRs are expressed on non-GABAergic neurons releasing glutamate or other neurotransmitters. Thus, different doses of GRPR agonists could induce differential effects on excitatory and inhibitory transmission, either stimulating or inhibiting synaptic plasticity and memory. Our finding that low and high doses of BB induced opposite effects on memory consolidation, as well as our recent observation that high doses of the GRPR antagonist RC-3095 can enhance IA memory consolidation (Dantas et al., in press) support this possibility.

Although BB-like peptides and the GRPR have been previously implicated in memory formation, previous studies have not investigated the underlying molecular mechanisms. Extensive evidence indicates that the PKC, MAPK and PKA pathways are critical in mediating memory consolidation in the hippocampus (Bevilaqua et al., 1997; Izquierdo and Medina, 1997; McGaugh, 2000; Quevedo et al., 2004). Previous studies using cancer and neuroendocrine cells have suggested that intracellular responses to GRPR activation involve a GRPR-elicited [Ca^{2+}] increase and activation of the phospholipase C (PLC)/PKC pathway, which, in turn activates the MAPK/ERK pathway. Thus, cellular responses to GRPR agonists are blocked by PKC and MAPK inhibitors (Hellmich et al., 1999; Kim et al., 2000; Qu et al., 2002; Xiao et al., 2003; Chen and Kroog, 2004; Stangelberger et al., 2005; Thomas et al., 2005). Consistent with these findings, our results clearly indicate that memory modulation by the GRPR in the hippocampus requires both PKC and MAPK. In addition, our findings indicate that memory modulation by BB was blocked by an otherwise ineffective dose of a PKA inhibitor and potentiated by activators of the PKA pathway. These findings were somewhat unexpected because the GRPR is coupled to the G_q family of G proteins, which directly activates the PKC but not the PKA pathway (Chan and Wong, 2005). In addition, previous studies have indicated that PKA inhibition does not prevent GRPR-elicited cellular responses (Kim et al., 2000; Qu et al., 2002). However, a possible role for cAMP signaling in the effects of GRPR antagonists in human pancreatic adenocarcinoma has been suggested by Qin et al. (1995), and a recent study has described a complex interaction between the GRPR and the D1R/cAMP/PKA pathway in COS-7 cells, in which co-stimulation of the GRPR and D1R inhibits GRPR-triggered protein kinase activity (Chan and Wong, 2005). Several mechanisms involved in cross-talk among the PKC, MAPK and PKA pathways could explain the requirement of PKA for BB modulation of memory consolidation. For instance, MAPK/ERK activity is synergistically enhanced by Ca^{2+} and activators of the cAMP/PKA pathway in hippocampal neurons (Impey et al., 1998). One possibility is that a GRPR-triggered increase in [Ca^{2+}] leads to stimulation of Ca^{2+}-responsive adenylyl cyclase (AC), thus further enhancing the raise in cAMP levels induced by stimulators of the D1R/cAMP/PKA pathway. This would be consistent with the model recently proposed by Chan and Wong (2005), in which a rise in [Ca^{2+}] elicited by GRPR stimulation leads to increased AC activity and cAMP levels in COS-7 cells, and also with the finding that Ca^{2+}-stimulated AC in the dorsal hippocampus plays a critical role in synaptic plasticity and long-lasting memory (Wong et al., 1999). Fig. 6 shows a schematic for a proposed model of GRPR interactions with the PKC, MAPK, and PKA pathways in regulating memory consolidation in the hippocampus.

