mediated increase in $[Ca^{2+}]_i$ (Fig. 1E, F and H). Moreover, we showed the co-localization of the NK-1 receptor and GFAP, which is a defined marker for astrocytes, by immunofluorescence staining (Fig. 2). In addition, it was indicated that the NK-1 receptor is mostly expressed in the plasma membrane of spinal astrocytes. Taken together, these data suggest that SP induces the increase in $[Ca^{2+}]_i$ in spinal astrocytes through stimulation of the NK-1 receptor.

In addition, we examined the effect of GR73632, a selective agonist of the NK-1 receptor, on the increase in $[Ca^{2+}]_i$. As shown in Fig. 3A–C and E, treatment with GR73632 at a concentration range of 10–1000 nM evoked a transient and sustained increase in $[Ca^{2+}]_i$ in a dose-dependent manner. The Ca^{2+} response activated by GR73632 was similar to that following SP exposure. In addition, preincubation with CP-96346 specifically blocked the GR73632-induced increase of $[Ca^{2+}]_i$ (Fig. 3D and F). As these data suggest that the NK-1 receptor contributes to the mobilization of $[Ca^{2+}]_i$, GR73632 was used for further investigation of the NK-1 receptor-mediated increase in $[Ca^{2+}]_i$ by spinal astrocytes.

3.2. Regulation of both Ca^{2+} release from Ca^{2+} stores and the influx of extracellular Ca^{2+} in spinal astrocytes by activation of the NK-1 receptor

As the regulation of $[Ca^{2+}]_i$ is associated with both the release of Ca^{2+} from intracellular Ca^{2+} stores and the influx of extracellular Ca^{2+}, the involvement of both of these processes in the GR73632-induced increase in $[Ca^{2+}]_i$, was examined by stimulating spinal astrocytes with 1000 nM GR73632 in Hanks' buffer with or without Ca^{2+}. In the presence of extracellular Ca^{2+} (1.3 mM), GR73632 induced both a transient and sustained increase of $[Ca^{2+}]_i$ as shown in Fig. 4A. On the other hand, in the absence of extracellular Ca^{2+}, only a transient increase in $[Ca^{2+}]_i$, which rapidly peaked and returned toward the basal level within 2 min after $[Ca^{2+}]_i$ reached to a peak, was observed after treatment with GR73632 (Fig. 4B). In addition, the increase in $[Ca^{2+}]_i$ induced by GR73632 was completely attenuated in the Ca^{2+}-free Hanks' buffer by preincubation with thapsigargin (1 μM), which depletes Ca^{2+} in intracellular Ca^{2+} stores by inhibiting Ca^{2+}-ATPase (Fig. 4C). Taken together, these data suggest that GR73632 induces both Ca^{2+} release from Ca^{2+} stores and also Ca^{2+} influx. Therefore, we defined the change in $[Ca^{2+}]_i$ at the transient peak to be the result of the Ca^{2+} release from Ca^{2+} stores (Fig. 4D). On the other hand, the change in $[Ca^{2+}]_i$ (compared to baseline) 2 min after the peak $[Ca^{2+}]_i$ was defined as the extent of Ca^{2+} influx (Fig. 4E).

3.3. Influence of intracellular signaling molecules on the GR73632-induced increase of $[Ca^{2+}]_i$ by spinal astrocytes

It is well known that stimulation of the NK-1 receptor activates PLC, which produces both inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) by the breakdown of phosphatidylinositol

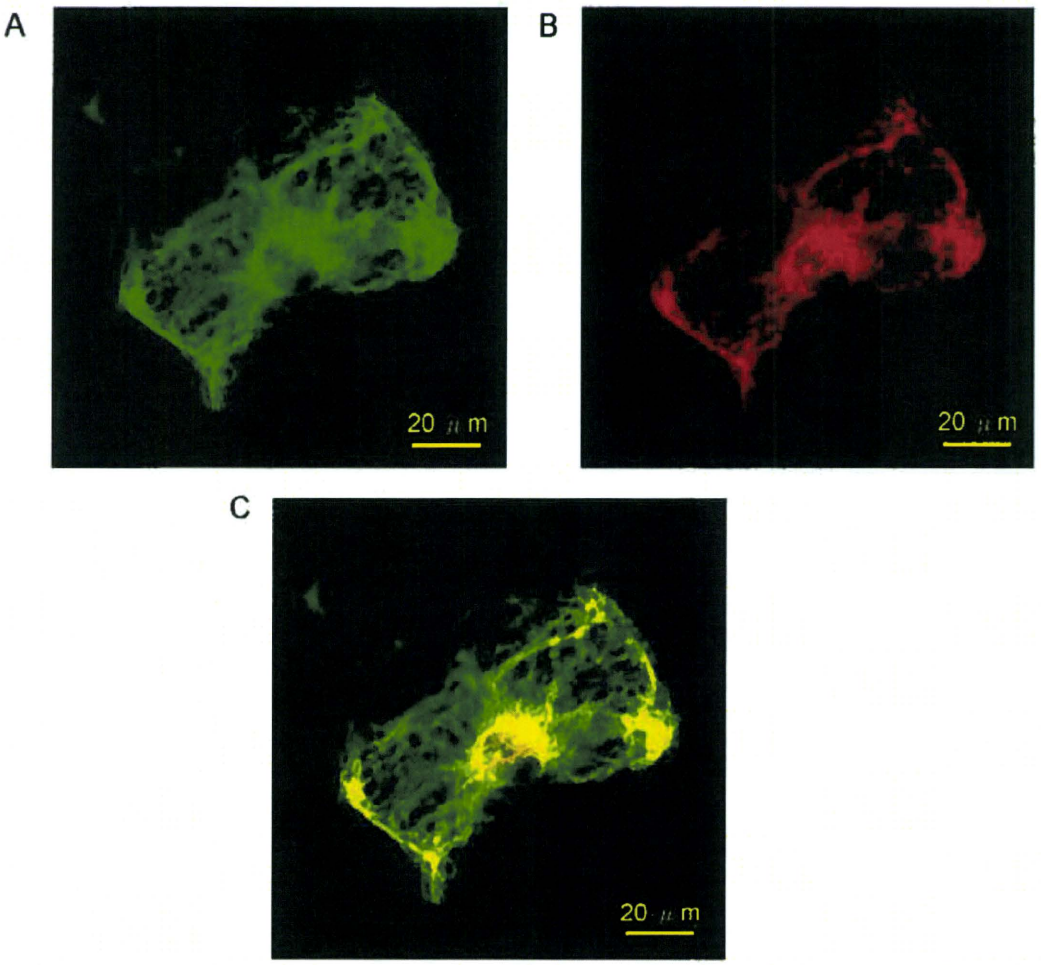

Fig. 2. Spinal astrocytes express the NK-1 receptor. Immunofluorescent analysis of GFAP (green; A) and the NK-1 receptor (red; B) expression in cultured spinal astrocytes. The expression of the NK-1 receptor was found in GFAP-labeled cells (C).

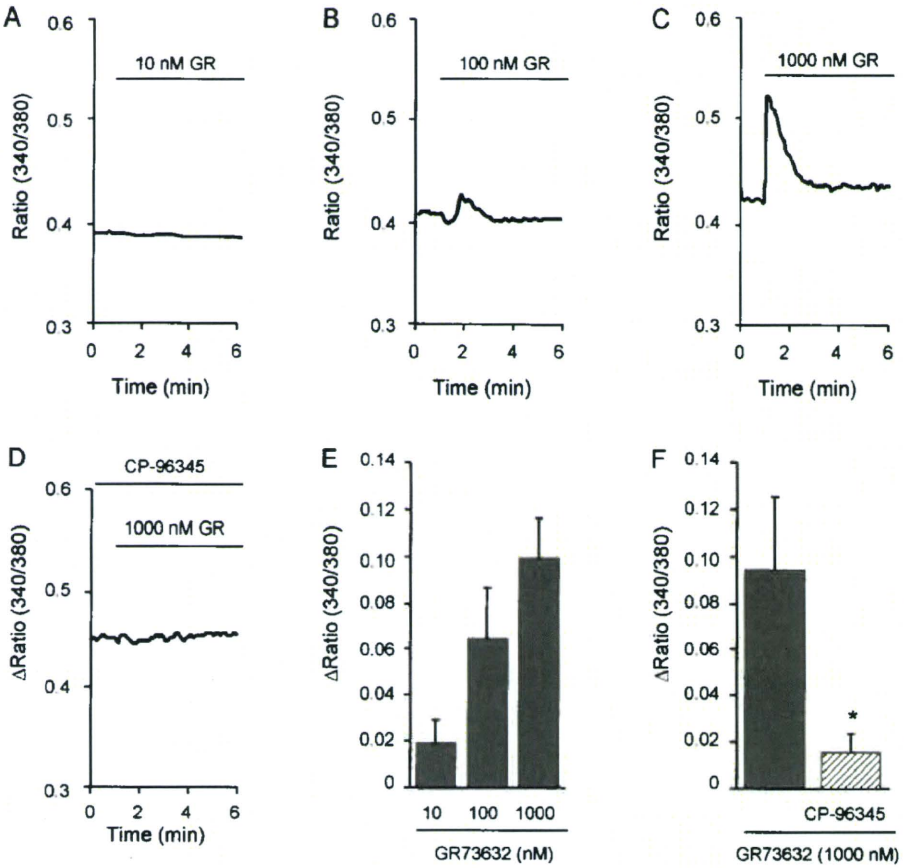

Fig. 3. Mobilization of $[Ca^{2+}]_i$ in spinal astrocytes stimulated with GR73632. The trace in each graph (A–D) shows the representative mean $[Ca^{2+}]_i$ in randomly selected cells. The fura-2-loaded cells were treated with 10–1000 nM of GR73632 (GR) in Hanks' buffer (A–C). After the cells were pretreated with 10 μM of CP96345 (D) for 20 min, then cells were stimulated with 1000 nM of GR73632. The extent of the increase in $[Ca^{2+}]_i$ induced by GR73632 was quantified by determining the differences between the ratio (340/380) of the basal and the peak level obtained after GR73632 treatment (E and F). The data are expressed as the means ± S.E.M. (*bars*) of separate experiments. $^*p < 0.05$ in comparison with the value for the cells treated with GR73632 alone.

