◆使用時のポイント
・抗がん剤の副作用である末梢神経障害を予防あるいは軽減し、発現を遅延させることができる。特に術後補助化学療法(FOLFOXおよびXELOX療法)の完遂率を高められる可能性がある。
・牛車腎気丸はオキサリプラチンの抗腫瘍効果に影響しないことが確認されている。
・独特の臭いと味があるために服薬コンプライアンスが低下することがある。特に抗がん剤などを使用している場合は、患者に十分説明することが必要である。
・牛車腎気丸は畜尿症状が強い排尿障害患者に用いる。閉塞症状を訴える場合や浮腫のない場合には八味地黄丸がよい。

◆牛車腎気丸・豆知識
牛車腎気丸は、加齢に伴う諸症状に頻用される八味地黄丸(別名：腎気丸)に牛膝(ヒナタイノコズチの根)と車前子(オオバコの種子)を加え、附子を倍量にして作用を増強したものである。"腎"は排尿経路全体を意味する。

◆文献
●薬効薬理
1) Suzuki, Y. et al. Meth Find Exp Clin Pharmacol. 1998, 20, p.321.
2) Suzuki, Y. et al. Jpn J Pharmacol. 1999, 79, p.387.
3) Tawata, M. et al. Diabetes Res Clin Pract. 1991, 26, p.121.
4) 鹿野昌彦ほか. 和漢医薬学会誌. 1988, 5, p.378.
5) 鹿野昌彦ほか. 和漢医薬学会誌. 1990, 7, p.442.
6) Ushio, S. et al. Eur J Cancer. 2012, 48, p.1407.
7) Hashimoto, K. et al. J Osaka Dent Univ. 2006, 40, p.47.
8) 鈴木孝憲ほか. 泌尿紀要. 1996, 42, p.951.
9) Gotoh, A. et al. J Pharmacol Sci. 2004, 96, p.115.
10) Nishijima, S. et al. J Urology. 2007, 177, p.762.
11) Imamura, T. et al. Neurourology Urodynamics. 2008, 27, p.832.
12) Zhang, X. et al. Am J Chin Med. 2006, 34, p.285.
●臨床効果
13) 進藤吉明ほか. 癌と化学療法. 2008, 35, p.863.
14) Kono, T. et al. Evid Based Complement Alternat Med. 2011, doi:10.1093/ecam/nep200.
15) Kono, T. et al. Jpn J Clin Oncol. 2009, 39, p.847.
16) Nishioka, M. et al. The Int J Clin Oncol. 2011, 16, p.322.
17) 深澤一昭ほか. 医療薬学. 2011, 37, p.625.
18) 関根秀明. 癌の臨床. 2005, 51, p.56.
19) 高島　勉. 癌の臨床. 2005, 51, p.58.
20) 田畑　務. 産婦人科漢方研究のあゆみ. 2006, 23, p.12.
21) 山本智也ほか. 癌と化学療法. 2009, 36, p.89.
22) 和田大樹ほか. 外科治療. 2009, 100, p.734.
23) Kaku, H. et al. Exp Ther Med. 2012, 3, p.60.
24) 阿部吉伸. 漢方医学. 2002, 25, p.284.

芍薬甘草湯(しゃくやくかんぞうとう)

国際名　shakuyakukanzoto

・タキサン系抗がん剤による末梢神経障害(筋肉痛)に有効で、頻用されている。
・急激に起こる骨格筋および平滑筋(消化管、胆道、尿路など)の痙攣とそれに伴う疼痛に有効で、速効性がある。
・筋弛緩の作用機序として、芍薬の主要成分ペオニフロリンによるカルシウムの細胞内への流入抑制、甘草の主要成分グリチルリチンによるカリウムの細胞外流出促進作用の相互作用が報告されている。
・骨格筋弛緩作用だけでなく、直接的な消化管平滑筋弛緩作用があることが報告されており、上部・下部消化管内視鏡検査時に前処置として使用している施設もある。
・肝硬変症、糖尿病性神経障害、血液透析による有痛性筋痙攣にも有効であることが報告されている。

◆構成生薬
シャクヤク(芍薬)、カンゾウ(甘草)

◆投与方法
7.5g/日を経口投与(通常食前または食間分3)
ただし、通常は頓服2.5g(1包)～5.0g(2包)で処方されることが多い。
【GF、CF検査時などの内視鏡的投与】内視鏡検査時は2.5g(1包)を250ccの微温湯で溶解し、内視鏡の鉗子口から収縮部位に直接噴霧する。抗コリン薬が使えない場合でも使用可能。

◆実地臨床における応用例
・タキサン系抗がん剤による末梢神経障害(筋肉痛・関節痛)
・化学療法による吃逆

図1　芍薬甘草湯の筋弛緩作用機序(文献15)より改変)

・消化管内視鏡検査時における消化管平滑筋異常収縮(挿入困難例、ブチルスコポラミン臭化物禁忌例)

◆現在までに得られているEBM
●薬効薬理
①筋弛緩作用
1)神経筋シナプス遮断作用[1]
2)消化管平滑筋弛緩作用[2]
②抗侵害受容(鎮痛)作用[3]
③筋疲労抑制作用[4]
④パクリタキセル投与に伴う末梢神経障害を緩和[5]

●臨床効果
①パクリタキセル投与に伴う筋肉痛を緩和[6)7)8)9)10)]
②化学療法による吃逆を改善[11]
③消化管検査の前処置に有用[12]
④大腸内視鏡検査の苦痛を軽減)[13]
⑤内視鏡的逆行性胆道膵管造影法(ERCP)による十二指腸スパスムを抑制[14]

◆使用時のポイント

・がん化学療法による末梢神経障害（筋肉痛）の予防に用いる場合は、開始2～3日前から芍薬甘草湯の投与を開始するとよい。

・グリチルリチンを主成分とする甘草を含有するため、低カリウム血症が引き起こされる場合がある。長期運用による呼吸筋、心筋などへの影響はこれまでほとんど報告されていないが、2週間以上長期連用する場合には定期的に血中カリウム値を測定することが望ましい。特に高齢者など生理機能が低下している場合は注意が必要である。

◆芍薬甘草湯・豆知識

芍薬と甘草の2生薬で構成されていることから名付けられた。漢方薬の構成生薬の数は処方ごとに異なるが、少なければ少ないほど速効性があると言われている。芍薬甘草湯は古くから腓腹筋の筋痙攣（こむら返り）に対する速効性が知られており、ゴルフ・テニスなど、急な運動をする機会には1包持参することが勧められる。

◆文献

●薬効薬理

1）Kimura, M. et al. Jpn J Pharmacol. 1984, 39, p.275.
2）Kurosawa, S. et al. Gastroenterology. 2000, 118, p.A221.
3）Omiya, Y. et al. J Pharmacol Sci. 2005, 99, p.373.
4）中井由佳ほか．和漢医薬学雑誌．1996, 13, p.356.
5）Hidaka, T. et al. Euro J Pain. 2009, 13, p.22.

●臨床効果

6）Yamamoto, K. et al. Gynecol Oncol. 2001, 81, p.333.
7）山本嘉一郎ほか．産婦人科漢方研究のあゆみ．2001, 18, p.101.
8）長谷川幸清ほか．癌と化学療法．2002, 29, p.569.
9）藤井和之ほか．癌と化学療法．2004, 31, p.1537.
10）Yoshida, T. et al. Support Care Cancer. 2009, 17, p.315.
11）松波馨士ほか．漢方医学．2012, 36, p.50.
12）Ai, M. et al. World J Gastroenterol. 2006, 12, p.760.
13）新井一信ほか．日本東洋医学雑誌．1994, 44, p.385.
14）Sakai, Y. et al. J Nat Med. 2009, 63, p.200.
15）木村正康ほか．漢方医学．2011, 35, p.154.

六君子湯（りっくんしとう）

国際名　rikkunshito

・上部消化管手術後および抗がん剤治療に伴う諸種の原因による食欲不振、胃部不快感、胸やけ、嘔吐、下痢といった消化管機能異常に使用されている。

・多施設二重盲検群間比較試験にて運動不全型NUD（non-ulcer dyspepsia）、すなわち現在は機能性ディスペプシア（FD）と呼ばれる胃腸症に対する臨床的有用性が証明されている。

・作用機序として、食欲亢進ホルモンであるグレリンの分泌促進作用が報告されており、食欲不振の改善効果が特に期待されている。

・胃食道逆流症（GERD）に有効であることは知られているが、小児のGERD患者の食道クリアランス改善作用が報告され、小児科領域でも注目されている。

◆構成生薬

ソウジュツ（蒼朮）、ニンジン（人参）、ハンゲ（半夏）、ブクリョウ（茯苓）、タイソウ（大棗）、チンピ（陳皮）、カンゾウ（甘草）、ショウキョウ（生姜）

◆投与方法

7.5g/日を経口投与（通常食前または食間分3）

◆実地臨床における応用例

・抗がん剤による悪心・食欲不振
・術後の機能性ディスペプシア（FD）
・胃食道逆流症（GERD）

◆現在までに得られているEBM

●薬効薬理

図1　六君子湯の薬理作用

①消化管運動に対する作用
　1）消化管運動促進作用[1)]
　2）胃排出能促進作用[2) 3)]
　3）胃適応性弛緩に対する作用[4) 5) 6) 7)]
②胃粘膜保護作用[8)]
③胃粘膜血流改善作用[9) 10)]
④食道クリアランス改善作用[11)]
⑤食道粘膜バリア機能改善作用（タイトジャンクション蛋白減少改善作用）[12)]
⑥グレリンに対する作用
　1）分泌に関する作用[13) 14) 15) 16) 17) 18) 19)]
　2）受容体に関する作用[20) 21)]
　3）代謝酵素に対する作用[22)]
⑦ストレスに対する作用[17) 18)]

●臨床効果

①がん化学療法施行患者での食欲不振を改善[23)]
②がん化学療法施行患者での悪心とQOLを改善[24)]

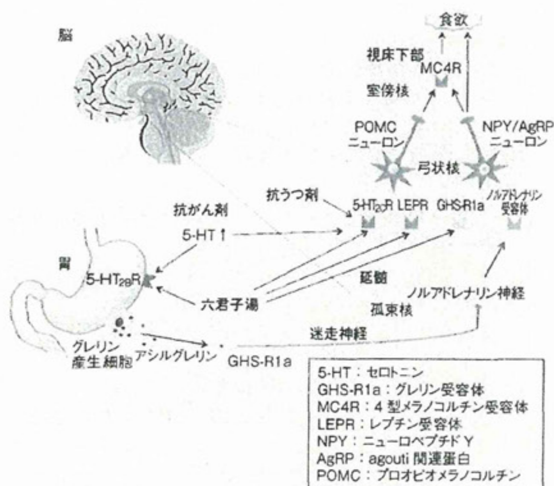

図2　六君子湯によるグレリン分泌機序(文献38)より改変)

③PPI抵抗性GERD患者の症状を改善[25) 26)]
④FD患者のグレリン分泌を促進[27)]
⑤幽門輪温存胃切除術後の停滞症状を改善[28)]
⑥SSRI投与による消化管症状の発生を低下[29)]
⑦胃切除後の逆流性食道炎の予防に有効[30)]
⑧咽喉頭逆流症(LPRD)の咽喉頭症状を改善[31) 32)]
⑨神経性食欲不振症患者の消化器症状(食欲不振、もたれなど)を改善[33) 34)]
⑩ストレス誘発性胃部膨満感抑制効果[35)]
⑪NSAIDsによる消化器症状を改善[36)]
⑫小児の消化器外科術後の上腹部不定愁訴を改善[37)]

◆使用時のポイント
・化学療法や機能性ディスペプシア(FD)による食欲不振を中心とした胃部症状、特に食後早期の満腹感に有効である。
・嘔気・嘔吐で食物摂取や服用が困難な場合は、溶かした六君子湯をアイスボール・氷漢方にして服用してもらう方法もある(他の処方でも応用可能)。
・慢性疾患、病後、手術後などによる体力低下、食欲不振には補中益気湯を用いるとよい。

◆六君子湯・豆知識
「君子危うきに近寄らず」と言われるように"君子"は人格者の意味であり、消化器系に働く重要な6つの生薬が配合されていることで「六君子湯」の名が付いたとされているが、日本で独自に発展し、8つの生薬が配合されている点は興味深い。

◆文献
●薬効薬理
1) 村岡　均ほか. 日本東洋医学雑誌. 1992, 43, p.255.
2) Kido, T. et al. J Pharmacol Sci. 2005, 98, p.161.
3) Kawahara, H. et al. Pediatr Surg Int. 2009, 25, p.987.
4) Hayakawa, T. et al. Drugs Exp Clin Res. 1999, 25, p.211.
5) 楠　裕明. 日本東洋心身医学研究. 2007, 22, p.5.
6) Kusunoki, H. et al. Intern Med. 2010, 49, p.2195.
7) Kito, Y. et al. Am J Physiol Gastrointest Liver Physiol. 2010, 298, p.G755.
8) Arakawa, T. et al. Drugs Exp Clin Res. 1999, 25, p.207.
9) 川合　満ほか. Ther Res. 1993, 14, p.2061.