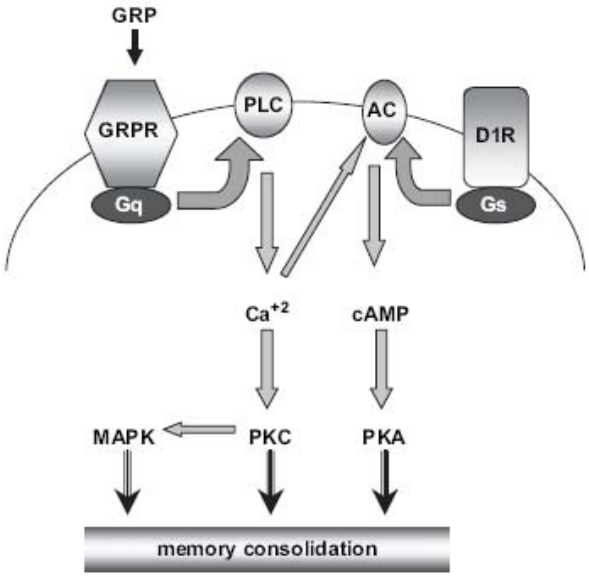

Fig. 6. Schematic diagram for a model of cellular signaling mechanisms mediating the regulatory actions of GRPR on memory consolidation in the hippocampus. Gastrin-releasing peptide (GRP) released from synaptic terminals binds to the G_q protein (G_q)-coupled GRP receptor (GRPR) at postsynaptic sites. GRPR activation induces an increase in [Ca^{2+}] and triggers activation of the phospholipase C (PLC)/protein kinase C (PKC) pathway, which, in turn, can activate mitogen-activated protein kinase (MAPK) (Hellmich et al., 1999). The dopamine D1/D5 receptor (D1R) is coupled to G_s protein (G_s) and adenylyl cyclase (AC) activation. The D1R-induced cAMP signal might be synergistically potentiated by [Ca^{2+}]-induced stimulation of [Ca^{2+}]-responsive types of AC (Wong et al., 1999; Chan and Wong, 2005), leading to increased activation of protein kinase A (PKA).

Several lines of evidence have indicated that the GRPR might be involved in cognitive dysfunctions associated with AD and other neurodegenerative and psychiatric disorders (for a review, see Roesler et al., 2006). For instance, alterations in GRPR density and dysfunctions in BB-elicited Ca^{2+} signaling have been described in fibroblasts and leucocytes from patients with AD (Ito et al., 1994; Gibson and Huang, 2005). These data, together with the present finding that BB might enhance memory retention by stimulating protein kinase pathways critically involved in mediating synaptic plasticity suggest that the GRPR could be considered a molecular target for the development of novel cognitive enhancers. Consistent with the view that GRPR agonists can display cognitive-enhancing properties in models of amnesia, Santo-Yamada et al. (2001) have shown that systemic administration of GRP attenuated scopolamine-induced memory impairment in mice. Based on previous electrophysiological (Saleshando and O'Connor, 2000; Freir et al., 2001; Costello and Herron,

2004) and behavioral (Chen et al., 1996; Stepanichev et al., 2005) experiments, in the present study we used a single intrahippocampal infusion of a low dose of Abeta (25–35) in rats as a model of memory dysfunction associated with AD. Administration of Abeta (25–35) to the hippocampus in vitro or in vivo can impair synaptic plasticity through a mechanism involving the MAPK pathway (Saleshando and O'Connor, 2000; Freir et al., 2001; Costello and Herron, 2004). Our finding that pretraining administration of an otherwise ineffective dose of BB prevented the Abeta (25–35)-induced impairment of IA retention supports the view that the GRPR is a molecular target for the development of cognitive enhancers for treatment of memory dysfunction associated with AD and other neuropsychiatric disorders.

In summary, the present results suggest that the GRPR regulates memory consolidation in the hippocampus through a mechanism involving the PKC, MAPK and PKA signaling pathways. In addition, administration of the GRPR agonist BB prevented memory impairment induced by Abeta (25–35) in the hippocampus. This is the first study investigating the molecular mechanisms mediating memory modulation by the GRPR.

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5 DISCUSSÃO

Bombesina e GRP participam da regulação de diversos processos fisiológicos tais como secreção hormonal e de ácidos gástricos (CARROLL *et al*., 1999), contração do músculo esquelético, modulação neuronal, controle da temperatura corporal (MARKI *et al*., 1981), regulação do ritmo circadiano (ALBERS *et al*., 1991), saciedade (McCOY & AVERY, 1990) e formação da memória (LIEBOW *et al.,* 1994; CARROLL *et al.,* 2000, CASSANO et al., 2001; CASANUEVA *et al*., 1996; ROESLER *et al*., 2006).

Além disso, estão associados ao desenvolvimento de doenças neurodegenerativas, neuropsiquiátricas (ITO *et al*., 1994; ISHIKAWA-BRUSH *et al*., 1997; MELLER *et al*., 2004; ROESLER *et al*., 2004a; GIBSON & HUANG, 2005; ROESLER *et al*., 2006), processos inflamatórios (DAL-PIZZOL *et al*., 2006) e neoplásicos (PRESTON *et al*., 1996).