4,5-bisphosphates. Therefore, we investigated the involvement of PLC and/or the IP_3 receptor in the increase of $[Ca^{2+}]_i$ following treatment with 1000 nM of GR73632. Pretreatment with U73122 (10 μM), a PLC inhibitor, or xestospongin C (1 μM), an inihibitor of the IP_3 receptor, completely inhibited the GR73632-induced increase in $[Ca^{2+}]_i$ (Fig. 5A–C). In addition, 2-APB, which inhibits both the IP_3 receptor and the subsequent Ca^{2+} influx (Zhou et al., 2007), also significantly suppressed the action of GR73632 (Fig. 5D). Quantitative data showed that all of these inhibitors blocked both Ca^{2+} release from intracellular Ca^{2+} stores and extracellular Ca^{2+} influx caused by GR73632 (Fig. 5G and H). These data suggest that the GR73632-mediated increase in $[Ca^{2+}]_i$ involves the activation of PLC and the IP_3 receptor.

Since activation of the NK-1 receptor is likely to be coupled to both Gq- and Gs-proteins (Holst et al., 2001), PKA may also be activated by stimulation of the NK-1 receptor. Therefore, we compared the influence of inhibitors of either PKA or PKC on the GR73632-induced increase in $[Ca^{2+}]_i$. Preincubation with H89 (10 μM), a PKA inhibitor, attenuated the GR73632-mediated increase in $[Ca^{2+}]_i$ (Fig. 5F). In contrast, pretreatment with BIM (10 μM), a PKC inhibitor, significantly enhanced the effect of GR73632 (Fig. 5E). Quantitative analysis data indicated that H89 significantly blocked the GR73632-induced Ca^{2+} release from intracellular Ca^{2+} stores, but did not affect the influx of extracellular Ca^{2+} (Fig. 5G and H). On the other hand, BIM (10 μM) markedly enhanced the GR73632-mediated Ca^{2+} influx without affecting the Ca^{2+} release from intracellular stores (Fig. 5G and H).

To further elucidate the involvement of these intracellular signaling molecules in the GR73632-mediated increase in $[Ca^{2+}]_i$, we investigated the effects of 2-APB, H89 or BIM on the GR73632-induced changes in $[Ca^{2+}]_i$ under Ca^{2+} free conditions, and following the addition of Ca^{2+} in the buffer. As shown in Fig. 6B, after a rapid and transient increase in $[Ca^{2+}]_i$ induced by GR73632 in Ca^{2+}-free Hanks' buffer, the addition of $CaCl_2$ led to a sustained increase in $[Ca^{2+}]_i$, indicating that this response was due to the influx of extracellular Ca^{2+}. Pretreatment with 2-APB inhibited both components (release from stores and extracellular influx) evoked by GR73632 treatment (Fig. 6C, F and G). H89 significantly suppressed only the release of Ca^{2+} from intracellular stores (Fig. 6E–G). In contrast, pretreatment with BIM enhanced only the GR73632-induced Ca^{2+} influx, but not Ca^{2+} release (Fig. 6D, F and G). Taken together, these data suggest that PKA regulates the GR73632-induced Ca^{2+} release from intracellular Ca^{2+} stores, whereas PKC has a negative impact on the GR73632-induced influx of extracellular Ca^{2+}.

3.4. Involvement of TRPC channels in the GR73632-induced increase in $[Ca^{2+}]_i$ by spinal astrocytes

TRPC, non-selective cation channels, are classified into TRPC1–7 (Venkatachakam and Montell, 2007). As we found that TRPC1, 3, 4, 5, and 6 channels were expressed on spinal astrocytes using RT-PCR (Fig. 7A), we examined which subtypes of TRPC channel are involved in the GR73632-induced increase in $[Ca^{2+}]_i$ by using TRPC channel inhibitors. Either BTP2 (10 μM), a general blocker of TRPC

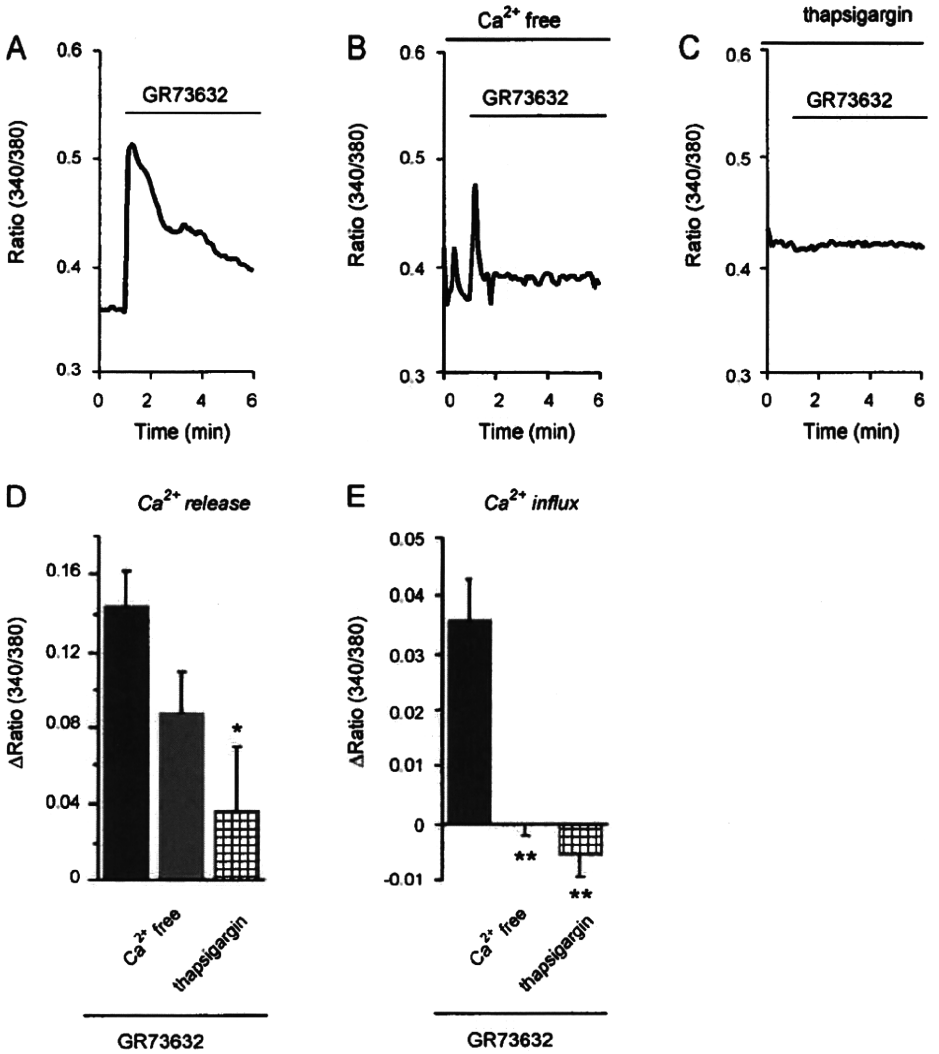

Fig. 4. Influence of both Ca^{2+} store and extracellular Ca^{2+} on the GR73632-induced increase in $[Ca^{2+}]_i$ in spinal astrocytes. The trace in each graph (A–C) shows the representative mean $[Ca^{2+}]_i$ in randomly selected cells. The fura-2-loaded cells were treated with 1000 nM of GR73632 in the presence (A) or absence (B and C) of Ca^{2+} in Hanks' buffer, respectively. After pre-treatment with 1 μM of thapsigargin (C) for 20 min in Ca^{2+}-free Hanks' buffer, the cells were stimulated with GR73632. The extent of Ca^{2+} release from intracellular Ca^{2+} stores induced by GR73632 was quantified by determining the differences between the ratio (340/380) of the basal and the peak level obtained after GR73632 treatment (D). The extent of the extracellular Ca^{2+} influx induced by GR73632 was quantified by determining the differences between the ratio (340/380) of the basal level and the level at 2 min after a peak $[Ca^{2+}]_i$ (E). The data are expressed as the means ± S.E.M. (*bars*) of separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ in comparison with the value for the cells treated with GR73632 alone.

channels, or Pyr3 (3 μM), a selective TRPC3 antagonist (Kiyonaka et al., 2009) inhibited only the transient increase of $[Ca^{2+}]_i$ as shown in Fig. 8A–C. Quantitative analysis data confirmed that these inhibitors completely inhibited the GR73632-mediated influx of extracellular Ca^{2+} without affecting the release of Ca^{2+} from intracellular Ca^{2+} stores (Fig. 8D and E). Furthermore, we found by immunofluorescence that the immunostaining for TRPC3 was co-localized with that of GFAP (Fig. 7B–D), thus indicating that TRPC3 is mostly expressed in the plasma membrane of spinal astrocytes. These findings suggest that the NK-1 receptor-stimulated extracellular Ca^{2+} influx might be mediated by TRPC3.