10) Kurose, I. et al. Pathophysiology. 1995, 2, p.153.
11) Kawahara, H. et al. Pediatr Surg Int. 2007, 23, p.1001.
12) Miwa, H. et al. J Gastroenterol. 2010, 45, p.478.
13) Takeda, H. et al. Gastroentrology. 2008, 134, p.2004.
14) Fujitsuka, N. et al. Biol Psychiatry. 2009, 65, p.748.
15) Yakabi, K. et al. Regulatory Peptides. 2010, 161, p.97.
16) Yakabi, K. et al. Endocrinology. 2010, 151, p.3773.
17) Saegusa, Y. et al. Am J Physiol Endocrinol Metab. 2011, 301, p.E685.
18) Yakabi, K. et al. Am J Physiol Endocrinol Metab. 2011, 301, p.E72.
19) Matsumura, T. et.al. J Gastroenterol. 2010, 45, p.300.
20) Takeda, H. et al. Endocrinology. 2010, 151, p.244.
21) Fujitsuka, N. et al. Translational Psychiatry. 2011, 1, p.e23.
22) Sadakane, C. et al. Biochem Biophys Res Commun. 2011, 412, p.506.
●臨床効果
23) Ohno, T. et al. Clin Exp Gastroenterol. 2011, 4, p.291.
24) Seike, J. et al. Int J Surg Oncol. 2011, doi:10.1155/2011/715623.
25) Koide, A. et al. Gastroenterology. 2005, 128, p.A530.
26) Tominaga, K. et al. J Gastroenterol. 2012, 47, p.284.
27) Arai, M. Hepato-Gastroenterology. 2012, 59, p.62.
28) Takahashi, T. et al. World J Surg. 2009, 33, p.296.
29) Oka, T. et al. Biopsychosoc Med. 2007, 1, p.21.
30) 水野修吾ほか. Prog Med. 2001, 21, p.1366.
31) 渡嘉敷亮二. MB ENT. 2010, 110, p.59.
32) Oridate, N. et al. J Gastroenterol. 2008, 43, p.519.
33) 松林　直ほか. 心身医学. 1995, 35, p.519.
34) 鈴木(堀田)眞理. 日本東洋心身医学研究. 2007, 22, p.18.
35) Shiratori, M. et al. Neurogastroenterol Motil. 2011, 23, p.323.
36) 田中政彦ほか. 日本東洋医学雑誌. 1993, 44, p.1.
37) Yagi, M. et al. Pediatr Surg Int. 2004, 19, p.760.
38) 中里雅光. 漢方医学. 2010, 34, p.254.

半夏瀉心湯（はんげしゃしんとう）

国際名　hangeshashinto

・がん化学療法による下痢および口内炎を軽減することが報告されている。
・心窩部に膨満感があって、悪心・嘔吐、食欲不振などがあり、軟便や下痢を伴う場合に用いられることが多い。
・基礎的研究から抗炎症作用、大腸水分吸収亢進作用、止瀉作用、胃粘膜防御作用などが知られている。

◆構成生薬
ハンゲ(半夏)、オウゴン(黄芩)、カンキョウ(乾姜=しょうが)、カンゾウ(甘草)、タイソウ(大棗)、ニンジン(人参)、オウレン(黄連)

◆投与方法
7.5g/日を経口投与(通常食前または食間分3)、含嗽使用

◆実地臨床における応用例
・腸液分泌型下痢
・イリノテカン塩酸塩による下痢
・がん化学療法による口内炎(含嗽使用が推奨)
・悪心・嘔吐、下痢、食欲不振

◆現在までに得られているEBM
●薬効薬理
①抗炎症作用[1) 2) 3) 4)]
②大腸水分吸収亢進作用[1) 5)]
③止瀉作用[1) 6)]
④制吐作用[7)]
⑤胃粘膜防御作用[8) 9)]

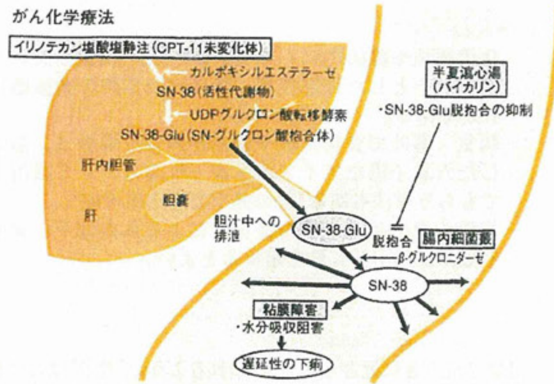

図1　イリノテカン塩酸塩（CPT-11）による遅発性下痢と半夏瀉心湯の作用機序（文献21）より改変）

●臨床効果

①イリノテカン塩酸塩による下痢を軽減[11) 12) 13) 14)]
②がん化学療法による口内炎を改善[15) 16) 17)]
③口内炎を改善[18) 19)]
④胃切除後の消化器症状（外科的NUD）を改善[20)]

◆使用時のポイント

・半夏瀉心湯は、その薬理機序からプロスタグランジンE_2が関与する腸液分泌型の下痢に効果がある。
・イリノテカン塩酸塩による下痢を予防するためには、イリノテカン塩酸塩投与数日前から使用するとよい。
・半夏瀉心湯はイリノテカン塩酸塩の抗腫瘍効果に影響しないことが確認されている。
・がん化学療法による口内炎には、コップ半分程度に1包を撹拌し、数回に分けて口腔に含んで1回5秒以上ゆすいでもらう局所（含嗽）療法が速効性もあり有効である。抗がん剤による吐き気がある場合でも可能な方法であり、患者に推奨しやすい。
・潰瘍部に直接塗布した場合は、短時間の刺激感を伴う場合がある。

◆半夏瀉心湯・豆知識

主薬である半夏と、薬効である瀉心（心窩部の痞（つか）え感を取り除く）から名付けられた。「湯」は水に溶解することを意味し、水溶成分が有効であることを示している。口内炎に服用しても効果はあるが、含嗽することで局所に対して成分を高濃度にして効果を高め、除痛効果の速効性を期待している。

◆文献

●薬効薬理
1) Kase, Y. et al. Jpn J Pharmacol. 1997, 75, p.407.
2) Kase, Y. et al. Biol Pharm Bull. 1998, 21, p.117.
3) Kase, Y. et al. Biol Pharm Bull. 1998, 21, p.1277.
4) 池田孔己ほか. 和漢医薬学雑誌. 1998, 15, p.390.

5) Kase, Y. et al. Biol Pharm Bull. 1997, 20, p.954.
6) Kase, Y. et al. Biol Pharm Bull. 1996, 19, p.1367.
7) Kase, Y. et al. Biol Pharm Bull. 1997, 20, p.1155.
8) 坂上　博ほか. 消化器科. 1990, 12, p.183.
9) 緒方優美ほか. 薬理と治療. 1993, 21, p.1747.
10) 原澤　茂ほか. Prog Med. 1993, 13, p.2533.
●臨床効果
11) 森　清志ほか. 癌と化学療法. 1998, 25, p.1159.
12) 村越　誉ほか. 産婦人科漢方研究のあゆみ. 1999, 16, p.114.
13) 鎌滝哲也ほか. 産婦人科漢方研究のあゆみ. 2000, 17, p.14.
14) Mori, K. et al. Cancer Chemother and Pharmacol. 2003, 51, p.403.
15) Kono, T. et al. World J Oncol. 2010, 1, p.232.
16) 柳瀬　徹. 漢方医学. 2010. 34. p.293.
17) 水田成彦ほか. 漢方医学. 2011. 35. p.167.
18) 石井正光. Medical Kanpo. 1995, 7, p.12.
19) 小林永治. 漢方の臨床. 2007, 54, p.208.
20) 合地　明ほか. Prog Med. 1999, 19, p.891.
21) 鎌滝哲也. 漢方医学. 2010, 34, p.46.

補中益気湯（ほちゅうえっきとう）

国際名　hochuekkito

・補中益気湯は、十全大補湯と同様に古くから病後の気力低下、全身倦怠感に対して有効とされてきた。
・基礎研究では、NK細胞などの細胞性免疫機能の活性化、栄養状態の改善作用を介して生体防御能を向上させることが報告されている。
・臨床では術後や担がん患者を中心に頻用されている。
・抗ウイルス作用（インフルエンザなど）、抗菌作用（MRSAなど）が報告されており、術後や担がん患者の感染、さらには院内感染に対する予防効果も期待されている。

◆構成生薬

オウギ（黄耆）、ソウジュツ（蒼朮）、ニンジン（人参）、トウキ（当帰）、サイコ（柴胡）、タイソウ（大棗）、チンピ（陳皮）、カンゾウ（甘草）、ショウマ（升麻）、ショウキョウ（生姜）

◆投与方法

7.5g/日を経口投与（通常食前または食間分3）

◆実地臨床における応用例

・がん化学療法に伴う食欲不振、全身倦怠感
・術後の全身倦怠感、QOLの低下
・各種感染症

◆現在までに得られているEBM

●薬効薬理

①免疫抑制状態改善作用[1)]
②発がん抑制作用[2)]
③NK細胞活性増強作用[3)]

④担がん状態の生体防御機構の修復作用[1]
⑤免疫機能回復による抗腫瘍効果[5]
⑥ストレスによるT細胞の抗腫瘍反応抑制の修復作用[6]
⑦抗がん剤投与マウスにおけるNK活性・白血球数の改善作用[7,8]
⑧放射線療法の副作用軽減[9]
⑨ウイルス感染に対する防御作用[10,11,12,13,14,15,16]
⑩胃切除後の骨代謝障害抑制作用[17]
⑪酸化ストレス減少作用[18]
⑫抗炎症作用[19]

●臨床効果

①がん化学療法に伴う食欲不振、全身倦怠感を改善[20,21,22]
②高齢者の生体防御機能を賦活[23]
③術後ストレスを抑制[24]
④手術ストレスによる免疫抑制を予防[25]
⑤肺がん患者の延命効果[26]
⑥がん患者の精神的苦痛を緩和[27]
⑦進行胃がん、大腸がん術後感染に対する防御能を増強[28]
⑧救急時のMRSA感染予防や免疫栄養指数を有意に改善[29]
⑨褥瘡に経腸栄養と併用して褥瘡面積が有意に減少、栄養状態も改善[30,31]
⑩消化器がん術後の窒素代謝を改善[32]
⑪胃切除後骨障害を改善[33,34]
⑫がん性疲労を改善[35]

◆使用時のポイント

・十全大補湯と同様に、病後や術後の全身状態の改善に使用するとともに、がん化学療法や放射線療法時の副作用軽減、がん再発予防にも使用される。
・両者の使い分けのポイントは、PS0から1では補中益気湯、PS2以上では十全大補湯を用いるとよい。
・甘草の成分であるグリチルリチン酸は尿細管でのカリウム排泄促進作用があるため、長期連用の場合は、低カリウム血症によるミオパシー、偽アルドステロン症、さらには間質性肺炎による発熱、咳嗽、呼吸困難に注意する必要がある。

◆補中益気湯・豆知識

"中"とは消化管を意味し、病後や術後の全身倦怠感に対して胃腸の働きを高め、体力を補い元気をつけるというのが名前の由来。

◆文献

●薬効薬理

1) Utsuyama, M. et al. Mech Ageing Dev. 2001, 122, p.341.
2) Onogi, K. Oncol Rep. 2006, 16, p.1343.
3) 大野修嗣. アレルギー. 1988, 37, p.107.
4) 久保千春ほか. 漢方と免疫・アレルギー. 1988, 1, p.50.
5) Harada, M. et al. Immunopharmacol Immunotoxicol. 1995, 17, p.687.
6) Li, T. et al. Immunopharmacology. 1999, 43, p.11.
7) 前村和也ほか. 漢方と免疫・アレルギー. 1990, 3, p.108.
8) Kaneko, M. et al. Immunopharmacology. 1999, 44, p.223.
9) 細川　康. 痛の臨床. 1993, 39, p.1655.
10) Mori, K. et al. Antiviral Res. 1999, 44, p.103.

11) Hossain, MS. et al. Immunopharmacology. 1999, 41, p.169.
12) Kido, T. et al. Anticancer Res 2000, 20, p.4109.
13) Yamaoka, Y. et al. Immunopharmacology 2000, 48, p.35.
14) Kiyohara, H. et al. Evid Based Complement. Alternat Med. 2006, 3, p.459.
15) Yamaya, M. et al. Br J Pharmacol. 2007, 150, p.702.
16) Ishii, A. et al. J Nat Med. 2007, 61, p.280.
17) 鈴木　裕ほか. Prog Med. 1999, 19, p.965.
18) 赤松浩彦ほか. 和漢医薬学雑誌. 1998, 15, p.348.
19) 磯濱洋一郎. 厚生労働科学研究費補助金（長寿科学総合研究事業）分担研究報告書. 2007, p.48.