Alguns estudos usando linhagens celulares tumorais e neuroendócrinas indicam que as respostas celulares mediadas por GRPR ativam as vias da PKC e MAPK (HELLMICH *et al*., 1999; KIM *et al*., 2000; QU *et al*., 2002; XIAO *et al*., 2003; CHEN & KROOG, 2004; SCHWARTSMANN *et al*., 2005; STANGELBERGER *et al*., 2005; THOMAS *et al*., 2005). Todavia, a interação entre GRPR e a via cAMP/PKA é bastante controversa (KIM *et al*., 2000; QU *et al*., 2002) e, os mecanismos de sinalização intracelulares e moleculares acionados por este peptídeo no cérebro e na formação da memória também não estão completamente entendidos.

É sabido que a via da PKA é importante para funções celulares tais como motilidade, adesão, interação célula a célula, captação e transdução de

sinais externos (KONDRASHIN *et al.*, 1999), entretento a relação desta via e a ativação de GRPRs ainda não está completamente entendida.

Algumas evidências sugerem que a via da PKA poderia interagir com a proliferação de células tumorais, pois ativadores de cAMP/PKA, como forskolin, 8-Br-cAMP e rolipram, diminuem a proliferação celular, aumentam a diferenciação e induzem apoptose na linhagem de glioblastoma humano, A-172 (CHEN *et al*., 1998; 2002).

Há cerca de quatro décadas, Walter e colaboradores (1977) descreveram o envolvimento de PKA na superfície externa de células de glioma de ratos, C6. Mais tarde, inibidores dessa via foram usados, resultando no aumento da proliferação celular desta mesma linhagem (HELMBRECH & RENSING, 1999).

Outros indícios mencionam que a inibição da PKA não previne mecanismos de transdução de sinal mediados por GRPRs (KIM *et al*., 2000; QU *et al*., 2002). Porém, PERRY e colaboradores (2004) indicam que a PKA parece estar relacionada à proliferação da linhagem celular, U-87MG.

No presente estudo, nós avaliamos as interações entre GRP e a sinalização mediada por cAMP/PKA, na linhagem celular de glioblastoma humano, U-138MG. Também examinamos a expressão e a funcionalidade do GRPR nestas células.

Nossos resultados, *in vitro,* mostram que GRP combinado com três diferentes ativadores da via de sinalização cAMP/PKA estimulam a proliferação de U-138MG, entretanto nenhum dos compostos avaliados teve efeito quando aplicados sozinhos nas concentrações usadas em nossoss experimentos.

Este foi o primeiro trabalho a mostrar que a co-ativação de GRPR e a cascata de sinalização de cAMP/PKA podem atuar em sinergismo para a promoção da proliferação celular em glioblastomas humanos *in vitro*.

Resultados semelhantes foram relatados por QIN e colaboradores (1995) na linhagem celular de adenocarcinoma pancreático, SW-1990, onde a inibição do crescimento tumoral foi relacionada à redução da produção de cAMP.

É importante observar, que o tratamento das células de U-138MG, apenas com GRP na concentração de 0,1 μM não estimulou a proliferação celular em comparação ao controle.

Outros estudos já haviam mostrado que GRP pode estimular apenas discretamente a proliferação celular de linhagens de gliomas, como U-87MG e U-373MG (em torno de 15%). E, a bombesina sozinha também pode falhar na indução da proliferação de células cancerígenas sensíveis aos efeitos inibitórios de antagonistas de GRPR (LIEBOW *et al.,* 1994).

Talvez essa modesta estimulação da proliferação celular induzida por GRP/BB exógenos esteja relacionada à produção celular de GRP/BB endógenos (PINSKI *et al*., 1994a; LIEBOW *et al.,* 1994*)*. Mais experimentos usando novas doses de GRP são necesários para verificar se GRP sozinho pode ou não estimular a proliferação da linhagem U-138MG e, se há produção de GRP endógeno nessas células.

Nós mostramos ainda que, tanto a proteína de GRPR quanto o mRNA são expressos em U-138MG.