As mentioned above, PKC was thought to participate in the influx of extracellular Ca^{2+}. To confirm this hypothesis, further investigation was preformed to elucidate the relationship between PKC and TRPC channels. Co-treatment with either BTP2 or Pyr3 and BIM completely inhibited the GR73632-indeuced in $[Ca^{2+}]_i$ (Fig. 9A–D). Quantitative data indicated that not only BTP2, but also Pyr3, significantly blocked the GR73632-induced Ca^{2+} influx by BIM (Fig. 9E and F). Taken together, these data suggest that the GR73632-induced influx of extracellular Ca^{2+} through TRPC3 is negatively regulated by PKC.

4. Discussion

In this study, we found that SP evoked an increase in $[Ca^{2+}]_i$ in spinal astrocytes by the activation of PLC and the IP_3 receptor through the NK-1 receptor. This increase in $[Ca^{2+}]_i$ by stimulation of the NK-1 receptor was composed of both Ca^{2+} release from IP_3-sensitive intracellular Ca^{2+} store and extracellular Ca^{2+} influx through TRPC channels. Furthermore, we found that the former was positively modulated by PKA, and the latter was negatively regulated by PKC.

In our study, CP-96345 (a NK-1 receptor antagonist) was the most effective compound found to inhibit the SP-induced increase in $[Ca^{2+}]_i$. We also demonstrated that the NK-1 receptor is expressed in spinal astrocytes by staining with an NK-1 receptor antibody.

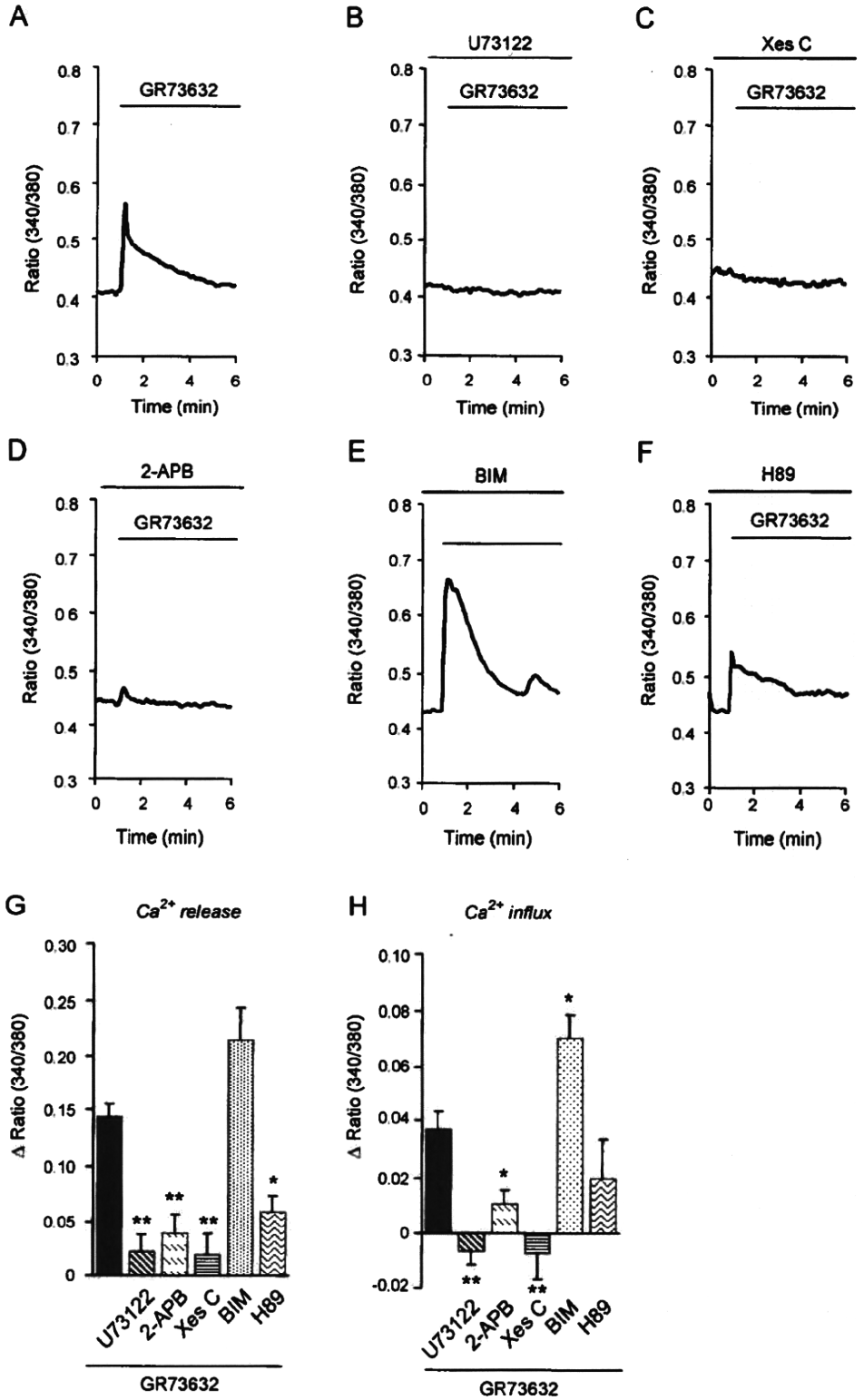

Fig. 5. Effects of inhibitors of several intracellular signaling molecules on the GR73632-induced increase in $[Ca^{2+}]_i$ in spinal astrocytes. The trace in each graph (A–F) shows the representative mean of $[Ca^{2+}]_i$ in randomly selected cells. The fura-2-loaded cells were treated with 1000 nM of GR73632 in Hanks' buffer (A–F). After the cells were pretreated with 10 μM U73122 (B), 1 μM xestospongin C (Xes C) (C), 100 μM 2-APB (D), 10 μM BIM (E) or 10 μM H89 (F) for 20 min, they were stimulated with GR73632. The extent of Ca^{2+} release from the intracellular Ca^{2+} stores induced by GR73632 was quantified by determining the differences between the ratio (340/380) of the basal and the peak level obtained after GR73632 treatment (G). The extent of extracellular Ca^{2+} influx induced by GR73632 was quantified by determining the differences between the ratio (340/380) of the basal level and the level at 2 min after a peak $[Ca^{2+}]_i$ (H). The data are expressed as the means ± S.E.M. (*bars*) of separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ in comparison with the value for the cells treated with GR73632 alone.