●臨床効果

20) 阿部憲司. Prog Med. 1989, 9, p.2916.
21) 森　清志ほか. Biotherapy. 1992, 6, p.624.
22) 上田晴彦ほか. 産婦人科漢方研究のあゆみ. 2008, 25, p.46.
23) Kuroiwa, A. et al. E Int Immunopharmacol. 2004, 4, p.317.
24) Satoh, N. et al. Phytomedicine. 2005, 12, p.549.
25) Kimura, M. et al. Surg Today. 2008, 38, p.316.
26) Satoh, H. et al. J Altern Complement Med. 2002, 8, p.107.
27) 篠崎　徹. 痛みと漢方. 2003, 13, p.49.
28) 齋藤信也ほか. 日臨外会誌. 2006, 67, p.568.
29) 北原正和ほか. Biotherapy. 2002, 16, p.261.
30) 鈴木　裕ほか. 漢方医学. 1999, 23, p.192.
31) 鈴木　裕. 第16回臨床東洋医学研究会講演記録集. 2001, 16, p.27.
32) 仲　秀司ほか. Prog Med. 2002, 22, p.1360.
33) 加藤一彦ほか. 日本東洋医学雑誌. 1992, 43, p.309.
34) 杉山　貢. 現代医療学. 1995, 10, p.210.
35) Jeong, JS. et al. Integrative Cancer Therapies. 2010, 9, p.331.

十全大補湯（じゅうぜんたいほとう）

国際名　juzentaihoto

・補中益気湯と同様に諸種の原因による体力低下、食欲不振、全身倦怠感に対して有効とされている。
・作用機序に関する基礎的研究では、免疫賦活作用によるがん転移抑制作用、再発抑制作用が期待され、臨床的にも応用されている。
・骨髄幹細胞に対する直接刺激作用が報告され、がん化学療法時の骨髄抑制（特に血小板減少、赤血球減少）に対して頻用されている。
・慢性肝炎、肝硬変症のALT値を低下させ、クッパー細胞の酸化ストレス抑制による肝がん発生を抑制することが報告されている。

◆構成生薬

オウギ（黄耆）、ケイヒ（桂皮）、ジオウ（地黄）、シャクヤク（芍薬）、センキュウ（川芎）、ソウジュツ（蒼朮）、トウキ（当帰）、ニンジン（人参）、ブクリョウ（茯苓）、カンゾウ（甘草）

◆投与方法

7.5g/日を経口投与（通常食前または食間分3）

◆実地臨床における応用例

・がん化学療法時の骨髄抑制
・術後の貧血、食欲不振、全身倦怠感など

◆現在までに得られているEBM

●薬効薬理

①免疫増強作用[1]
②免疫抑制状態改善作用[2]
③感染予防効果[3]
④発がん抑制作用[4]

⑤がんの増殖・転移抑制効果[5) 6) 7) 8) 9) 10) 11)]
⑥抗がん剤の副作用軽減[12) 13)]
⑦貧血改善作用[14)]
⑧肝切除後の血中アンモニア濃度上昇抑制[15)]

●臨床効果

①免疫・栄養状態を改善[16)]
②悪性腫瘍術後の抗がん剤療法による食欲不振を改善[17)]
③悪性腫瘍術後の抗がん剤療法による細胞性免疫抑制を軽減[18)]
④進行乳がん患者の白血球減少を改善[19)]
⑤がん化学療法による白血球減少を予防[20)]
⑥放射線治療と併用して子宮頸がん患者に延命効果[21)]
⑦婦人科悪性腫瘍の術後化学療法による骨髄抑制を有意に軽減[22) 23) 24)]
⑧肝硬変から肝がんへの移行を抑制[25)]
⑨肝細胞がん術後患者の無再発生存率を改善[26)]
⑩褥瘡を改善し、MRSA菌量が有意に減少[27)]
⑪貧血を改善[28) 29) 30)]
⑫婦人科悪性腫瘍患者の術前自己血貯血におけるヘモグロビンが増加[31)]
⑬婦人科悪性腫瘍患者の術前自己血貯球数、ヘマトクリット値の減少幅を有意に抑制[32)]

◆使用時のポイント

・補中益気湯と同様に、病後や術後の全身状態の改善に使用するとともに、がん化学療法や放射線療法時の骨髄抑制、特に血小板や赤血球の減少に効果がある。
・全身倦怠感や食欲不振に、貧血、顔色不良、皮膚枯燥などの病態が加わった場合には、補中益気湯から十全大補湯に変更する。
・甘草の成分であるグリチルリチン酸は尿細管でのカリウム排泄促進作用があるため、長期連用の場合は、低カリウム血症によるミオパシー、偽アルドステロン症、さらには間質性肺炎による発熱、咳嗽、呼吸困難などに注意する必要がある。

◆十全大補湯・豆知識

"十全"とは完全無欠の意で「幅広く大いに補う」というのが名前の由来。人参はウコギ科のオタネニンジン(朝鮮人参とも言われる)のことで、野菜の人参(セリ科)とは全く異なる植物である。江戸時代に朝鮮から日本に導入され、その後、徳川吉宗が御薬園から諸藩に種を分けて栽培を奨励したことから"御種人参"と呼ばれるようになった。

◆文献

●薬効薬理

1) Fujiki, K. et al. Int J Mol Sci. 2008, 9, p.1142.
2) 李　愛麗ほか. 感染症学雑誌. 1996, 70, p.717.
3) Abe, S. et al. Immunopharmacol Immunotoxicol. 1998, 20, p.421.
4) Tagami, K. et al. Biol Pharm Bull. 2004, 27, p.156.
5) Takahashi, H. et al. Int J Immunother. 1995, 11, p.65.

6) Ohnishi, Y. et al. Jpn J Cancer Res. 1996, 87, p.1039.
7) Ohnishi, Y. et al. Jpn J Cancer Res. 1998, 89, p.206.
8) Saiki, I. Biol Pharm Bull. 2000, 23, p.677.
9) Chino, A. et al. Int Immunopharmacol. 2005, 5, p.871.
10) Matsuda, T. et al. Evid Based Complement Alternat Med. 2011, doi:10.1093/ecam/nen081.
11) Ishikawa, S. et al. Evid Based Complement Alternat Med. 2012, doi:10.1155/2012/945714.
12) Sugiyama, K. et al. Biol Pharm Bull. 1995, 18, p.544.
13) Kiyohara, H. et al. Planta Med. 1995, 61, p.531.
14) Hisha, H. et al. Blood. 1997, 90, p.1022.
15) 栗原直人ほか. 漢方と最新治療. 2008, 17, p99.

●臨床効果

16) 青行俊郎ほか. Prog Med. 2002, 22, p.1358.
17) 黒川胤臣ほか. Biotherapy. 1989, 3, p.789.
18) 山田輝司. 和漢医薬学会誌. 1992, 9, p.157.
19) Adachi, I. Biomedical Research. 1990, 11, p.25.
20) 鈴木眞一ほか. Prog Med. 1995, 15, p.1968.
21) 竹川佳宏ほか. Biotherapy. 2006, 20, p.61.
22) 藤原道久ほか. 産婦人科漢方研究のあゆみ. 1998, 15, p.86.
23) 藤原道久ほか. 産婦中四会誌. 1999, 47, p153.
24) 藤原道久ほか. 産婦人科漢方研究のあゆみ. 2006, 23, p.24.
25) 樋口清博ほか. 肝胆膵. 2002, 44, p.341.
26) Tsuchiya, M. et al. Int J Cancer. 2008, 123, p.2503.
27) 永井弥生ほか. 漢方と最新治療. 2009, 18, p.143.
28) Hisha, H. et al. Stem Cells. 2002, 20, p.311.
29) Sho, Y. et al. C. J Gastroenterol. 2004, 39, p.1202.
30) Nakamoto, H. et al. Hemodial Int. 2008, 12, p.S9.
31) 青江尚志ほか. 自己血輸血1999, 12, p.100.
32) 青江尚志. Pharma Medica. 2007, 25, p.11.

抑肝散（よくかんさん）

国際名　yokukansan

・緩和ケアにおける麻薬(モルヒネ)による異常興奮、せん妄、不眠症などにも応用が可能である。
・作用機序として、脳内興奮性神経伝達物質であるグルタミン酸の放出抑制作用および過剰状態を制御するトランスポーター賦活作用、$5\text{-}HT_{1A}$受容体パーシャルアゴニスト作用、$5\text{-}HT_{2A}$受容体ダウンレギュレーションが報告されている。
・認知症の行動・心理症状(BPSD; Behavioral and Psychological Symptoms of Dementia)、すなわち妄想、幻覚、興奮/攻撃性、うつ、不安、焦燥感/易刺激性、睡眠障害などに用いられ、有効性が確認されている。
・抗精神病薬のような副作用(過鎮静に伴うふらつき、転倒、誤嚥など)がほとんど認められない。

◆構成生薬

ソウジュツ(蒼朮)、ブクリョウ(茯苓)、センキュウ(川芎)、チョウトウコウ(釣藤鈎)、トウキ(当帰)、サイコ(柴胡)、カンゾウ(甘草)

◆投与方法

7.5g/日を経口投与(通常食前または食間分3)

◆実地臨床における応用例

・がん患者のせん妄
・異常興奮、焦燥感、不眠などの精神神経症状

◆現在までに得られているEBM

●薬効薬理

①抗不安様作用[1) 2) 3)]

②グルタミン酸放出抑制作用および過剰状態を制御するグルタミン酸トランスポーター賦活作用により、脳内興奮性神経伝達物質であるグルタミン酸の細胞間隙量を減少[4) 5) 6) 7)]

③ 5-HT_{1A} 受容体パーシャルアゴニスト作用および 5-HT_{2A} 受容体ダウンレギュレーションにより異常行動を改善[8) 9) 10)]

④アルツハイマー病モデルマウスにおいて学習障害・脱抑制状態および過活動障害を改善[11)]

⑤アルツハイマー病モデルマウスにおける短期記憶障害を改善[12)]

⑥アルツハイマー病モデルマウスにおける記憶障害および異常な社会行動を改善[13)]

⑦チアミン欠乏マウスにおける攻撃行動の増大および社会行動の低下を改善[14)]

⑧統合失調モデルにおいて脳内グルタチオン濃度の低下を正常レベルまで回復させることにより病的症状を改善[15)]

⑨ベータアミロイド(A β)添加で低下するラット皮質ニューロン初代培養細胞の細胞毒性に対する神経保護作用[16)]

⑩ストレス負荷マウスの三相性皮膚反応を用量依存的に抑制[17)]

⑪隔離飼育NC/Ngaマウスにおけるアトピー性皮膚炎様病変の発現を抑制[18)]

●臨床効果

①末期がん患者の過活動型せん妄を改善[19)]

②総睡眠時間の増加、ノンレム睡眠時間の増加、睡眠中の周期性四肢運動の低下、レム睡眠障害の改善により睡眠障害を改善[20) 21) 22)]

③認知症の行動・心理症状(BPSD)を改善[20) 21) 23) 24) 25) 26) 27) 28) 29)]

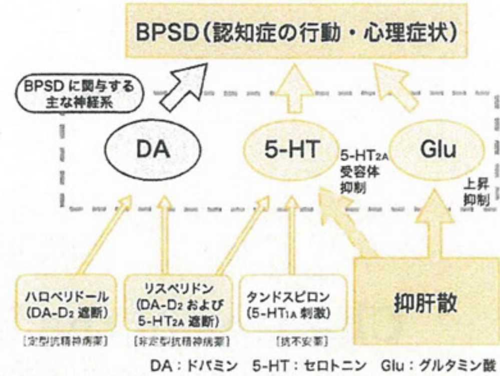

図1　認知症の行動・心理症状(BPSD)に対する抑肝散の薬理作用

◆使用時のポイント

- せん妄においては向精神薬の服用で過鎮静が起こった場合にも使用しやすい薬剤である。
- がん患者の高齢化が進み、がん患者にも認知症を併発するケースがある。認知症の行動・心理症状(BPSD)で頻用されており、ADLを低下させることなくBPSDの改善が期待できる。特に衝動性、攻撃性、興奮、イライラなどには速効性があり、感情のコントロールに有効である。
- 甘草の成分であるグリチルリチン酸は尿細管でのカリウム排泄促進作用があるため、長期に連用する場合は低カリウム血症によるミオパシー、偽アルドステロン症に注意する必要がある。

◆抑肝散・豆知識

漢方では"肝"は心や精神を主(つかさど)る働きを持つと言われている。肝の亢(たか)ぶりはイライラ、怒り、興奮などの精神神経症状をもたらすと考えられており、その精神神経症状を抑えることから抑肝散と名付けられた。

◆文献

●薬効薬理

1) 栗原　久ほか. 神経精神薬理. 1996, 18, p.179.
2) Mizoguchi, K. et al. J Ethnopharmacol. 2010, 127, p.70.
3) Nogami, A. et al. J Nat Med. 2011, 65, p.275.
4) Takeda, A. et al. Neutr Neurosci. 2008, 11, p.41.
5) Takeda, A. et al. Neurochem Int. 2008, 53, p.230.
6) Kawakami, Z. et al. Neuroscience. 2009, 159, p.1397.
7) Kawakami, Z. et al. Eur J Pharmacol. 2010, 626, p.154.
8) Egashira, N. et al. Prog Neuropsychopharmacol Biol Psychiatry. 2008, 32, p.1516.
9) Kanno, H. et al. J Pharm Pharmacol. 2009, 61, p.1249.
10) Terawaki, K. et al. J Ethnopharmacol. 2010, 127, p.306.
11) Tabuchi, M. et al. J Ethnopharmacol. 2009, 112, p.157.
12) Yamada, M. et al. J Ethnopharmacol. 2011, 135, p.737.
13) Fujiwara, H. et al. Neuroscience. 2011, 180, p.305.
14) Yamaguchi, T. et al. Phytopharmacology. 2011, 1, p.123.
15) Makinodan, M. et al. J Brain Disease. 2009, 1, p.1.
16) Tateno, M. et al. Prog Neuro-Psychopharmacol Biol Psychiatry. 2008, 32, p.1704.
17) Saiki, I. J Trad Med. 2004, 21, p.51.
18) Funakushi, N. et al. Arch Dermatol Res. 2011, 303, p.659.