Peptídeos como bombesina e GRP são listados por participarem de mecanismos de memória e aprendizado, pois administrações sistêmicas destes

peptídeos promovem melhoramento destes processos em ratos e camundongos (FLOOD & MORLEY, 1988; RASHIDY-POUR & RAZVANI, 1998; SANTO- YAMADA *et al*., 2001).

Outros estudos corroboram com esses resultados, demonstrando que administrações sistêmicas ou diretamente sobre as áreas cerebrais do hipocampo dorsal ou amígdala basolateral de antagonistas de GRPR, prejudicam a memória (SANTO-YAMADA *et al.,* 2003; ROESLER *et al.,* 2003; 2004b; 2004c; MARTINS *et al.,* 2005; VENTURELLA *et al*., 2005).

Nossos resultados *in vivo,* apontam que baixas doses de BB na área CA1 do hipocampo melhoram a consolidação da memória de ratos Wistar. Esse efeito foi potencializado pela estimulação da via D1R e prevenido pela inibição da via de sinalização da PKA em teste de condicionamento aversivo (esquiva inibitória).

É sabido que o agonista do receptor de dopamia 1 (D1R), SKF 38393, se liga a adenilil ciclase, aumentando os níveis de cAMP intracelulares (BALMFORTH et al., 1988). Um estudo recente descreveu a interação entre GRPR e a via do D1R em células de fibroblastos de macacos, COS-7, onde a ativação de GRPR e D1R inibiu a atividade de proteínas quinases (CHAN & WONG, 2005).

Kelly e colaboradores (2008) mostraram que a formação da memória requer sinalização de cAMP, pois com a ativação de subunidades α de proteínas G (Gαs) em neurônios de camundongos recém-nascidos, houve um aumento da atividade de AC, e diminuição dos níveis de cAMP no córtex e hipocampo através da ativação de PDE. Existiu um aumento compensatório pela atividade de PDE1/cAMP e uma tendência a aumento pela PDE4,

sugerindo que a inibição da atividade de PKA em camundongos pode estar relacionada ao risco de déficit de memória de curta e longa duração em tarefas de condicionamento de medo.

Com nossos resultados *in vitro* e *in vivo,* sugerimos que além das vias da PKC e MAPK, a via de sinalização cAMP/PKA também interage na regulação de funções cerebrais de ratos Wistar e na proliferação celular de glioblastomas humanos. Porém, segundo CHAN & WONG (2005), receptores de GRP/BB são acoplados a uma proteina Gq, que ativa diretamente a via da PKC, mas não a via da PKA.

Todavia, muitos mecanismos de interação entre as vias da PKC, MAPK e PKA poderiam explicar a participação da via da PKA na proliferação celular de gliomas e na modulação da consolidação da memória provocada por GRP/BB. Por exemplo, a atividade de MAPK/ERK atua em sinergismo com o aumento de Ca^{+2} e ativadores da via do cAMP/PKA em neurônios no hipocampo (IMPEY *et al*., 1998). Então, a ativação de GRPRs poderia aumentar os níveis de Ca^{+2} intracelular, causando uma estimulação da resposta de adenilil ciclase (AC) e desta forma, elevando os níveis de cAMP e induzindo a via do D1R em ambos os processos.

Monje e colaboradores (2006) investigaram os efeitos de cAMP na ativação das vias ERK e PI3K/Akt, usando culturas primárias de células de Schwann (SC). Eles mostraram que quando os níveis de cAMP são aumentados (por forskolin ou um análogo de cAMP) sinergisticamente há um aumento da fosforilação de ERK para Akt. Além disso, estudos com inibidores de MEK, superestimularam ERK e PI3K/Akt, indicando que vias mediadas por

cAMP aceleram a progressão da fase do ciclo celular G1-S por polongar a ativação de ERK e Akt.

Chan & Wong (2005) sugeriram recentemente, que a estimulação de GRPR aumentaria a atividade de AC e os níveis de cAMP em células COS-7, agindo na plasticidade sináptica no hipocampo dorsal e na memória de longa duração.

Nosso grupo propôs um modelo no qual GRPR interage tanto com as vias PKC e MAPK quanto com a via da PKA na regulação da consolidação da memória no hipocampo. O mesmo modelo pode ser usado para explicar nossos resultados na proliferação celular de glioblastomas humanos.