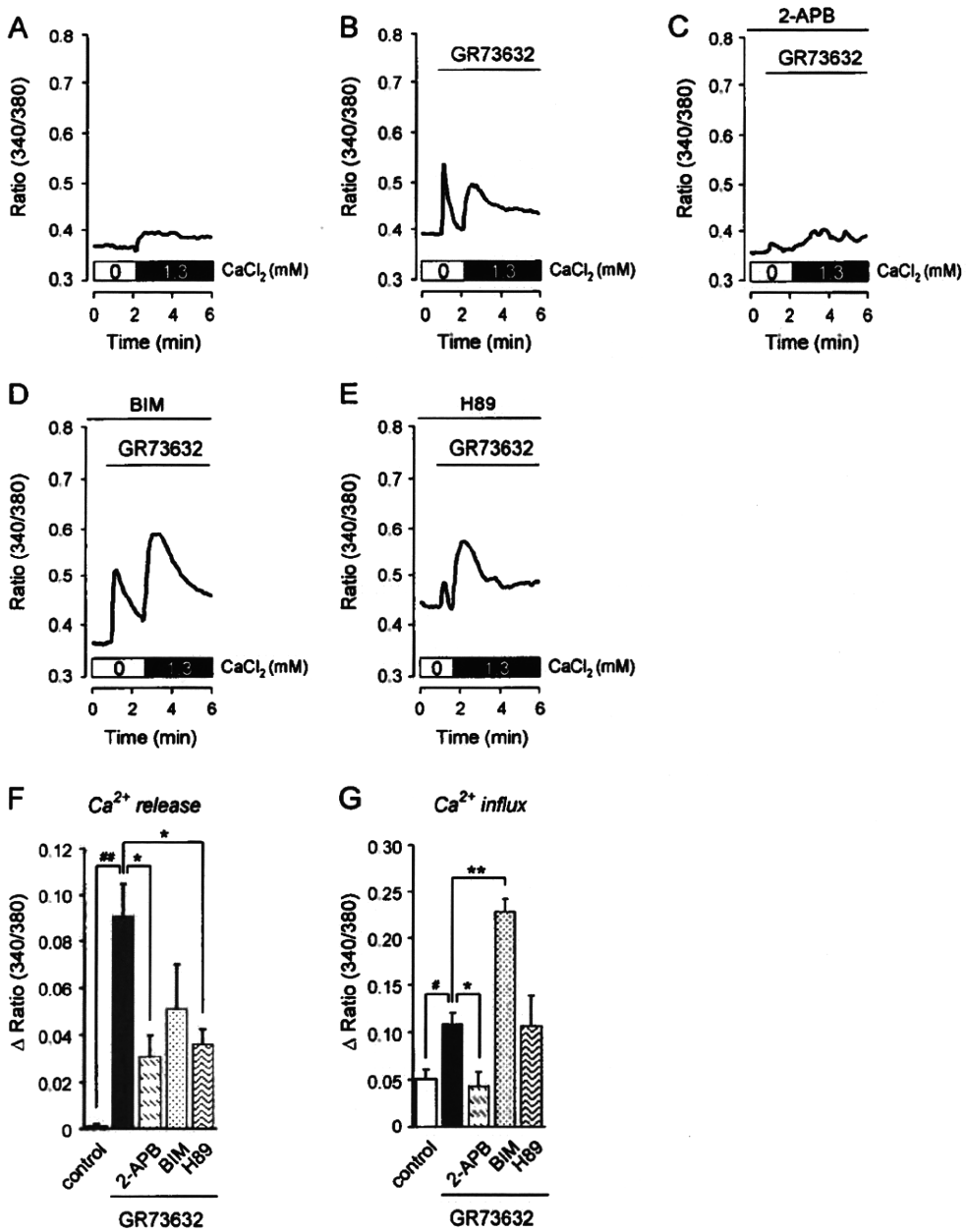

Fig. 6. Effects of 2-APB, BIM and H89 on both Ca^{2+} release and extracellular Ca^{2+} influx induced by GR73632 in spinal astrocytes. The trace in each graph (A–E) shows the representative mean $[Ca^{2+}]_i$ in randomly selected cells. The fura-2-loaded cells were either untreated (A) or were treated with (B–E) 1000 nM of GR73632 in Ca^{2+}-free Hanks' buffer, followed by the subsequent addition of 1.3 mM $CaCl_2$. After the cells were pretreated with 100 μM 2-APB (C), 10 μM BIM (D) or 10 μM H89 (E) for 20 min in Ca^{2+}-free Hanks' buffer, they were stimulated with GR73632. The extent of Ca^{2+} release from the intracellular Ca^{2+} stores induced by GR73632 was quantified by determining the differences between the ratio (340/380) of the basal and the peak level obtained after GR73632 treatment (G). The extent of extracellular Ca^{2+} influx induced by GR73632 was quantified by determining the differences between the ratio (340/380) of the level before addition of $CaCl_2$ and the peak level obtained after the addition (H). The data are expressed as the means ± S.E.M. (*bars*) of separate experiments. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ in comparison with the value for control. $^{*}p < 0.05$, $^{**}p < 0.01$ in comparison with the value for the cells treated with GR73632 alone.

According to radioligand binding studies in humans, the level of SP binding to the NK-1 receptor in spinal astrocytes was about six times that in brain astrocytes (Palma et al., 1997). Indeed, even 100 nM of SP did not have any effect on $[Ca^{2+}]_i$ in brain astrocytes (data not shown). Therefore, we would conclude that the SP-NK-1 receptor interaction might be strongly involved in the regulation of spinal astroglial functions, including $[Ca^{2+}]_i$ mobilization in the spinal cord. Moreover, GR73632, a selective NK-1 agonist which did not affect either NK-2 or -3 receptors (Maggi, 1995), was used to reveal the mechanisms of the NK-1 receptor-stimulated increase in $[Ca^{2+}]_i$ of spinal astrocyte in the present study. Hagan et al. (1991) and Meini et al. (1995) have demonstrated that GR73632 possess a higher constrictive effect than SP in both the rat urinary bladder and the guinea-pig trachea. In this study, however, the extent of Ca^{2+} response evoked by 1000 nM GR73632 was as same as that by 10 nM SP, indicating that a hundredfold dose of GR73632 is need to mimic the Ca^{2+} response by SP. In addition, there was a tendency for GR94800 (a NK-2 receptor antagonist) and SB222200 (a NK3 receptor antagonist) to attenuate the SP-evoked increase in $[Ca^{2+}]_i$. These data show the possibility that the SP-induced increase in $[Ca^{2+}]_i$ is involved in NK-2 and -3 receptors, although NK-1 receptor plays the most important role for its action in spinal astrocytes.

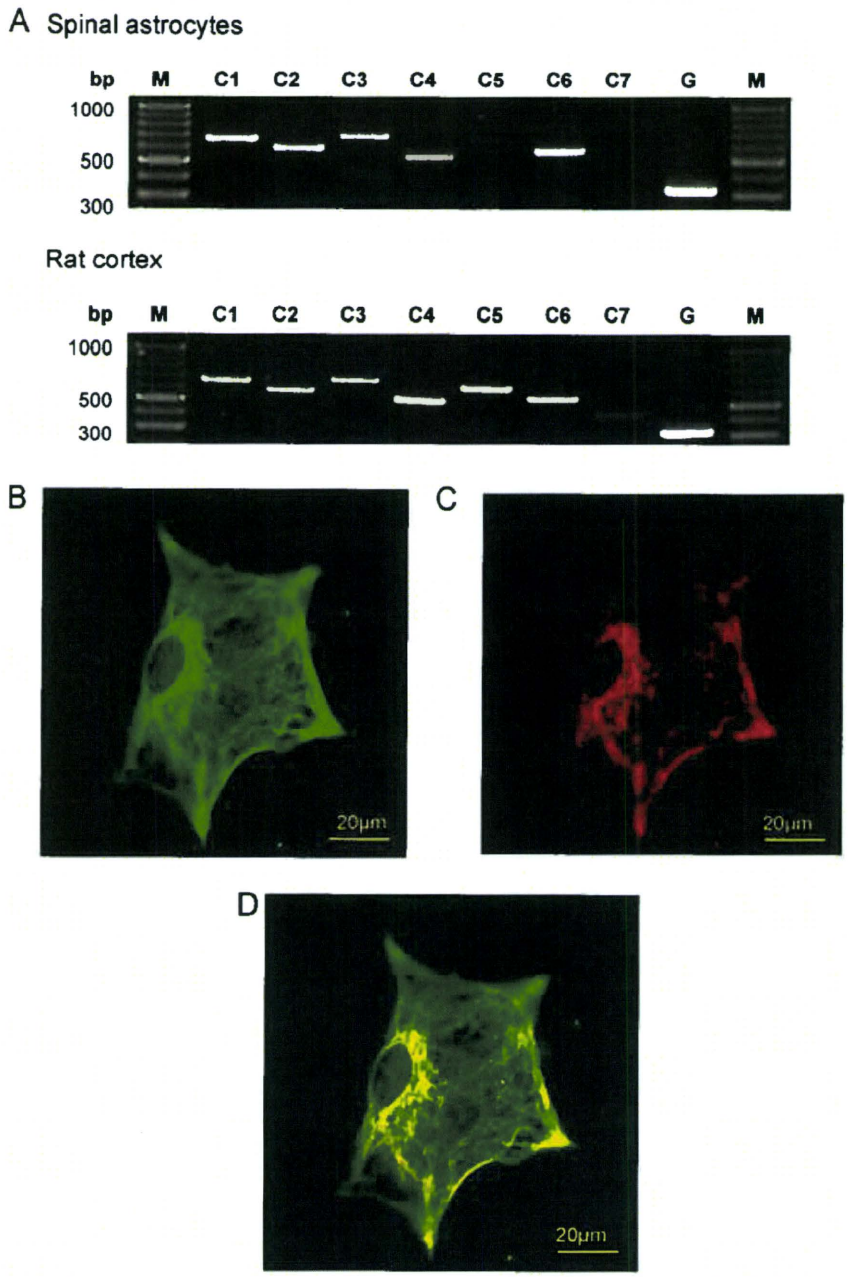

Fig. 7. TRPC3 is expressed in cultured spinal astrocytes. (A) RT-PCR analysis of TRPC channels mRNA expression in cultured rat spinal astrocytes. Each lane represents the cDNA fragments of TRPC1–7 (C1–C7) and GAPDH (G) amplified from the RNA of cultured spinal astrocytes (the upper panel) or rat cortex (the lower panel), respectively. The sizes of fragments and sequences of primers used are indicated in Table 1. The products of PCR from rat cortex are shown as a positive control. Lane M indicates the size marker. (B–D) An immunofluorescence analysis of GFAP (green; B) and TRPC3 (red; C) expression in cultured spinal astrocytes. The expression of TRPC3 was found in GFAP-labeled cells (D).