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19) 井上潤一ほか. ペインクリニック. 2009, 30, p.525.
20) Shinno, H. et al. Prog Neuro-Psychopharmacol Biol Psychiatry.
21) Shinno, H. et al. Prog Neuro-Psychopharmacol Biol Psychiatry. 2008, 32, p.1749.
22) Shinno, H. et al. Prog Neuro-Psychopharmacol Biol Psychiatry. 2007, 31, p1543.
23) Iwasaki, K. et al. J Clin Psychiatry. 2005, 66, p.248.
24) Mizukami, K. et al. Int J Neuropsychopharmacol. 2009, 12, p.191.
25) Monji, A. et al. Prog Neuro-Psychopharmacol Biol Psychiatry. 2009, 33, p.308.
26) Kimura, T. et al. Psychogeriatrics. 2009, 9, p.38.
27) Kimura, T. et al. Psychiatry Clin Neurosci. 2010, 64, p.207.
28) Iwasaki, K. et al. J Am Geriatr Soc. 2011, 59, p.936.
29) Sumiyoshi, H. et al. Am J Geriatr Psychiatry. 2011, 19, p.906.

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Short Communication

Changes in the Melanocortin Receptors in the Hypothalamus of a Rat Model of Cancer Cachexia

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KEY WORDS stage-dependent cancer cachexia; central melanocortin system; hypothalamus; rat

Cancer cachexia affects up to 80% of patients with advanced cancers and accounts for nearly 30% of cancer-related deaths (Acharyya et al., 2005; Fearon, 2008; Tisdale, 2009). The close association between chronic illness such as cancer and the deterioration of nutritional status not only impairs the quality of life but also increases the risk for morbidity and mortality (Norman et al., 2008). Cancer cachexia syndrome is characterized by anorexia and a loss of body weight associated with reduced muscle mass and adipose tissue. In addition to a variable contribution from decreased energy intake, the resting energy expenditure can be elevated in cancer cachexia. It is well known that advanced stages of cancer are scarcely responsive to available pharmacological and nutritional treatments, which suggests that, once a critical point is reached, it may be difficult to improve the metabolism and the availability of nutrients. Accordingly, clinicians require more effective treatments.

Recently, cachexia and precachexia were defined by the Special Interest Group on cachexia–anorexia in chronic wasting diseases. This group advocates the new concept that the mechanisms that ultimately lead to severe wasting in cachexia, that is, late-stage cachexia, are occurring early during the natural history of the disease, which suggests that appropriate interventions in early-stage cachexia might be effective for preventing the onset of this syndrome (Muscaritoli et al., 2010). This underscores the need for appropriate pharmacological and nutritional interventions to prevent precachexia from progressing to cachexia.

The central melanocortin system directly controls nutrient intake and energy metabolism (Garfield et al., 2009). Melanocortins (α-, β-, and γ-melanocyte-stimulating hormones and adrenocorticotropic hormone) are peptides derived from the proteolytic cleavage of pro-opiomelanocortin (POMC). Five melanocortin receptor (MC1R–MC5R) subtypes mediate the diverse actions of these melanocortins. Of these five MCRs, MC3R and MC4R are predominantly expressed in the brain and are involved in regulating energy homeostasis. Extensive localization studies have shown that MC3R and MC4R are highly expressed in the paraventricular nucleus (PVN), including both parvicellular and magnocellular neurons, of the hypothalamus. The activation of MC4R results in decreased food intake and increased energy expenditure by inducing the release of brain-derived

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neurotropic factor (BDNF) in the hypothalamus, which suggests that BDNF is a downstream mediator of MC4R signaling (Xu et al., 2003).

TABLE I. Primer sequences for the rat genes characterized in this experiment

Gene	Primers
GAPDH	Forward primer 5′CCCCCAATGTATCCGTTGTG 3′
	Reverse primer 5′TAGCCCAGGATGCCCTTTAGT 3′
MC4R	Forward primer 5′GCTGCAGGAAGATGAACTCC 3′
	Reverse primer 5′TCCAGAGGTGGAGGGAAGTA 3′
MC3R	Forward primer 5′CCGCCGATAACCATGAACT 3′
	Reverse primer 5′GTTAGGCAGCGTCGGATAAG 3′
POMC	Forward primer 5′TCCTCAGAGAGCTGCCTTTC 3′
	Reverse primer 5′TGTAGCAGAATCTCGGCATCT 3′
BDNF	Forward primer 5′AGCGCGAATGTGTTAGTGGT 3′
	Reverse primer 5′GCAATTGTTTGCCTCTTTTTCT 3′

In the present study, we identified changes in the expression of MC3R, MC4R, POMC, and BDNF in the rat hypothalamus in both an early- and late-stage cancer cachexia model. The present findings may provide new insight into the mechanisms that underlie stage-dependent cancer cachexia.

All experiments were conducted in accordance with the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and approved by the Committee for Ethics of Animal Experimentation of National Cancer Center. Efforts were made to minimize the numbers and any suffering of animals used in the following experiments. The human gastric cancer cell-line 60As6, originating from the human scirrhous gastric cancer cell line HSC60, was established as described previously (Yanagihara et al., 2004). Seven-week-old male F344/N-mu (mu/mu) rats were used. Rats were purchased from CLEA Japan (Tokyo, Japan) and held under standard laboratory conditions (23°C ± 1°C with a 12-h light/dark cycle). Food and water were available ad libitum. Rats were housed singly and, after a 2-week adaptation period, randomly assigned to a tumor or control group. For tumor implantation, rats were anesthetized with isoflurane. Approximately 1×10^6 60As6 cells suspended in phosphate-buffered saline (PBS) were then implanted between the peritoneum and the abdominal wall of the central abdomen. Non-tumor controls underwent the same procedure but only received PBS injection. Body weight and food consumption were measured 2, 4, 6, 8, 10, 12, 14, and 16 weeks after tumor implantation. Rats were decapitated at both 8 weeks, as early-stage cancer cachexia, and 16 weeks, as late-stage cancer cachexia. The weights of epididymal fat and the gastrocnemius muscle were measured 8 and 16 weeks after tumor implantation. For real-time reverse transcription PCR, brains were rapidly removed, and the hypothalamic area was dissected on an ice-cold metal plate. Total RNA was isolated from the hypothalamic area using ISOGEN (Niponjine) according to the manufacturer's instructions. First-strand cDNA was reverse transcribed from 5 μg of total RNA by using a SuperScript™ first-strand synthesis system (Invitrogen) in a final volume of 100 μL. Diluted cDNA (2 μL) was amplified in a rapid thermal cycler (LightCycler; Roche Diagnostics) in 10 μL of LightCycler 480 SYBR Green I Master (Roche) and each oligonucleotide. Primer sequences for the genes of interest [MC4R, MC3R, POMC, BDNF, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] are shown in Table I. To quantify the PCR products, LightCycler 480 quantification software was used to analyze the exponential phase of amplification and the melting curve, as recommended by the manufacturer. The amount of target mRNA in the experimental group relative to that in the control was determined from the resulting fluorescence and threshold values (C_T) using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

All data are presented as the mean ± SEM. All the statistical parameters used in the experiments were calculated using GraphPad PRISM. The statistical significance of differences between groups was assessed with Student's *t*-test.

As shown in Figure 1a, tumor-bearing rats showed a significant decrease in body weight from 8 to 16 weeks after the inoculation of tumor cells ($*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. control group). Consistent with the data, food intake in tumor-bearing rats at 8 and 16 weeks was 11 and 23% lower than that in control rats ($*P < 0.05$ and $**P < 0.01$ vs. control group; Fig. 1b). At 8 weeks after inoculation, the weight of epididymal fat tissue, but not that of gastrocnemius muscle, was significantly reduced compared to that in control rats ($*P < 0.05$ vs. control group; Figs. 1c and 1d). A 73% decrease in the weight of epididymal fat tissue and a 31% decrease in the weight of gastrocnemius muscle were observed in tumor-bearing rats 16 weeks after tumor inoculation ($***P < 0.01$ vs. control group; Figs. 1c and 1d). At 8 weeks after inoculation, when rats exhibited slight anorexia and weight loss, the mRNA levels of MC4R, MC3R, and BDNF were significantly decreased in the hypothalamus of tumor-bearing rats compared to those in control rats (8 weeks, $*P < 0.05$ and $***P < 0.001$ vs. control group; Figs. 2a, 2b, and 2d). In contrast, the expression of these receptors and BDNF was dramatically increased in the hypothalamus of tumor-bearing rats 16 weeks after inoculation, when rats exhibited severe wasting associated with a significant reduction of both adipose tissue and muscle mass (16 weeks, $***P < 0.001$ vs. control group; Figs. 2a, 2b, and 2d). In addition, while the mRNA level of POMC, which encodes α-MSH, in tumor-bearing mice was not different from that in the control at 8 weeks after inoculation, a remarkable decrease in POMC expression was observed in the hypothalamus of tumor-bearing rats

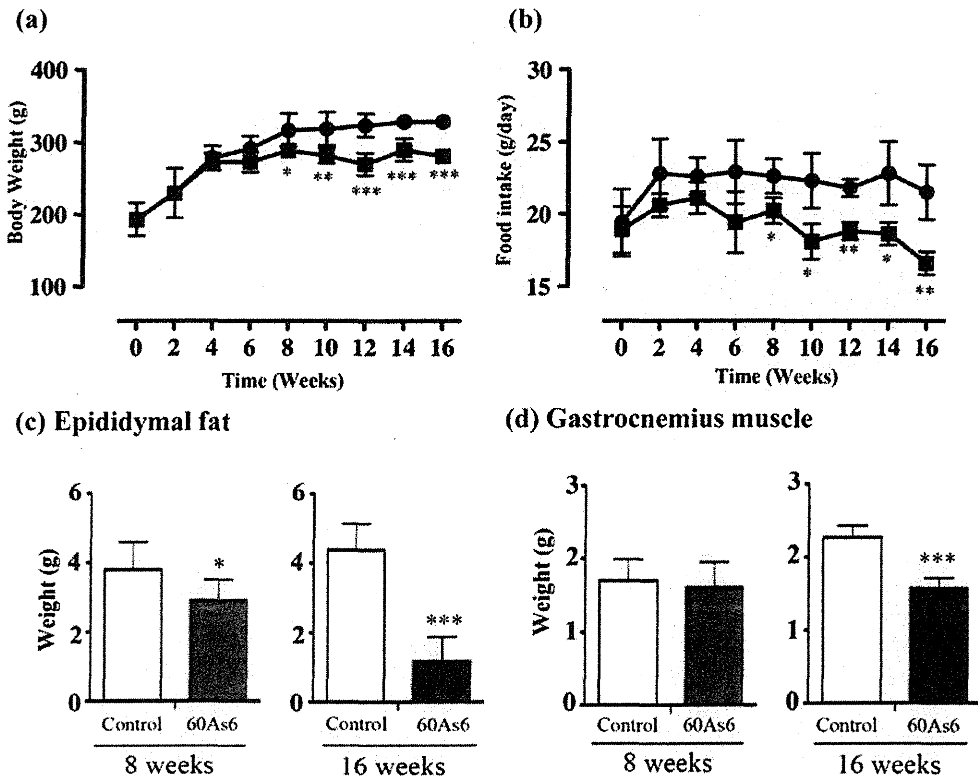

Fig. 1. Body weight and food intake of tumor-inoculated rats. (**a**) Body weight in 60As6 tumor-bearing (■) or PBS-control rats (●). (**b**) Food intake in 60As6 tumor-bearing (■) or PBS-control rats (●). The results represent the mean ± SEM of five to seven rats. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control group. (**c**) Epididymal fat weight in 60As6 tumor-bearing or PBS-control rats at 8 and 16 weeks after the inoculation of tumor cells. (**d**) Gastrocnemius muscle weight in 60As6 tumor-bearing or PBS-control rats at 8 and 16 weeks after the inoculation of tumor cells. Each column represents the mean ± SEM of five to seven rats. *$P < 0.05$, ***$P < 0.001$ versus control group.