Acredita-se que a via da PKA é maior mediadora da transdução de sinal de cAMP em células de mamíferos (KREBS & BEAVO, 1979; BEEBE & CORBIN, 1986), por isso muitas pesquisas estão sendo desenvolvidas para melhor entender o seu funcionamento.

Portanto, cabe ressaltar que esta enzima consiste de dímeros de duas subunidades: a catalítica (C) e a regulatória (R). A ativação da PKA ocorre quando duas moléculas de cAMP se ligam em cada subunidade R, resultando na liberação das subunidades C (BEEBE & CORBIN, 1986).

A PKA é formada por duas isoformas, o tipo I (PKA-I) e o tipo II (PKA-II), que compartilham a subunidade C, mas contém diferentes subunidades R (RI e RII, respectivamente). Estudos bioquímicos e de clonagem gênica, identificaram quatro isoformas das subunidades R (RIa, RIb, RIIa e RIIb) (McKNIGHT *et al.*, 1988).

Variações nas proporções das duas isoformas estão vinculadas ao crescimento e à diferenciação celular (LOHMANN & WALTER, 1984; CHO-

CHUNG 1990; TORTORA & CIARDIELLO, 2003). Deste modo, enquanto a expressão aumentada de RI/PKA-I é relatada por ativar crescimento e transformação celular, a redução de RI/PKA-I e o aumento de RII/PKA-II são relacionadas à inibição de crescimento, diferenciação e maturação celular (CHO-CHUNG 1990; LOHMANN & WALTER, 1984).

A expressão de PKA-II é localizada principalmente em tecidos normais. Entretanto, PKA-I e/ou a subunidade RIa são encontradas em linhagens celulares neoplásicas e tumores primários. PKA-I e RIa são relacionadas à estimulação da proliferação celular, favorecendo a propagação de sinalização mitogênica, induzindo a transformação de fatores de crescimento e oncogenes, e são ainda incluídas em piores prognósticos de diferentes tipos de cânceres bem como à resistência múltipla a drogas (MILLER ET AL., 1993; BRADBURY *et al*., 1994; CHO-CHUNG *et al.,* 1995; TORTORA & CIARDIELLO, 2002).

A expressão excessiva da subunidade RIIb é bastante comum em linhagens celulares de diversos cânceres, resultando na notável mudança na distribuição, detenção de crescimento, diferenciação e transformação reversa da isoenzima da PKA (CHO-CHUNG 1990; BUDILLON *et al.,* 1995; NESTEROVA *et al.,* 1995).

Chen e colaboradores (2002) demonstraram que a apoptose foi aumentada em gliomas malignos pela ativação da isoenzima tipo II da PKA, mas não pelo tipo I.

Os resultados divergentes existentes entre a ativação e inibição da via da cAMP/PKA e sua interação com GRPR podem estar relacionados com a razão entre as subunidades RI/RII. O GRP pode estar agindo sobre a razão das subunidades da PKA, e talvez por este motivo, ele sozinho não provoque

proliferação celular em U-138MG, mas em sinergismo com ativadores da PKA isso ocorra significativamente. Por isso, para a continuidade desse estudo, é necessário dar esse novo enfoque que certamente proporcionará o melhor entendimento desses efeitos.

O uso de técnicas sofisticadas poderá auxiliar na compreensão dos resultados obtidos neste trabalho, como por exemplo, a técnica de transferência de energia de ressonância fluorescente (FRET), que permite a detecção de alterações bioquímicas rápidas em células vivas isoladas, possibilitando a visualização das mudanças de concentrações intracelulares de segundos mensageiros tais como Ca^{+2} e cAMP ou da atividade de proteínas quinases como a PKA.

Poderemos, ainda, avaliar a razão entre as subunidades regulatórias da PKA pelo uso de anticorpos específicos para RI e RII (BD Transduction Laboratories, Lexington, KY), através das técnicas de imuno-histoquímica, Western blot ou PCR em tempo real (MANTOVANI *et al*., 2005).

Novos testes serão necessários para examinar se a inibição da via da PKA sozinha ou combinada a antagonistas de GRPR será capaz de prevenir ou minimizar a proliferação celular em U-138MG.