In this study, U73122 completely attenuated the GR73623-treated increase of $[Ca^{2+}]_i$ in spinal astrocytes, thus suggesting that PLC activation is indispensable for the increase in $[Ca^{2+}]_i$ initiated by stimulation of the NK-1 receptor. In addition, inhibitory effects of thapsigargin and xestospongin C on the GR73632-induced increase in $[Ca^{2+}]_i$ indicates that IP_3-sensitive intracellular Ca^{2+} stores are involved in the increase of $[Ca^{2+}]_i$ initiated by stimulation of the NK-1 receptor. These data suggest that GR73632 could produce IP_3 by the activation of PLC, which then triggers Ca^{2+} release through the IP_3 receptor expressed on Ca^{2+} stores. This is in agreement with a previous report by Palma et al. (1997) who showed that SP caused accumulation of IP_3 in spinal astrocytes.

Furthermore, Holst et al. (2001) demonstrated that not only Gq, but also Gs, proteins are associated with the NK1 receptor, and that the cAMP-PKA cascade contributes to these receptor-mediated functions. In fact, we elucidated that PKA regulates the GR73632-induced Ca^{2+} release (Figs. 4F and 5D), although the present study did not clarify how PKA modulated the Ca^{2+} release through the IP_3 receptor. Bezprozvanny (2005) or Volpe and Alderson-Lang (1990) reported that the activation of PKA enhanced the Ca^{2+} release from IP_3-sensitive Ca^{2+} stores. Furthermore, the IP_3 receptor possesses sites that are phosphorylated by PKA. Mutation of these sites attenuated Ca^{2+} release from IP_3-sensitive Ca^{2+} stores (Wagner et al., 2008). Therefore, these findings suggest that the phosphorylation of the IP_3 receptor by PKA would affect the sensitivity of the

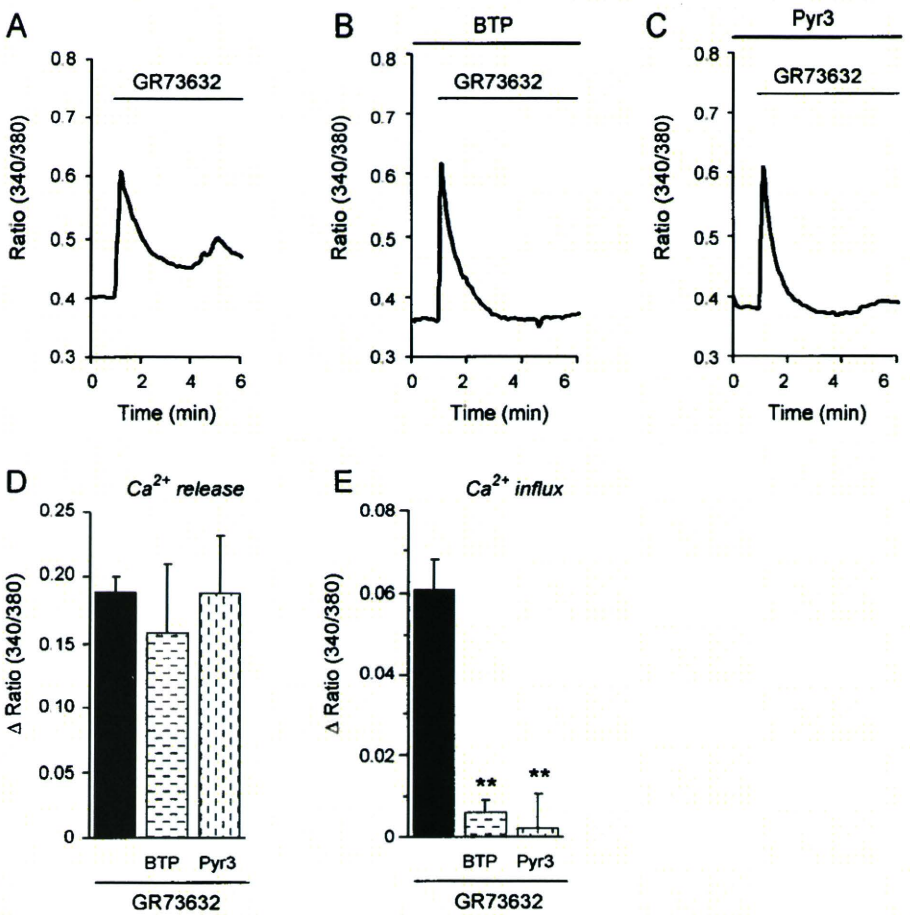

Fig. 8. Effects of inhibitors of TRPC channels on the GR73632-induced increase in $[Ca^{2+}]_i$ by spinal astrocytes. The trace in each graph (A–C) shows the representative mean $[Ca^{2+}]_i$ in randomly selected cells. The fura-2-loaded cells were treated with 1000 nM of GR73632 in Hanks' buffer (A–C). After the cells were pretreated with 10 μM BTP2 (BTP) (B) or 3 μM Pyr3 (C) for 20 min, they were stimulated with GR73632. The extent of Ca^{2+} release from the intracellular Ca^{2+} stores induced by GR73632 was quantified by determining the differences between the ratio (340/380) of the basal and the peak level obtained after GR73632 treatment (D). The extent of the extracellular Ca^{2+} influx induced by GR73632 was quantified by determining the differences between the ratio (340/380) of the basal level and the level at 2 min after a peak of $[Ca^{2+}]_i$ (E). The data are expressed as the means ± S.E.M. (*bars*) of separate experiments. $^{**}p < 0.01$ in comparison with the value for the cells treated with GR73632 alone.

receptor. Taken together, it appears that the activation of PKA by stimulation of the NK1 receptor might enhance the sensitivity of the IP_3 receptor. However, further investigations are needed to determine whether PKA activation by GR73632 leads to phosphorylation of the IP_3 receptor.

In terms of TRPC cation channels, extracellular Ca^{2+} influx through the TRPC channels is known to mainly occur through two pathways: (1) influx induced by depletion of intracellular Ca^{2+} stores (capacitative Ca^{2+} entry: CCE); (2) DAG-sensitive influx (non-CCE: NCCE) (Large et al., 2009). Therefore, TRPC channels are divided into two types (Venkatachalam and Montell, 2007; Large et al., 2009). The DAG-insensitive channels, which are associated with CCE, include TRPC1, 4 and 5. The others are DAG-sensitive channels, and include TRPC2, 3, 6 and 7.

To the best of our knowledge, there have been no previous reports about either the expression or activity of the TRPC channels in spinal astrocytes. The present study is the first to demonstrate that spinal astrocytes express TRPC1, 3, 4 and 6 (Fig. 6) and that Pyr3, which is a selective TRPC3 antagonist (Kiyonaka et al., 2009), completely suppressed the GR73632-induced influx of extracellular Ca^{2+} without affecting GR73632-induced Ca^{2+} release from intracellular Ca^{2+} stores. The inhibitory effects of Pyr3 on the Ca^{2+} influx were similar to those of BTP2 (Fig. 7B and C). Based on these data, we concluded that (1) activation of TRPC3 is responsible for the Ca^{2+} influx induced after the stimulation of the NK-1 receptor, because the 3 μM Pyr3 used in this study had no effect on the activity of other types of TRPC channels (Kiyonaka et al., 2009); (2) the increase in $[Ca^{2+}]_i$ by stimulation of the NK-1 receptor is due to CCE, because xestospongin C completely inhibited the GR73632-induced Ca^{2+} influx. Our hypothesis is consistent with recent reports showing that TRPC3 can not only induce CCE through the IP_3 receptor, but can also function as non-DAG-sensitive channels (Venkatachalam et al., 2003; Woodard et al., 2010). Furthermore, a recent report indicated that TRPC6 evokes CCE by associating with TRPC1, suggesting that DAG-sensitive TRPC channels are involved in not only NCCE, but also CCE (Jardin et al., 2009). Further investigations are needed to better characterize TRPC3 in spinal astrocytes.

In the present study, the BIM-potentiated increase in $[Ca^{2+}]_i$ after stimulation with GR73632 was completely inhibited by Pyr3 suggesting that PKC regulates the activity of TRPC3. Trebak et al. (2005) demonstrated that NCCE induced by OAG, a membrane-permeability analogue of DAG, was suppressed by phosphorylation of Ser^{712} in TRPC3. In addition, they showed that a mutation at Ser^{712} in TRPC3 potentiated the influx of extracellular Ca^{2+} caused by methacholine, an agonist of the muscarinic acetylcholine receptor (Trebak et al., 2005). These findings suggest that the phosphorylation of TRPC3 by PKC is crucial for inhibition of extracellular Ca^{2+} influx. Thus, the activation of TRPC3 after stimulation of the NK-1 receptor might be negatively regulated by the simultaneous activation of PKC.