16 weeks after inoculation (16 weeks, ***$P < 0.001$ vs. control group; Fig. 2c).

The key finding in the present study was that dramatic increases in the expression of both MC3R and MC4R were observed in the hypothalamus of tumor-bearing rats 16 weeks after tumor inoculation, when rats exhibited severe wasting associated with a significant reduction of both adipose tissue and muscle mass, that is, so-called late-stage cachexia. In addition, a remarkable increase in BDNF expression and a decrease in POMC expression in the hypothalamus of tumor-bearing rats were also observed. Central MC3R and MC4R are involved in the regulation of body weight and have been shown under many paradigms to reduce food intake and/or increase energy expenditure (De Jonghe et al., 2011). BDNF in the hypothalamus is thought to regulate the energy balance as a downstream effector of MC4R (Xu et al., 2003). In our preliminary experiments, we found that the locomotor activity of tumor-bearing rats during the dark period was significantly decreased without any changes in the resting energy expenditure, which suggests that the energy expenditure in the dark period was increased in rats with late-stage cachexia (data not shown). Furthermore, MC4R-knockout mice have been shown to exhibit marked hyperphagia and obesity (Huszar et al., 1997), which were blocked by selective MC4R re-expression in the PVN and amygdala (Balthasar et al., 2005). In addition, mice with a double knockout of both MC3R and MC4R exhibit a higher weight gain than those with the deletion of either receptor alone (Chen et al., 2000). Taken together, our present findings raise the possibility that the dramatic up-regulation of MC3R/MC4R signaling, possibly due to the down-regulation of POMC in the hypothalamus, may contribute to the metabolic alterations and reduced availability of nutrients under late-stage cancer cachexia.

At 8 weeks after tumor inoculation, when rats exhibited slight anorexia and weight loss, so-called early-stage cachexia, the expression levels of MC3R, MC4R, and BDNF in the hypothalamus were significantly reduced without any changes in POMC expression. The "adiposity negative-feedback" model of energy homeostasis is based on the premise that circulating signals inform the brain of changes in body

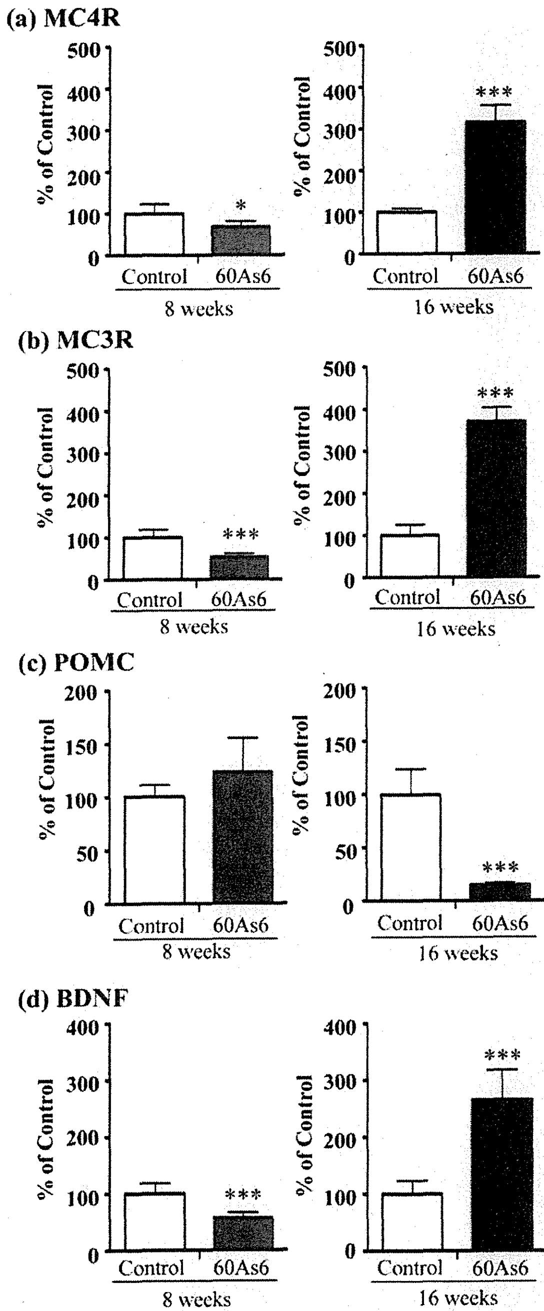

Fig. 2. Transcriptional regulation of MC4R, MC3R, POMC, and BDNF in the hypothalamus induced by tumor inoculation. Expression of MC4R (**a**), MC3R (**b**), POMC (**c**), and BDNF (**d**) in the hypothalamus of tumor-bearing rats on 8 and 16 weeks. The mRNA levels were normalized to those for GAPDH (housekeeping gene), and the results are presented as the mean ± SEM of five to seven mice. $^{*}P < 0.05$, $^{***}P < 0.001$ versus control group.

fat mass and, in response to this input, the brain makes adaptive adjustments to the energy balance to stabilize fat stores (Rousso-Noori et al., 2011). The criteria that have been proposed for a negative-feedback signal include that it circulates at levels proportionate to body fat content and that it promotes weight loss by acting on neuronal systems implicated in energy homeostasis. Many hormones have been implicated in the regulation of central melanocortin signaling, including many nutrients (free fatty acids, glucose), cytokines (interleukin-6, tumor necrosis factor-α), and the adipocyte-secreted hormone leptin (Deboer and Marks, 2006). In the present model, early-stage cachexia model rats exhibited a significant but slight decrease in epididymal fat. Although additional studies will be required to clarify the mechanism of the downregulation of MC3R, MC4R, and BDNF in early-stage cachexia model rats, decreased MC3R/MC4R signaling in the hypothalamus may be responsible for the adiposity negative-feedback signal due to a slight decrease in body fat mass.

In conclusion, our present data provide the first evidence that opposite changes in MC3R/MC4R expression and possible downstream signaling in the hypothalamus occur between the early and late stages in the development of cancer cachexia. Our findings may be important for studying the pathogenesis of stage-dependent cancer cachexia and for the future development of effective treatments for cancer cachexia.

REFERENCES

Acharyya S, Butchbach ME, Sahenk Z, Wang H, Saji M, Carathers M, Ringel MD, Skipworth RJ, Fearon KC, Hollingsworth MA, Muscarella P, Burghes AH, Rafael-Fortney JA, Guttridge DC. 2005. Dystrophin glycoprotein complex dysfunction: A regulatory link between muscular dystrophy and cancer cachexia. Cancer Cell 8:421–432.

Balthasar N, Dalgaard LT, Lee CE, Yu J, Funahashi H, Williams T, Ferreira M, Tang V, McGovern RA, Kenny CD, Christiansen LM, Edelstein E, Choi B, Boss O, Aschkenasi C, Zhang CY, Mountjoy K, Kishi T, Elmquist JK, Lowell BB. 2005. Divergence of melanocortin pathways in the control of food intake and energy expenditure. Cell 123:493–505.

Chen AS, Marsh DJ, Trumbauer ME, Frazier EG, Guan XM, Yu H, Rosenblum CI, Vongs A, Feng Y, Cao L, Metzger JM, Strack AM, Camacho RE, Mellin TN, Nunes CN, Min W, Fisher J, Gopal-Truter S, MacIntyre DE, Chen HY, Van der Ploeg LH. 2000. Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. Nat Genet 26:97–102.

De Jonghe BC, Hayes MR, Bence KK. 2011. Melanocorotin control of energy balance: Evidence from rodent models. Cell Mol Life Sci 68:2569–2588.

Deboer MD, Marks DL. 2006. Cachexia: Lessons from melanocortin antagonism. Trends Endocrinol Metab 17:199–204.

Fearon KC. 2008. Cancer cachexia: Developing multimodal therapy for a multidimensional problem. Eur J Cancer 44:1124–1132.

Garfield AS, Lam DD, Marston OJ, Przydzial MJ, Heisler LK. 2009. Role of central melanocortin pathway in energy homeostasis. Trends Endocrinol Metab 20:203–215.

Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F. 1997. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 88:131–141.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C_T)$ method. Methods 25:402–408.

Muscaritoli M, Anker SD, Argiles J, Aversa Z, Bauer JM, Biolo G, Boirie Y, Bosaeus I, Cederholm T, Costelli P, Fearon KC, Laviano

A, Maggio M, RossiFanelli F, Schols A, Sieber CC. 2010. Consensus definition of sarcopenia, cachexia and pre-cachexia: Joint document elaborated by Special Interest Groups (SIG) "cachexia-anorexia in chronic wasting diseases" and "nutrition in geriatrics." Clin Nutr 29:154–159.

Norman K, Pichard C, Lochs H, Pirlich M. 2008. Prognostic impact of disease-related malnutrition. Clin Nutr 27:5–15.

Rousso-Noori L, Knobler H, Levy-Apter E, Kuperman Y, Neufeld-Cohen A, Keshet Y, Akepati VR, Klinghoffer RA, Chen A, Elson A. 2011. Protein tyrosine phosphatase epsilon affects body weight by downregulating leptin signaling in a phosphorylation-dependent manner. Cell Metab 13:562–572.

Tisdale M. 2009. Mechanisms of cancer cachexia. Physiol Rev 89:381–410.

Xu B, Goulding EH, Zang K, Cepoi D, Cone RD, Jones KR, Tecott LH, Reichardt LF. 2003. Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. Nat Neurosci 6:763–742.

Yanagihara K, Tanaka H, Takigahira M, Ino Y, Yamaguchi Y, Toge T, Sugano K, Hirohashi S. 2004. Establishment of two cell lines from human gastric scirrhous carcinoma that possess the potential to metastasize spontaneously in nude mice. Cancer Sci 95:575–582.

Zimmermann M. 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16:109–110.

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GABA$_B$ Receptors Do Not Internalize After Baclofen Treatment, Possibly Due to a Lack of β-Arrestin Association: Study With a Real-Time Visualizing Assay

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KEY WORDS β-arrestin; GABA$_B$ receptor; internalization; endocytosis; GRK

ABSTRACT The mechanism of agonist-induced GABA$_B$ receptor (GABA$_B$R) internalization is not well understood. To investigate this process, we focused on the interaction of GABA$_B$R with β-arrestins, which are key proteins in the internalization of most of the G protein-coupled receptors, and the agonist-induced GABA$_B$R internalization and the interaction of GABA$_B$R with β-arrestin1 and β-arrestin2 were investigated in real time using GABA$_B$R and β-arrestins both of which were fluorescent protein-tagged. We then compared these profiles with those of μ-opioid receptors (μOR), well-studied receptors that associate and cointernalize with β-arrestins. When stimulated by the specific GABA$_B$R agonist baclofen, GABA$_B$R composed of GABA$_{B1a}$R (GB$_{1a}$R) and fluorescent protein-tagged GABA$_{B2}$R-Venus (GB$_2$R-V) formed functional GABA$_B$R; they elicited G protein-activated inwardly rectifying potassium channels as well as nontagged GABA$_B$R. In cells coexpressing GB$_{1a}$R, GB$_2$R-V, and β-arrestin1-Cerulean (βarr1-C) or β-arrestin2-Cerulean (βarr2-C), real-time imaging studies showed that baclofen treatment neither internalized GB$_2$R-V nor mobilized βarr1-C or βarr2-C to the cell surface. This happened regardless of the presence of G protein-coupled receptor kinase 4 (GRK4), which forms a complex with GABA$_B$R and causes GABA$_B$R desensitization. On the other hand, in cells coexpressing μOR-Venus, GRK2, and βarr1-C or βarr2-C, the μOR molecule formed μOR/βarr1 or μOR/βarr2 complexes on the cell surface, which were then internalized into the cytoplasm in a time-dependent manner. Fluorescence resonance energy transfer assay also indicated scarce association of GB$_2$R-V and β-arrestins-C with or without the stimulation of baclofen, while robust association of μOR-V with β-arrestins-C was detected after μOR activation. These findings suggest that GABA$_B$Rs failure to undergo agonist-induced internalization results in part from its failure to interact with β-arrestins. **Synapse 66:759–769, 2012.** © 2012 Wiley Periodicals, Inc.