Nos últimos anos, receptores acoplados à proteína G e vias de sinalização de proteínas quinases têm sido propostos como novos alvos terapêuticos anticâncer e contra doenças neurodegenerativas (CHEN *et al*., 1998; 2002; HELMBRECH & RENSING, 1999; PATEL *et al*., 2006; CORNÉLIO *et al.,* 2007; DORSAM & GUTKIND, 2007; ROBERTS & DER, 2007).

Em estudos clínicos de fase I, RC-3095 demonstrou baixa toxicidade em pacientes com tumores sólidos avançados e refratários, indicando ser uma

molécula segura e precursora para o tratamento de neoplasias (PINSKI *et al.*, 1994b; KIARIS *et al*., 1999; KIM *et al.*, 2000; THOMAS *et al*., 2005; SCHWARTSMANN *et al*., 2005; 2006; ZHANG *et al*., 2007). Além disso, BB, GRP e antagonistas de GRPR estão sendo propostos para ações terapêuticas no sistema nervoso central, incluindo o tratamento de desordens cerebrais (ROESLER *et al*., 2007).

Portanto, nossos resultados sugerem que a interação entre GRPR e a via de sinalização cAMP/PKA pode ser uma nova estratégia para o tratamento de tumores cerebrais e patologias relacionadas ao sistema nervoso central.

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7 APÊNDICE

CARTA DE ACEITE DO ARTIGO 1:

----- Original Message -----
From: <ocl@karger.ch>
To: rroesler@terra.com.br
Sent: Monday, December 31, 2007 11:26 AM
Subject: Ms. No. 200710038, Oncology

Oncology
Ms. No. 200710038
Title: Stimulation of Proliferation of U138-MG Glioblastoma Cells by Gastrin-Releasing Peptide in Combination with Agents that Enhance cAMP Signaling

Dear Dr. Roesler,
Thank you for submitting a revised version of your manuscript to Oncology. On behalf of the Editor-in-Chief, Dr. Maurie Markman, I am pleased to inform you that it has now been accepted for publication and forwarded to the Editing and Production Department at S. Karger AG - Medical and Scientific Publishers - who will be contacting you shortly.
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CURRICULUM VITÆ
resumido

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- Investigação de Processos Neurotóxicos Mediado pelo Glutamato em Linhagens Derivadas de Gliomas Humanos;
- Investigação de Processos Neurotóxicos Mediados pelo Glutamato;
- Investigação dos Mecanismos Moleculares Associados à Radioresistência de Gliomas Humanos;
- Investigação de Mecanismos Moleculares Associados à Progressão dos Gliomas Humanos.

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- Mecanismos de Sinalização Celular Envolvidos no Crescimento Tumoral Induzido pela Ativação de Receptores GRP em Gliomas;
- Avaliação da Atividade da Telomerase em Sangue de Pacientes com o Diagnóstico de Tumores da Família do Sarcoma de Ewing;
- Avaliação da Atividade da Telomerase em Sangue de Pacientes com o Diagnóstico de Leucemia Linfóide Aguda;
- Estudo Genético Epidemiológico em Sarcoma de Ewing: Genes Candidatos a Polimorfismo;
- Análise da Diferença de Expressão Gênica entre Mucosa Gástrica Normal, Metaplasia e Câncer Gástrico por cDNA Microarray;
- Avaliação do Efeito de Antagonistas NMDA sobre Linhagens Tumorais Pediátricas Humanas.

5. ARTIGOS COMPLETOS PUBLICADOS

FERNANDO, Augusta; FARIAS, Caroline B.; ROESLER, Rafael. e SCHWARTSMANN, Gilberto. Targeting the Epidermal Growth Factor Receptor in Colorectal Câncer: A Potential Therapeutic Role for Gastrin-Releasing Peptide Receptor Antagonist. Oncology. Letter to Editor.

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6. RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS

FARIAS, Caroline Brunetto de**; LIMA, Rodrigo Cruz; LIMA, Luciana Otero; FLORES, Débora Gazzana; BRUNETTO, Algemir Lunardi; SCHWARTSMANN, Gilberto; ROESLER, Rafael; Mecanismo De Sinalização Celular Envolvido No Crescimento Tumoral Induzido Pela Ativação De Receptores GRP Em Gliomas, FESBE, 2007

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