In conclusion, stimulation of the NK-1 receptor causes Ca^{2+} release from IP_3-sensitive intracellular Ca^{2+} stores via PLC activation, and thereby induces the influx of extracelluar Ca^{2+}

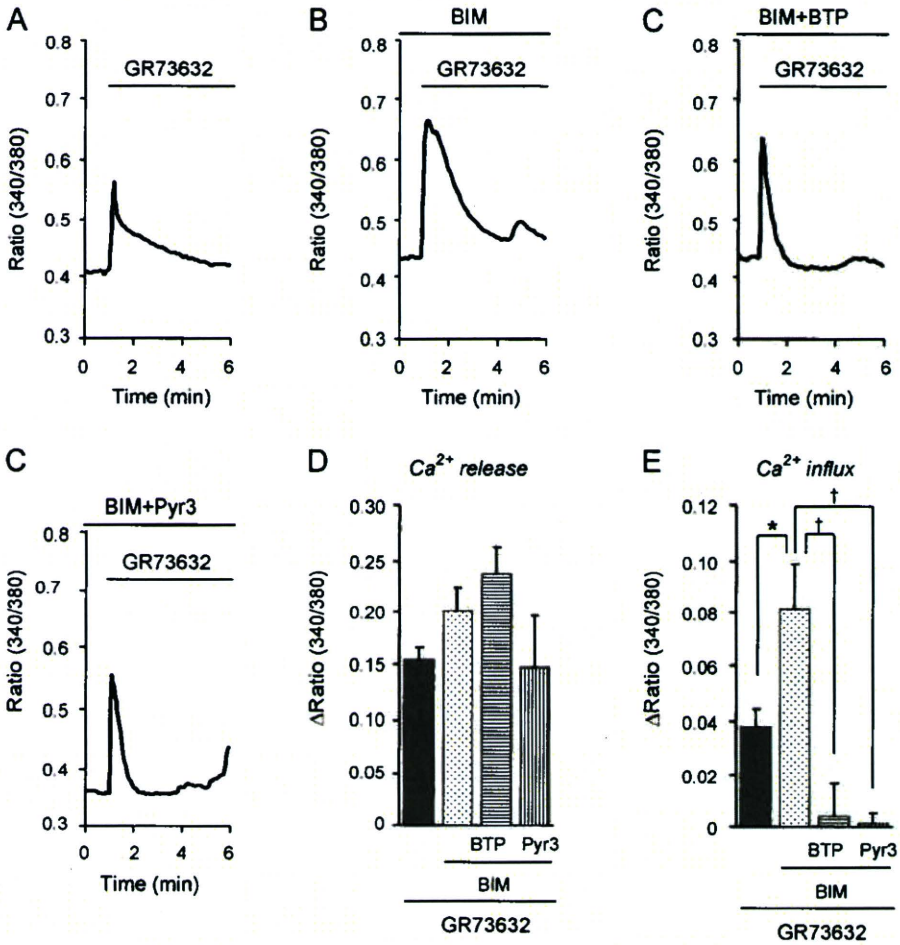

Fig. 9. Involvement of PKC in the GR73632-induced increase in $[Ca^{2+}]_i$ through TRPC channels in spinal astrocytes. The trace in each graph (A–D) shows the representative mean $[Ca^{2+}]_i$ in randomly selected cells. The fura-2-loaded cells were treated with 1000 nM of GR73632 (GR) in Hanks' buffer (A–D). After the cells were pretreated with 10 μM BIM (B), a combination of 10 μM of BIM and BTP2 (BIM + BTP) (C) or 10 μM BIM and 3 μM Pyr3 (BIM + Pyr3) (D) for 20 min, they were stimulated with GR73632. The extent of Ca^{2+} release from the intracellular Ca^{2+} stores induced by GR73632 was quantified by determining the differences between the ratio (340/380) of the basal and the peak level obtained after GR73632 treatment (E). The extent of the extracellular Ca^{2+} influx induced by GR73632 was quantified by determining the differences between the ratio (340/380) of the basal level and the level at 2 min after a peak of $[Ca^{2+}]_i$ (F). The data are expressed as the means ± S.E.M. (*bars*) of separate experiments. $^{*}p < 0.05$ in comparison with the value for the cells treated with GR73632 alone. $^{\dagger}p < 0.05$ in comparison with the value for the cells pretreated with BIM + GR73632.

through the TRPC3 in spinal astorcytes. In addition, PKA activation by SP potentiates Ca^{2+} release, while PKC down-regulates the Ca^{2+} influx through TRPC3. Our results indicate that SP binding to the NK-1 receptor in not only neurons but also in spinal astrocytes plays an essential role in spinal synaptic transmission. As a result, these observations provide valuable new insights into the regulation of pain transduction.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) to Nakata (No. 21590280) and the Japanese Smoking Research Association. We thank Dr. Brian Quinn (Kyushu University, Japan) for his critical reading of the manuscript. We also thank the Analysis Center of Life Science, Hiroshima University for the use of an Argus Hisca color image processor.

References

Barajas, M., Andrade, A., Hernandez-Hernandez, O., Felix, R., Arias-Montano, J.A., 2008. Histamine-induced Ca^{2+} entry in human astocytoma U373 MG cells: evidence for involvement of store-operated channels. J. Neurosci. Res. 86, 3456–3468.

Bezprozvanny, I., 2005. The inositol 1,4,5-triphosphate receptors. Cell Calcium 38, 261–272.

Fields, R.D., Stevens-Graham, B., 2002. New insights into neuron-glia communication. Science 298, 556–562.

Hagan, R.M., Ireland, S.J., Jordan, C.C., Beresford, I.J.M., Deal, M.J., Ward, P., 1991. Receptor-selective, peptidase-resistant agonists at neurokinin NK-1 and NK-2 receptors: new tools for investigating neurokinin function. Neuropeptides 19, 127–135.

Hirota, N., Kuraishi, Y., Hino, Y., Sato, Y., Satoh, M., Takagi, H., 1985. Met-enkephalin and morphine but not dynorphin inhibit noxious stimuli-induced release of substance P from rabbit dorsal horn in situ. Neuropharmacology 24, 567–570.

Holst, B., Hastrup, H., Raffetseder, U., Martini, L., Schwartz, T.W., 2001. Two active molecular phenotypes of the tachykinin NK1 receptor revealed by G-protein functions and mutagenesis. J. Biol. Chem. 276, 19793–19799.

Jardin, I., Gomez, L.J., Salido, G.M., Rosado, J.A., 2009. Dynamic interaction of hTRPC6 with the Orai1-STIM1 complex or hTRPC3 mediates its role in capacitative or non-capacitative Ca^{2+} entry pathway. Biochem. J. 420, 267–276.

Kiyonaka, S., Kato, K., Nishida, M., Mio, K., Numaga, T., Sawaguchi, Y., Yoshida, T., Wakamori, M., Mori, E., Numata, T., Ishii, M., Takemoto, H., Ojida, A., Watanabe, K., Uemura, A., Kurose, H., Morii, T., Kobayashi, T., Sato, Y., Sato, C., Hamachi, I., Mori, Y., 2009. Selective and direct inhibiton of TRPC3 channels underlies biological activities of a pyrazole compound. Proc. Natl. Acad. Sci. U.S.A. 106, 5400–5405.

Large, W.A., Saleh, S.N., Albert, A.P., 2009. Role of phosphoinositol 4,5-bisphosphate and diacylglycerol in regulating native TRPC channel proteins in vascular smooth muscle. Cell Calcium 45, 574–582.

Maggi, C.A., 1995. The mammalian tachykinin receptors. Gen. Pharmacol. 26, 911–944.

Maggi, C.A., Schwartz, T.W., 1997. The dual nature of the tachykinin NK_1 recetor. TiPS 18, 351–353.

Marriott, D.R., Wilkin, G.P., Wood, J.N., 1991. Substance P-induced release of prostaglandins from astrocytes: regional specialisation and correlation with phosphoinositol metabolism. J. Neurochem. 56, 259–265.

Meini, S., Patacchini, R., Lecci, A., Poulos, C., Rovero, P., Maggi, C.A., 1995. GR73,632 and [Glu(OBzl)^{11}] substance P are selective agonists for the Septide-sensitive tachykinin NK_1 receptor in the rat urinary bladder. Neuropeptides 28, 99–106.

Miyano, K., Tang, H.B., Nakamura, Y., Morioka, N., Inoue, A., Nakata, Y., 2009. Paclitaxel and vinorelbine, evoked the release of substance P from cultured rat dorsal root ganglion cells through different PKC isoform-sensitive ion channels. Neuropharmacology 57, 25–32.

Morioka, N., Tanabe, H., Inoue, A., Dohi, T., Nakata, Y., 2009. Noradrenaline reduces the ATP-stimulated phosphorylation of p38 MAP kinase via β-adrenergic receptors–cAMP–protein kinase A-dependent mechanism in cultured rat spinal microglia. Neurochem. Int. 55, 226–234.

Nakajima, Y., Tsuchida, K., Negishi, M., Ito, S., Nakanishi, S., 1992. Direct linkage of three tachykinin receptors to stimulation of both phosphatidylinositol hydrolysis and cyclic AMP cascades in transfected Chinese hamster ovary cells. J. Biol. Chem. 267, 2437–2442.

Nakao, K., Shirakawa, H., Sugishita, A., Matsutani, I., Niidome, T., Nakagawa, T., Kaneko, S., 2008. Ca^{2+} mobilization mediated by transient receptor potential canonical 3 is associated with thrombin-induced morphological changes in 1321N1 human astrocytoma cells. J. Neurosci. Res. 86, 2722–2732.

Palma, C., Minghetti, L., Astolfi, M., Ambrosini, E., Silberstein, F.C., Manzini, S., Levi, G., Aloisi, F., 1997. Functional characterization of substance P receptors on cultured human spinal cord astrocytes: synergism of substance P with cytokines in inducing interleukin-6 and prostaglandin E2 production. Glia 21, 183–193.