INTRODUCTION

GABA$_B$ receptors (GABA$_B$Rs) play important roles in controlling inhibitory neurotransmission by GABA in the central and peripheral nervous system (Bettler et al., 2004). The receptors belong to the G protein-coupled receptor (GPCR) family and are the first discovered obligatory heterodimer consisting of GABA$_{B1}$ receptor (GB$_1$R) and GABA$_{B2}$ receptor (GB$_2$R) (Agnati

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et al., 2003; Bettler et al., 2004; Gainetdinov et al., 2004; Uezono et al., 2006). This GB_1R/GB_2R heterodimerization is indispensable for the molecule to locate within the plasma membrane (Couve et al., 1998). Additionally, the two molecules have different functions within the dimers: agonists bind only to GB_1R, while GB_2R transduces the signal though the trimeric G proteins (Gα and Gβγ subunits) to downstream molecules (Galvez et al., 2001; Uezono et al., 2006).

Desensitization and subsequent internalization of these receptors by agonist stimulation is important to avoid their overstimulation and to terminate their agonist-induced signaling (Kelly et al., 2008). The initial step in this process is thought to involve phosphorylation of the receptors by G protein-coupled receptor kinases (GRKs) or second-messenger-regulated kinases, such as protein kinase C or A (Kelly et al., 2008). β-arrestins, composed of β-arrestin1 (βarr1) and β-arrestin2 (βarr2), were identified as proteins that have the ability to desensitize GPCR, and further recognized as endocytic adapters and trafficking mediators of a variety of cell-surface receptors, including GPCRs (Shenoy and Lefkowitz, 2011). Once receptors are phosphorylated by several kinases, βarr1 or βarr2 bind to the phosphorylated receptors, forming the receptor/β-arrestin complex, which is then internalized (Gainetdinov et al., 2004).

Phosphorylation of $GABA_BR$ is unique compared with that of common GPCRs such as β-adrenergic receptor and the μ-opioid receptors (μOR). We and others have previously shown that GRK4 and GRK5 but not GRK2, GRK3, or GRK6 are involved in the GABA- or baclofen-mediated $GABA_BR$ desensitization processes (Ando et al., 2011; Kanaide et al., 2007; Perroy et al., 2003). However, the receptors were not phosphorylated by these kinases (Kanaide et al., 2007; Perroy et al., 2003). Accordingly, GRK4 and GRK5 seem to function solely as anchoring proteins, not as kinases (Kanaide et al., 2007; Perroy et al., 2003; Terunuma et al., 2010).

There are contradictory reports regarding agonist-induced $GABA_BR$ internalization, with recent studies demonstrating that $GABA_BR$ is not internalized by agonist stimulation (Fairfax et al., 2004; Grampp et al., 2007; Perroy et al., 2003; Vargas et al., 2008), while earlier studies showed the opposite. In addition, one report has shown that $GABA_BR$ is constitutively internalized and that this process is accelerated by $GABA_BR$-agonist stimulation (Wilkins et al., 2008).

Although β-arrestins are key proteins in most GPCR internalization, baclofen did not recruit β-arrestins to the plasma membrane and failed to form a complex with $GABA_BR$ (Fairfax et al., 2004; Perroy et al., 2003). However, the studies reporting these results were not performed in real time. More recent reports have shown that $GABA_BR$ is constitutively internalized into the cytosol without receptor activation (Grampp et al., 2007; Vargas et al., 2008; Wilkins et al., 2008). Although investigation of activation and inactivation steps of $GABA_BR$ are necessary to understand GABA-induced regulation in the central and peripheral nervous systems, one of the most important receptor-mediated signaling such as interaction of $GABA_BR$ and β-arrestins is not well understood.

In this study, we focused on the interaction of $GABA_BR$ with β-arrestins. We used a real-time analysis with and without stimulation of the agonist. Additionally, we visually analyzed protein-complex formation using fluorescent protein-fused $GABA_BR$ with fluorescent protein-fused β-arrestins in a fluorescence resonance energy transfer (FRET) assay developed by our laboratory (Ando et al., 2011; Kanaide et al., 2007; Uezono et al., 2006). FRET is a useful tool to quantify molecular dynamics in biophysics and biochemistry, such as protein–protein interactions and protein conformational changes (Miyawaki and Tsien, 2000). We compared results from real-time visualization and FRET efficiency of fluorescent protein-tagged $GABA_BR$ complexed to β-arrestins with corresponding results from fluorescent protein-tagged μOR combined to β-arrestins. μOR is well known to interact with β-arrestins and consequently be internalized (Gainetdinov et al., 2004; Groer et al., 2011). This analysis showed that $GABA_BR$ did not associate with β-arrestin, regardless of agonist stimulation.

MATERIALS AND METHODS

Drugs and chemicals

Baclofen was purchased from Tocris Cookson (Bristol, UK). DAMGO ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin) was purchased from Sigma (St. Louis, MO). All other chemicals used were of analytical grade and were obtained from Nacalai Tesque (Kyoto, Japan).

Construction of cDNAs

Human $GB_{1a}R$ and GB_2R clones were generously provided by Dr. N.J. Fraser (Glaxo Wellcome, Stevenage, UK). Cerulean, a brighter variant of cyan fluorescent protein (Rizzo et al., 2004) was provided by Dr. D.W. Piston (Vanderbilt University, Nashville, TN), and Venus, a brighter variant of yellow fluorescent protein (Nagai et al., 2002), was provided by Dr. T. Nagai (Hokkaido Univ., Sapporo, Japan). Human GRK4 was provided by Dr. A. De Blasi (Neuromed, IRCCS, Pozzilli, Italy); bovine GRK2 were provided by Dr. J.L. Benovic (Thomas Jefferson University, Philadelphia, PA). Rat βarr1 and βarr2 were provided by Dr. Nagayama (Nagasaki Univ., Nagasaki, Japan). Rat μOR was provided by Dr. N. Dascal (Tel Aviv Univ., Tel Aviv, Israel). Venus-fused $GABA_BR$ and μOR, and Cerulean-fused βarr1 and βarr2 were created by ligating their cDNA sequences into the *Not*I I sites of the corresponding Venus or Cerulean sites, as reported previously (Ando

et al., 2011; Kanaide et al., 2007; Uezono et al., 2006). All cDNAs for transfection into cells were subcloned into pcDNA3.1 (Invitrogen, San Diego, CA).

Cell culture and transfection

BHK cells were grown in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C and 5% CO_2. For transfection experiments, BHK cells were seeded at a density of 1–2 × 10^5 cells/35-mm in glass-bottomed culture dishes (World Precision Instrument, Sarasota, FL) for 24 h. Transient transfection was then performed with Hilymax transfection reagent (Dojindo, Kumamoto, Japan) using a total of 0.6 μg cDNA, according to the manufacturer's protocol. Cells were analyzed under confocal microscopy 24 h after transfection.

Real-time monitoring of the mobilization of receptors and β-arrestins fused to fluorescence proteins

We constructed fluorescent-protein-fused GB_2R-V, μOR-V, βarr1-C, and βarr2-C molecules to visually monitor mobilization of receptors and β-arrestins. BHK cells that coexpressed GB_2R-V with βarr1-C or βarr2-C were treated with baclofen for the indicated periods, then placed in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline. Translocation or complex formation of $GB_{B2}R$-V and βarr-C was observed for 120 min. Cells coexpressing μOR-V and βarr1-C or βarr2-C were treated with the μOR agonist DAMGO in the same manner. For visualization, a 63× magnification and 1.25 numerical aperture oil immersion objective with pinhole was used. Both, Cerulean and Venus were excited by a 458-nm laser, and images were obtained by placing the dish onto a stage in a Zeiss LSM510 META confocal microscope (Carl Zeiss, Jena, Germany), as described previously (Kanaide et al., 2007; Uezono et al., 2006).

Confocal microscopy for FRET analysis

For the analysis of protein-complex formation of GB_2R-V and μOR-V with β-arr1-C or β-arr2-C, we used the FRET assay. $GB_{1a}R$ or GB_2R-V was coexpressed with either β-arr1-C or β-arr2-C in BHK cells. These BHK cells were cultured in 35-mm glass-bottomed dishes and cotransfected with each of DNA. Both, Cerulean and Venus were excited by a 458-nm laser, and images were obtained by placing the dish onto a stage in a Zeiss LSM510 META confocal microscope.

Photobleaching and calculation of FRET efficiency

To confirm FRET to find association of Cerulean- and Venus-fused proteins, we monitored acceptor photobleaching analysis in BHK cells that coexpressed $GB_{1a}R$, GB_2R-V, βarr2-C, and GRK4, or cells that coexpressed μOR-V, βarr2-C, and GRK2. FRET was measured by imaging Cerulean before and after photobleaching Venus with 100% intensity from a 514-nm argon laser for 1 min, a duration that efficiently bleached Venus with little effect on Cerulean. An increase of donor fluorescence (Cerulean) was interpreted as evidence of FRET from Cerulean to Venus. All experiments were analyzed from at least six cells with three independent regions from their plasma membranes. As a control, we examined the FRET efficiency of the unbleached area of plasma membranes from at least three areas in the same cell. In some cases, we performed the photobleaching assay using BHK cells coexpressing Venus + Cerulean, or GB1aR-C + GB2R-V + βarr2 +GRK4 as negative and positive controls of FRET, respectively. The photobleaching assay was performed as previously described (Kanaide et al., 2007).

FRET efficiency was calculated using emission spectra before and after acceptor photobleaching of Venus (Miyawaki and Tsien, 2000). According to this procedure, if FRET is occurring, then photobleaching of the acceptor (Venus) should yield a significant increase in fluorescence of the donor (Cerulean). Increase of donor spectra due to desensitized acceptor was measured by the Cerulean emission (at 488 nm) from spectra before and after acceptor photobleaching. FRET efficiency was then calculated using the equation $E = 1 - I_{DA}/I_D$, where I_{DA} is the peak of donor (Cerulean) emission in the presence of the acceptor, and I_D is the peak in the presence of the sensitized acceptor, as previously described (Riven et al., 2003). Before and after this bleaching, Cerulean images were collected to assess changes in donor fluorescence.

Statistical analysis

Data are expressed as mean ± SEM. Differences between two groups were examined for statistical significance using paired t test. GraphPad Prism software (San Diego, CA) was used to analyze data for statistical significance and to fit curves. For comparisons between multiple groups, one-way analysis of variance was used, followed by Scheffe's test. A P value of less than 0.05 was classified as statistically significant.

RESULTS

Fluorescence-tagged GABA$_B$R activation of GIRK channels

In this study, we used fluorescence protein-tagged GABA$_B$R instead of wild type GABA$_B$R. This is because, as we previously showed, fluorescence-tagged $GB_{1a}R$-Venus or GB_2R-Venus behave like non-tagged, wild type GABA$_B$R. That is, they display G

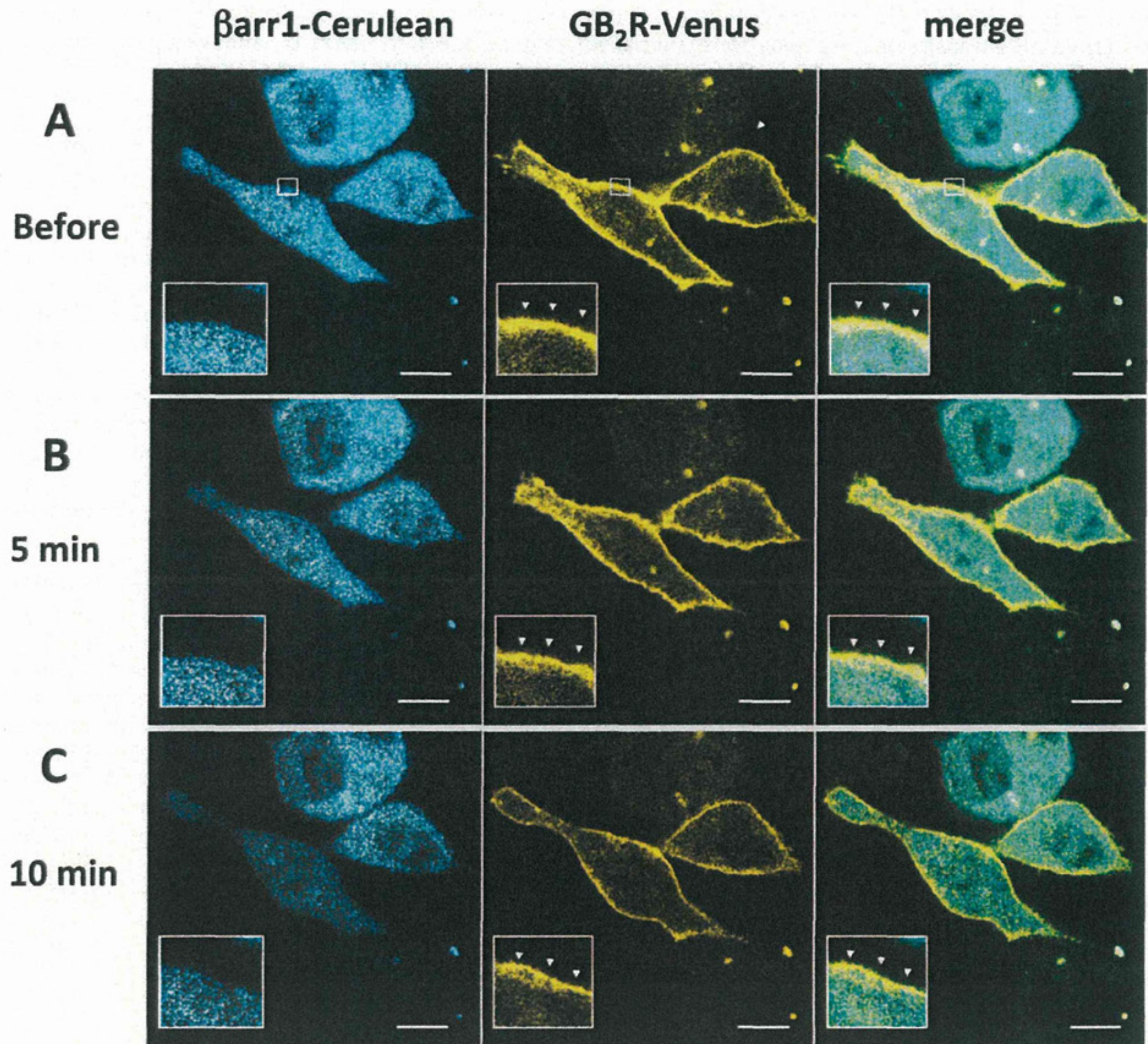

Fig. 1. Confocal imaging of the translocation of GB_2R-V and βarr2-C in BHK cells expressing $GB_{1a}R$, GB_2R-V, βarr2-C, and GRK4. Visualization of GB_2R-V and βarr2-C in BHK cells before (**A**), and 5 min (**B**) and 10 min (**C**) after stimulation with 10^{-4} M baclofen. Arrowheads show GB_2R-V on the plasma membranes. Similar results were obtained in at least six independent experiments. Calibration bar = 10 μm.

protein-activated inwardly rectifying potassium-channel activation, channels known to be activated by $GABA_BR$ stimulation (Ando et al., 2011; Kanaide et al., 2007; Uezono et al., 1998, 2006).

Activation of $GABA_BR$ did not induce receptor internalization

We first determined the distribution and translocation of functional $GABA_BR$ and β-arrestins in BHK cells and examined whether β-arrestins were able to associate with $GABA_BR$ in response to receptor stimulation. In living BHK cells, coexpressing $GB_{1a}R/GB_2R$-V and GRK4 with βarr1-C or βarr2-C, both βarr1 and βarr2 proteins were diffusely distributed in the cytosol (Figs. 1 and 2). GB_2R-V was expressed exclusively on the plasma membrane (Figs. 1 and 2). Although expression of $GB_{1a}R$ on the plasma membrane was not found, our previous reports showed that $GB_{1a}R$ and GB_2R formed heterodimers on the plasma membranes during the same experimental procedure (Uezono et al., 2006). In this study, when cells expressing $GB_{1a}R/GB_2R$-V, βarr2-C, and GRK4 received baclofen at 10^{-4} M for 5 min or GABA at 10^{-4} M for 5 min (data not shown), both agonists failed to mobilize βarr1-C from the cytosol to plasma

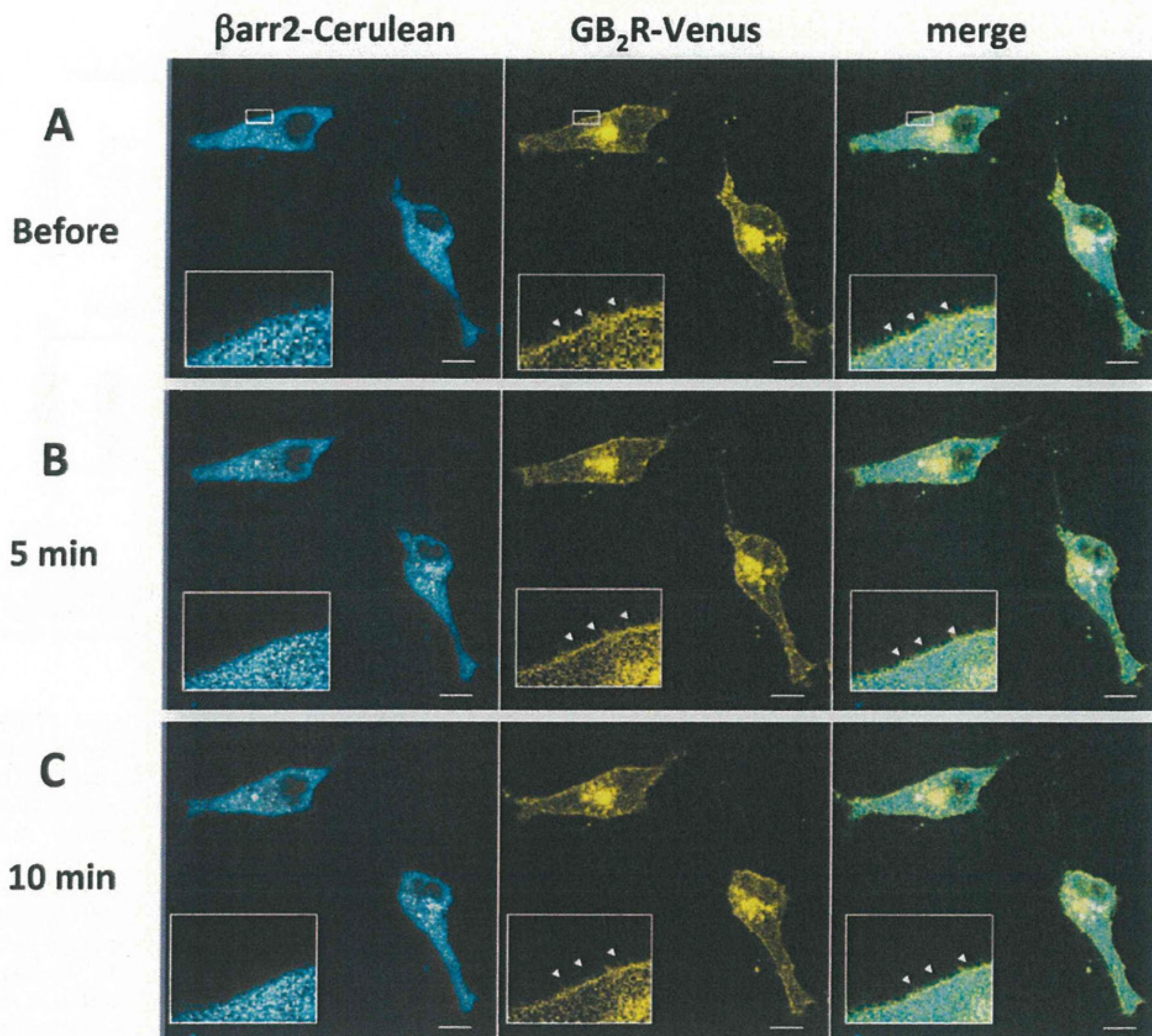

Fig. 2. Confocal imaging of the translocation of GB$_2$R-V and βarr1-C in BHK cells expressing GB$_{1a}$R, GB$_2$R-V, βarr1-C, and GRK4. Visualization of the GB$_2$R-V and βarr1-C in BHK cells before (**A**), and 5 min (**B**) and 10 min (**C**) after stimulation with 10^{-4}M baclofen. Arrowheads show GB$_2$R-V on the plasma membranes. Similar results were obtained in at least six independent experiments. Calibration bar = 10 μm.

membranes (Fig. 1) for up to 120 min (data not shown). The concentration of baclofen and GABA and duration chosen were those causing submaximal cellular responses and the translocation of GRK4 to the plasma membrane, with subsequent formation of the GB$_2$R/GRK4 complex that would desensitize GABA$_B$R (Ando et al., 2011; Kanaide et al., 2007; Uezono et al., 1998, 2006). Similar results were observed in cells coexpressing βarr2-C instead of βarr1-C (Fig. 2).

For the real-time critical measurement of the intensity of GB$_2$R-V combined with βarr2-C or βarr1-C, we calculated the intensity strength within the cytosolic area, shown as the red line in Figure 3A. Results showed that, when activated by baclofen at 10^{-4} M, intensities of both GB$_2$R-V and βarr2-C gradually decreased. This was possibly due to quenching by exposure to laser power (Fig. 3). As shown in Figure 3C, there were almost no changes, for up to 120 min, in the intensities of GB$_2$R-V and βarr2-C after stimulation by baclofen (data not shown), indicating no internalization of GB$_2$R-V into the cytosol and no movement of βarr2-C at any time examined. Similar results were observed in cells expressing GB$_2$R-V with βarr1-C (Fig. 3D). We also examined constitutive internalization of GB$_2$R in BHK cells coexpressing GB$_{1a}$R/GB$_2$R-V, βarr2-C, and GRK4; for up to 120 min, we were unable to detect any internalization profiles of GB$_2$R-V or any mobilization of βarr2-C (data not shown).

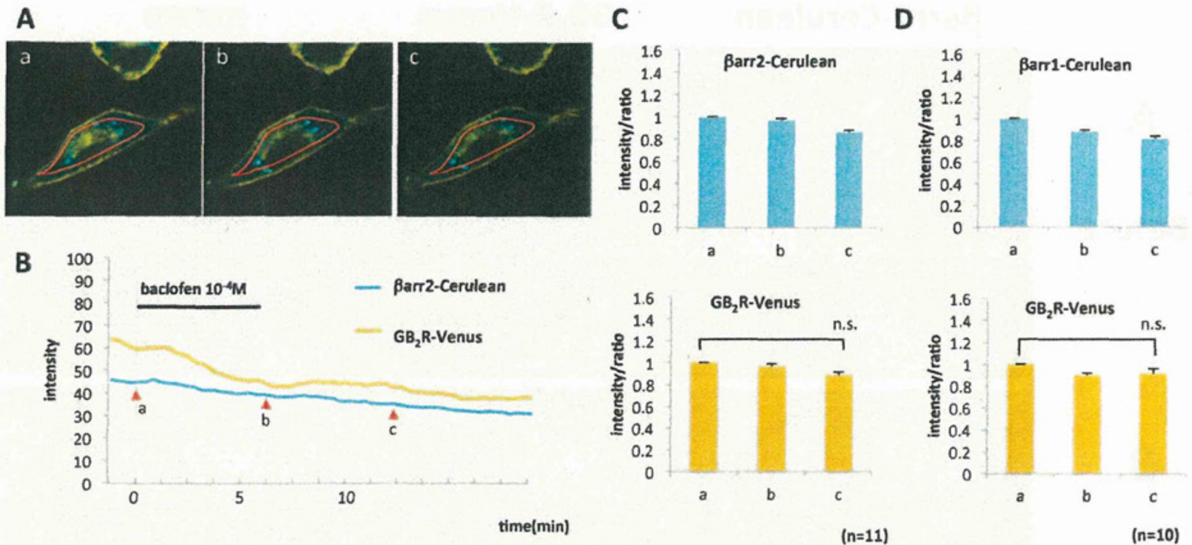

Fig. 3. Time courses of changes in intensities of GB_2R-V, βarr2-C, or βarr1-C in BHK cells. **A**: Confocal imaging of the BHK cells expressing $GB_{1a}R$, GB_2R-V, βarr2-C, and GRK4. For calculation, intensities of the areas within the red line (cytosol) were measured. **B**: Changes in intensities before (a), and 5 min (b) and 10 min (c) after stimulation of baclofen (10^{-4} M) in real time. **C**: Intensity ratio of βarr2-C and GB_2R-V at the indicated points as in (B). **D**: Intensity ratio of βarr1-C and GB_2R-V at the indicated points as in (B). Data were expressed as intensity ratio of the level at "b" or "c"/the level at "a."

We then investigated μOR internalization induced by the μOR agonist DAMGO. In cells expressing μOR-V, GRK2, and βarr1-C or βarr2-C, we applied DAMGO at 10^{-7} M for 5 min, the concentration and duration that cause submaximal μOR activation and receptor desensitization. This resulted in dramatic translocation of βarr1-C and βarr2-C into the plasma membranes and subsequent internalization with μOR-V (Figs. 4 and 5). In the same cells, βarr1-C or βarr2-C were translocated and concentrated into dot-like shapes on the plasma membranes for the first 4–5 min stimulation by DAMGO, as indicated by arrowheads. Consequently, βarr1-C or βarr2-C was eventually internalized into the cytosol (Figs. 4 and 5).

DAMGO-induced βarr1-C or βarr2-C internalization and translocation into the cells were also measured, as shown in Figure 6. The intensity of μOR-V was increased by application of DAMGO, and small granules were observed, shown within the area of the red line (Fig. 6A). In addition, corresponding decreases of βarr2-C intensity were also detected. As shown in Figures 6B and 6C, increases in the intensities of μOR-V and decreases in βarr2-C were detected in a time-dependent manner (Figs. 6B and 6C). Similar results were observed in cells expressing μOR-V, GRK2, and βarr1-C instead of βarr2-C (Fig. 6D).