Randic, M., Miletic, V., 1977. Effect of substance P in cat dorsal horn neurons activated by noxious stimuli. Brain Res. 128, 164–169.

Severini, C., Improta, G., Falconieri-Erspamer, G., Salvadori, S., Erspamer, V., 2002. The tachykinin peptide family. Pharmacol. Rev. 54, 285–322.

Snijdelaar, D.G., Dirksen, R., Slappendel, R., Crul, B.J., 2000. Substance P. Eur. J. Pain 4, 121–135.

Trebak, M., Hempel, N., Wedel, B.J., Smyth, J.T., Bird, J.S., Putney Jr., J.W., 2005. Negative regulation of TRPC3 channels by protein kinase C mediated phosphorylation of serine 712. Mol. Pharmacol. 67, 558–563.

Venkatachalam, K., Zheng, F., Gill, D.L., 2003. Regulation of canonical transient receptor potential (TRPC) channel function by diacylglycerol and protein kinase C. J. Biol. Chem. 278, 29031–29040.

Venkatachalam, K., Montell, C., 2007. TRP channels. Annu. Rev. Biochem. 76, 387–417.

Volpe, P., Alderson-Lang, B.H., 1990. Regulation of inositol 1,4,5-trisphosphate-induced Ca^{2+} release. II. Effect of cAMP-dependent protein kinase. Am. J. Physiol. 258, 1086–1091.

Wagner, L.E., Joseph, S.K., Yule, D.I., 2008. Regulation of single inositol 1,4,5-trisphosphate receptor channel activity by protein kinase A phosphorylation. J. Physiol. 586, 3577–3596.

Woodard, G.E., López, J.J., Jardín, I., Salido, G.M., Rosado, J.A., 2010. TRPC3 regulates agonist-stimulated Ca^{2+} mobilization by mediating the interaction between type I inositol 1,4,5-trisphosphate receptor, RACK1 and Orai1. J. Biol. Chem. 285, 8045–8053.

Zhou, H., Iwasaki, H., Nakamura, T., Nakamura, K., Maruyama, T., Hamano, S., Ozaki, S., Mizutani, A., Mikoshiba, T., 2007. 2-Aminoethyl diphenylborinate analogues: selective inhibition for store-operated Ca^{2+} entry. Biochem. Biophys. Res. Commun. 352, 227–282.

S(+)-Ketamine Suppresses Desensitization of γ-Aminobutyric Acid Type B Receptor-mediated Signaling by Inhibition of the Interaction of γ-Aminobutyric Acid Type B Receptors with G Protein–coupled Receptor Kinase 4 or 5

Yuko Ando, M.D.,* Minoru Hojo, M.D.,† Masato Kanaide, M.D., Ph.D.,‡ Masafumi Takada, M.D., Ph.D.,† Yuka Sudo, B.S.,§ Seiji Shiraishi, M.D., Ph.D.,‖ Koji Sumikawa, M.D., Ph.D.,# Yasuhito Uezono, M.D., Ph.D.**

ABSTRACT

Background: Intrathecal baclofen therapy is an established treatment for severe spasticity. However, long-term management occasionally results in the development of tolerance. One of the mechanisms of tolerance is desensitization of γ-aminobutyric acid type B receptor ($GABA_BR$) because of the complex formation of the $GABA_{B2}$ subunit (GB_2R) and G protein–coupled receptor kinase (GRK) 4 or 5. The current study focused on *S*(+)-ketamine, which reduces the development of morphine tolerance. This study was designed to investigate whether *S*(+)-ketamine affects the $GABA_BR$ desensitization processes by baclofen.

Methods: The G protein–activated inwardly rectifying K^+ channel currents induced by baclofen were recorded using *Xenopus* oocytes coexpressing G protein–activated inwardly rectifying K^+ channel 1/2, $GABA_{B1a}$ receptor subunit, GB_2R, and GRK. Translocation of GRKs 4 and 5 and protein complex formation of GB_2R with GRKs were analyzed by confocal microscopy and fluorescence resonance energy transfer analysis in baby hamster kidney cells coexpressing $GABA_{B1a}$ receptor subunit, fluorescent protein–tagged GB_2R, and GRKs. The formation of protein complexes of GB_2R with GRKs was also determined by coimmunoprecipitation and Western blot analysis.

Results: Desensitization of $GABA_BR$-mediated signaling was suppressed by *S*(+)-ketamine in a concentration-dependent manner in the electrophysiologic assay. Confocal microscopy revealed that *S*(+)-ketamine inhibited translocation of GRKs 4 and 5 to the plasma membranes and protein complex formation of GB_2R with the GRKs. Western blot analysis also showed that *S*(+)-ketamine inhibited the protein complex formation of GB_2R with the GRKs.

Conclusion: *S*(+)-Ketamine suppressed the desensitization of $GABA_BR$-mediated signaling at least in part through inhibition of formation of protein complexes of GB_2R with GRK 4 or 5.

What We Already Know about This Topic:

- Tolerance to intrathecal baclofen for treatment of spasticity is produced by desensitization of the γ-aminobutyric acid type B receptor ($GABA_BR$).

What This Article Tells Us That Is New:

- In cell culture, *S*(+)-ketamine suppressed the desensitization of $GABA_BR$-mediated signaling at least in part through inhibition of formation of protein complexes of $GABA_{B2}$ subunit (GB_2R) with GRK 4 or 5.

BACLOFEN, a selective γ-aminobutyric acid type B receptor ($GABA_BR$) agonist, has been widely used as an antispasticity agent. Intrathecal baclofen (ITB) therapy is an established treatment for severe spasticity of both spinal and

* Graduate Student, † Assistant Professor, ‡ Staff Member, # Professor, Department of Anesthesiology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan. § Graduate Student, Department of Molecular and Cellular Biology, Nagasaki University Graduate School of Biomedical Sciences, and Trainee, Cancer Pathophysiology Division, National Cancer Center Research Institute, Tokyo, Japan. ‖ Section Head, ** Chief, Cancer Pathophysiology Division, National Cancer Center Research Institute.

Received from the Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan. Submitted for publication December 27, 2009. Accepted for publication October 6, 2010. Supported by the Alumni Association of Nagasaki University School of Medicine, Nagasaki, Japan (Dr. Ando); grants for Scientific Research 20591834 (Dr. Hojo), 00404244 (Dr. Takada), 60028660 (Dr. Sumikawa), and 2160009 and 19500325 (Dr. Uezono) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan; the Public Health Research Foundation, Tokyo (Dr. Kanaide); Daiichi-Sankyo Co, Ltd, Tokyo (Drs. Sumikawa and Uezono); Grant-in-Aid 21150801 for the Third Term Comprehensive 10-Year Strategy for Cancer Control and Cancer Research and Grant-in-Aid for Cancer Research 21-9-1 from the Japanese Ministry of Health, Labor and Welfare, Tokyo (Dr. Uezono). Presented at the 55th Annual Meeting of the Japanese Society of Anesthesiologists, June 12, 2008, Yokohama, Japan, and the 60th Meeting of the Seinan Regional Chapter of Japanese Pharmacological Society, November 22, 2007, Miyazaki, Japan.

Address correspondence to Dr. Uezono: 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. yuezono@ncc.go.jp. Anesthesiology 2011; 114: 401–11

cerebral origin.[1] Recently, increasing reports have shown that ITB therapy has powerful antinociceptive effects in patients with spasticity and in patients without spasticity who experience chronic pain,[1] such as somatic pain,[2] central pain,[2,3] and complex regional pain syndrome.[4,5]

However, long-term management of ITB therapy occasionally results in the development of tolerance,[6] which makes treatment difficult with respect to both pain and spasticity. Such decreased responsiveness to baclofen, so-called baclofen tolerance, is, in part, because of the desensitization of $GABA_BR$.[7,8] In addition, the desensitization of $GABA_BR$ occurred by the formation of complexes of $GABA_BR$ and either G protein–coupled receptor kinase (GRK) 4[7,8] or 5,[7] which is a member of the GRK family consisting of GRKs 1 through 7.[9]

Until today, several agents (*e.g.*, morphine, baclofen, ketamine, clonidine, and local analgesics) have been administered intrathecally for effective chronic pain management or spinal anesthesia clinically.[10,11] Among them, intrathecal ketamine coadministration has a synergistic analgesic effect with opioids.[12] In addition, ketamine administration prevented the development of tolerance against morphine in several animal models,[13,14] although the mechanism has not yet been clearly elucidated. Regulation of tolerance of μ-opioid receptor–mediated cellular signaling, receptors to which morphine mainly act, is known to be mediated by GRKs, particularly GRK 2[15] or 3.[16,17] GRKs 2 and 3 are reported to play in desensitization processes of μ-opioid receptors[15,17] or development of tolerance to opioids in an animal model.[16] In case of $GABA_BR$, it was previously demonstrated that the desensitization of $GABA_BR$-mediated responses was associated with the formation of protein complexes of $GABA_{B2}$ receptor subunit (GB_2R) with GRK 4 or 5.[7] Our hypothesis is that ketamine would interact with GRK 4 or 5. Thus, we focused on the effects of ketamine on the modification of GRKs 4 and 5 in $GABA_BR$-mediated desensitization processes. Ketamine consists of two enantiomers, *S*(+)-ketamine and *R*(−)-ketamine, that have distinct pharmacologic properties.[18] *S*(+)-Ketamine has a three times higher anesthetic potency than that of the racemic mixture, the incidence of adverse effects is equal at the same concentration for both enantiomers,[18] and both are clinically available.[18] Thus, in the current study, we used *S*(+)-ketamine and investigated whether *S*(+)-ketamine has effects on $GABA_BR$ desensitization and the formation of complexes of $GABA_BR$ with GRK 4 or 5.