FRET and acceptor photobleaching analysis of BHK cells coexpressing $GB_{1a}R$ and GB_2R-V with βarr2-C

Because βarr1-C or βarr2-C was translocated into the plasma membranes following μOR activation, but not following $GABA_BR$ activation, we used FRET analysis to determine whether βarr2-C interacts and forms a protein complex with GB_2R. We used BHK cells that coexpressed $GB_{1a}R$, $GB_{B2}R$-V, βarr2-C, and GRK4 or cells that coexpressed μOR-V, βarr2-C, and GRK2.

In such cells, photobleaching analysis of baclofen-stimulated cells demonstrated that FRET efficiency at the cell membranes was not remarkably changed at 5 min after stimulation with baclofen at 10^{-4} M (Fig. 7A). FRET efficiency before stimulation, and 1, 2, 5, 10, 30, and 60 min (and 120 min, data not shown) after stimulation was not significantly changed (Fig. 7B). Similar results were obtained when we used βarr1-C instead of βarr2-C (data not shown). In contrast, photobleaching analysis of DAMGO-stimulated BHK cells demonstrated that FRET efficiency was increased 1 min after stimulation and reached maximal value at 5 min, then gradually decreased for 30 min (Fig. 7B), demonstrating that the receptor/β-arrestin complex was formed by stimulation of the agonist.

DISCUSSION

Recent study has shown that DAMGO activation of μOR recruited both βarr1 and βarr2 to the μOR then cause robust internalization in mouse embryonic fibroblasts (mef; Groer et al., 2011). They showed no internalization profiles of μOR by DAMGO in mef derived from βarr1/βarr2 knockout mice. Furthermore, βarr1 or βarr2 reintroduction into these mef

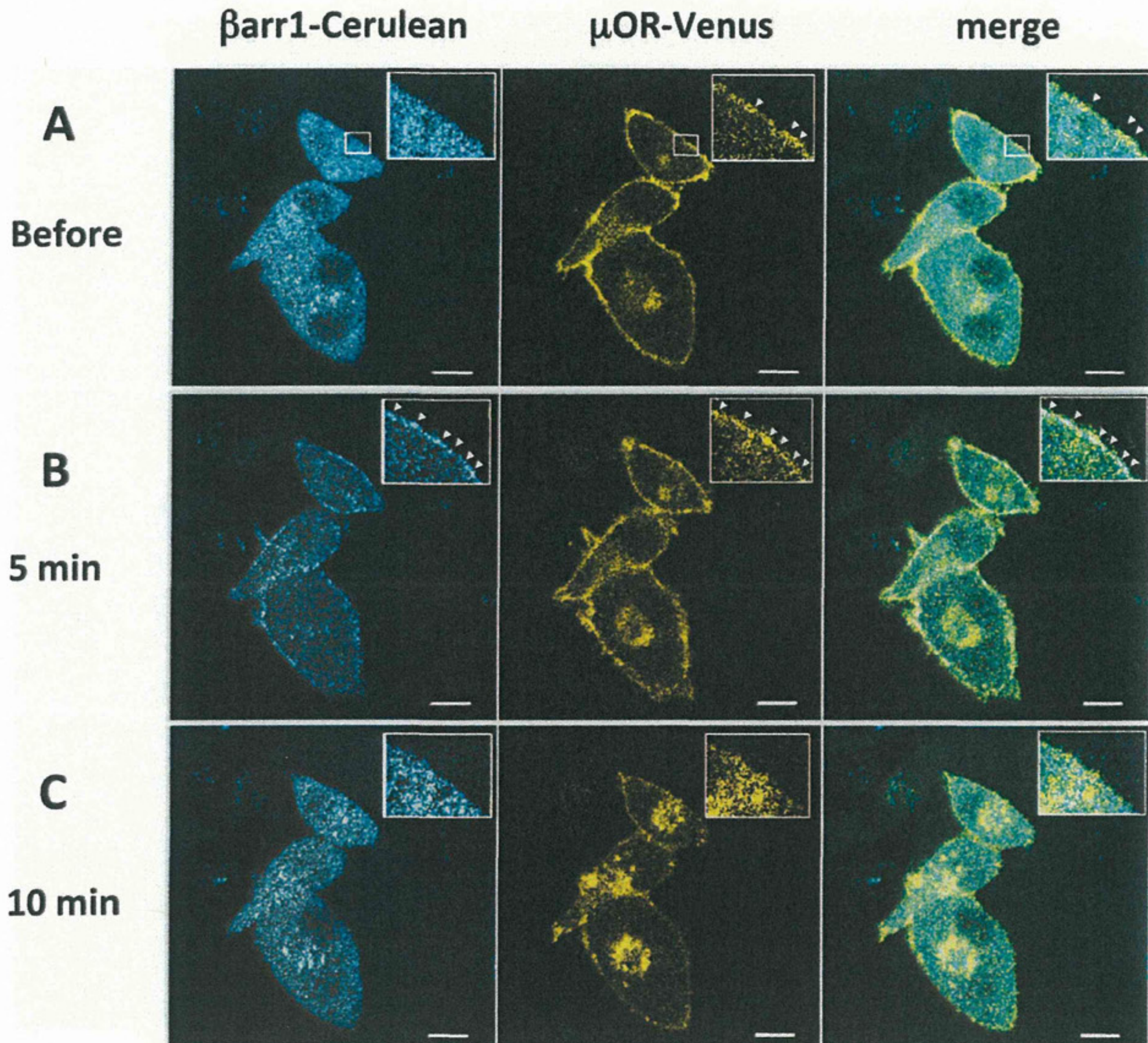

Fig. 4. Confocal imaging of the translocation of μOR-V and βarr1-C and in BHK cells expressing μOR-V, βarr1-C, and GRK2. Visualization of μOR-V and βarr1-C in BHK cells before (**A**) and 5 min (**B**) and 10 min (**C**) after stimulation with 10^{-7} M DAMGO. Arrowheads show μOR and βarr1-C on the plasma membranes. Similar results were obtained in at least six independent experiments. Calibration bar = 10 μm.

rescued DAMGO-induced μOR internalization (Groer et al., 2011), demonstrating that interaction of βarr1 or βarr2 with μOR is required for μOR internalization.

In this study, we showed that fluorescent protein-tagged GABA$_B$R failed to internalize on stimulation by the GABA$_B$R agonist baclofen or GABA. This was true in the presence of βarr1, βarr2, and even GRK4, one of the kinases that causes GABA$_B$R desensitization (Kanaide et al., 2007; Perroy et al., 2003). We also showed that neither βarr1 nor βarr2 were able to form a GB$_2$R-V/β-arrestins-C complex on the plasma membrane before or after stimulation with baclofen, which was determined by FRET analysis. By contrast, using the same experimental system, μOR-Venus was found to internalize to the cytosol on stimulation by μOR agonist DAMGO, with the formation of μOR/β-arrestin complex on the cell surface in the presence of GRK2, as previously shown by Groer et al. (2011). These results suggest lack of β-arrestin association with GABA$_B$R correlated with in the lack of internalization of GABA$_B$R by baclofen.

We further showed, with a real-time assay, that stimulation with baclofen for up to 120 min failed to cause the internalization of GABA$_B$R into the cytosol. This result is in accordance with several previous

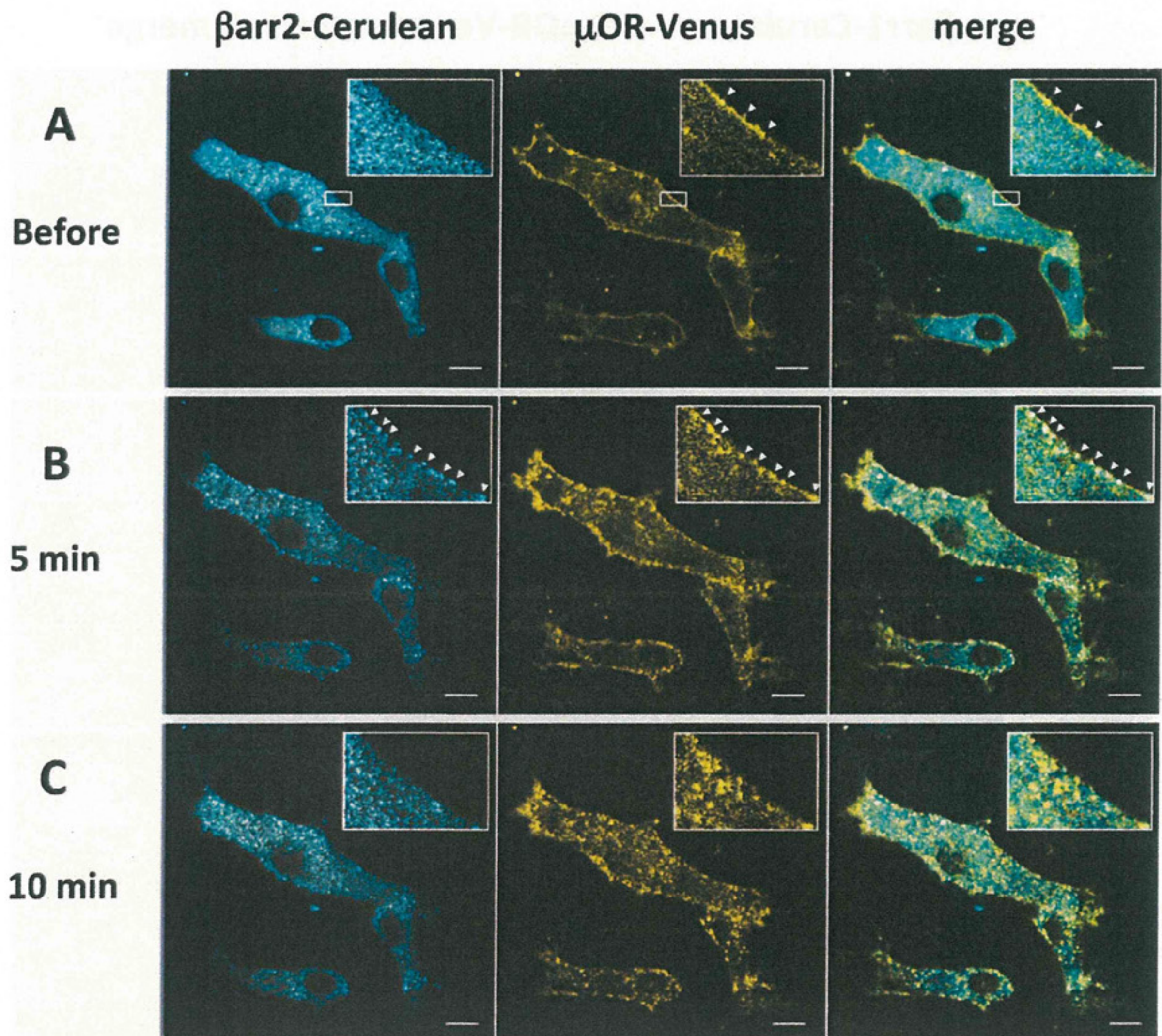

Fig. 5. Confocal imaging of the translocation of μOR-V and βarr2-C in BHK cells expressing μOR-V, βarr2-C, and GRK2. Visualization of μOR-V and βarr2-C in BHK cells before (**A**) and 5 min (**B**) and 10 min (**C**) after stimulation with 10^{-7} M DAMGO. Arrowheads show μOR and βarr2-C on the plasma membranes. Similar results were obtained in at least six independent experiments. Calibration bar = 10 μm.

reports that $GABA_BR$ does not internalize on stimulation with $GABA_BR$ agonists (Fairfax et al., 2004; Grampp et al., 2007; Laffray et al., 2007; Perroy et al., 2003). One early study, by contrast, showed that $GABA_{B1}R$ tagged with cyan fluorescent protein and $GABA_{B2}R$ tagged with yellow fluorescent protein were both internalized after treatment with baclofen. However, this happened at only one time point (2 h after stimulation of GABA at 10^{-4} M), and the study did not monitor the intensities of the fluorescence in both the plasma membrane and cytosol (González-Maeso et al., 2003). The discrepancy of this result compared with other studies probably results from the different cell types and experimental designs used.

We also studied the role of phosphorylation in these processes. Specifically, phosphorylation of GPCRs by several protein kinases, such as the GRKs, plays a role in the desensitization and internalization of most of these receptors (Kelly et al., 2008; Luttrell and Lefkowitz, 2002). $GABA_BR$ phosphorylation is unique in that, though some GRKs are involved in GABA- or baclofen-mediated $GABA_BR$ desensitization (especially GRK4 and 5 but not GRK2, 3, or 6), these kinases do not phosphorylate the receptors (Kanaide et al., 2007; Perroy et al., 2003). These results suggest that GRK4 and GRK5 may behave as anchoring proteins instead of as kinases (Kanaide et al., 2007; Perroy et al., 2003; Terunuma et al., 2010).