Materials and Methods

Drugs and Chemicals

Baclofen was purchased from Tocris Cookson, Bristol, United Kingdom; and *S*(+)-ketamine, gentamicin, and sodium pyruvate were obtained from Sigma, St Louis, MO. All other chemicals used were of analytic grade and were obtained from Nacalai Tesque, Kyoto, Japan.

Construction of Complementary DNA and Preparation for Complementary RNAs

Complementary DNA (cDNA) for rat G protein–activated inwardly rectifying K^+ channel (GIRK) 1 and mouse GIRK2 were provided by Henry A. Lester, Ph.D. (Professor of Biology, Caltech, Pasadena, CA). $GABA_{B1a}$ receptor subunit ($GB_{1a}R$), GB_2R, and anti-hemagglutinin (HA)–tagged GB_2R were provided by Niall. J. Fraser, Ph.D. (Glaxo Wellcome, Stevenage, United Kingdom). Cerulean, a brighter variant of cyan fluorescent protein, was obtained from David W. Piston, Ph.D. (Professor of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN); and Venus, a brighter variant of yellow fluorescent protein, was obtained from Takeharu Nagai, Ph.D. (Professor of Nanosystems Physiology, Hokkaido University, Sapporo, Japan). Human GRK4 was provided by Antonio De Blasi, Ph.D. (Professor of Istituto Neurologico Mediterraneo Neuromed, Pozzilli, Italy); and rat GRK5 was obtained from Yuji Nagayama, M.D., Ph.D. (Professor of Medical Gene Technology at Atomic Bomb Disease Institute, Nagasaki University, Nagasaki, Japan). For receptor construction, the N-DYKDDDDK-C (FLAG) epitope tag (5′-GAACAAAAACTCATCTCAGAAGAGGATGTG-3′) was engineered to ligate the N-terminus of GRK 4 or 5 by using standard molecular approaches that use polymerase chain reaction. Venus-fused GB_2R was created by ligating the receptor cDNA into *Hin*dIII sites into the corresponding sites of Venus cDNA. Venus- or Cerulean-fused GRKs 4 and 5 were created by ligating the GRK cDNA sequences into the *Not*I or *Bam*HI sites of corresponding Venus or Cerulean sites. All cDNAs for transfection in baby hamster kidney (BHK) cells were subcloned into pcDNA3.1 (Invitrogen, San Diego, CA). For expression in *Xenopus* oocytes, all cDNAs for the synthesis of complementary RNAs (cRNAs) were subcloned into the pGEMHJ vector, which provides 5′- and 3′-untranslated regions of the *Xenopus* β-globin RNA, ensuring a high concentration of protein expression in the oocytes.[19] Each of the cRNAs was synthesized with a messenger RNA kit (mCAP messenger RNA Capping Kit; Ambion, Austin, TX) and with a T7 RNA polymerase *in vitro* transcription kit (Ambion) from the respective linearized cDNAs.[20]

Oocyte Preparation and Injection

Immature V and VI oocytes from *Xenopus* were enzymatically dissociated, as previously described.[21,22] Isolated oocytes were incubated at 18°C in ND-96 medium (containing 96-mM NaCl, 2-mM KCl, 1-mM $CaCl_2$, 1-mM $MgCl_2$, and 5-mM HEPES, pH 7.4) containing 2.5-mM sodium pyruvate and 50-μg/ml gentamicin. For measurement of GIRK currents induced by baclofen, cRNAs of GIRKs 1 and 2 (0.2 ng each) and $GB_{1a}R$ and GB_2R (5 ng each) were coinjected into the oocytes, together with or without GRKs (4 or 5) or FLAG-tagged GRKs (FLAG-GRK4 or FLAG-GRK5) (3 ng each). The final injection volume was less than 50 nl in all

cases. Oocytes were incubated in ND-96 medium and used 3–8 days after injection, as previously reported.[21]

Electrophysiologic Recordings

Electrophysiologic recordings were performed using the two-electrode voltage clamp method with an amplifier (Geneclamp 500; Axon Instruments, Foster City, CA) at room temperature. Oocytes were clamped at −60 mV and continuously superfused with ND-96 medium or 49 mM K^+ (high potassium) solution, in which tonicity was adjusted to reduce concentrations of NaCl (48-mM NaCl, 49-mM KCl, 1-mM $CaCl_2$, 1-mM $MgCl_2$, and 5-mM HEPES, pH 7.4) in a 0.25-ml chamber at a flow rate of 5 ml/min. Then, baclofen alone or *S*(+)-ketamine and baclofen were added to the superfusion solution. Voltage recording microelectrodes were filled with 3 M potassium chloride, and their tip resistance was 1.0–2.5 MΩ. Currents were continuously recorded and stored with a data acquisition system (PowerLab 2/26; AD Instruments, Castle Hill, Australia) and a computer (Macintosh; Apple, Cupertino, CA), as previously described.[21,22] All test compounds applied to oocytes were dissolved into the ND-96 medium or 49-mM K^+ media.

Cell Culture and Transfection

The BHK cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. For confocal microscopic assay, BHK cells were seeded at a density of 1×10^5 cells/35-mm glass-bottomed culture dish (World Precision Instruments, Sarasota, FL) and cultured for 24 h. Transient transfection was then performed with a transfection reagent (Effectene; Qiagen, Tokyo, Japan) in 0.2 μg each cDNA, as previously described,[7,20] and according to the protocol provided by the manufacturer. Cells were used in confocal microscopy and fluorescence resonance energy transfer (FRET) analysis 16–24 h after transfection.

Confocal Fluorescence Microscopy

For translocation studies of GRKs and protein complex formation of $GABA_BR$ with each GRK (4 or 5) using confocal microscopy and the FRET assay, GB_2R and each of the GRKs (4 and 5) were fused through the carboxyl terminus to Cerulean or Venus. The BHK cells cultured in 35-mm glass-bottomed dishes were cotransfected with 0.2 μg Venus-fused $GABA_BR$ and Venus- or Cerulean-fused GRKs. A ×63 magnification 1.25–numerical aperture oil immersion objective was used with the pinhole for visualization. Both Venus and Cerulean were excited by a 458-nm laser, and images were obtained by placing the dish onto a stage in a confocal microscope (Zeiss LSM510 META; Carl Zeiss, Jena, Germany).

Photobleaching and Calculation of FRET Efficiency

To confirm FRET between Venus and Cerulean, we monitored acceptor photobleaching analysis in BHK cells that coexpressed $GB_{1a}R$, Venus-fused GB_2R, and Cerulean-fused GRKs. FRET was measured by imaging Cerulean before and after photobleaching Venus with the 100% intensity of 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 the evidence of FRET from Cerulean to Venus. All experiments were analyzed from at least six cells with three independent regions of interest. As a control, we examined the FRET efficiency of the unbleached area of membrane in the same cells in at least three areas. In some cases, we performed a photobleaching assay using fixed BHK cells. Cells were fixed as previously described.[23]

FRET efficiency was calculated using emission spectra before and after acceptor photobleaching of Venus.[24] 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 because of desensitized acceptor was measured by taking the Cerulean emission (at 488 nm) from spectra before and after acceptor photobleaching. FRET efficiency was then calculated using the following 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.[25] Before and after this bleaching, Cerulean images were collected to assess changes in donor fluorescence.

Coimmunoprecipitation and Western Blotting

Monoclonal anti–FLAG M2 was obtained from Sigma; monoclonal anti-HA (12CA5), from Roche, Mannheim, Germany; and polyclonal anti-HA (Y-11), from Santa Cruz Biotechnology, Santa Cruz, CA. The BHK cells were transiently cotransfected with each of the FLAG-tagged GRK cDNAs, HA-tagged GB_2R (HA-GB_2R), and nontagged $GB_{1a}R$ cDNAs. Twenty-four hours later, the cells were harvested, sonicated, and solubilized in a protein extraction buffer containing a combination of protease inhibitor cocktail (PRO-PREP; iNtRON Biotechnology, Sungnam, Korea) for 1 h at 4°C. The mixture was centrifuged (at 15,000 rpm for 30 min), and the supernatants were incubated with FLAG or HA (12CA5) antibody at 5 μg/ml overnight at 4°C. The mixture was centrifuged, and the pellets were washed five times by centrifugation and resuspension. Immunoprecipitated materials were dissolved in sample buffer (Lammeli) containing 0.1-M dithiothreitol subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and subjected to immunoblotting using monoclonal antibodies against FLAG (1:10,000) and polyclonal HA (Y-11) (1:10,000); then, bovine mouse or goat rabbit anti-IgG was conjugated with horseradish peroxidase at 1:5,000 and reacted with chemiluminescence Western blot detection reagents (Nacalai Tesque).

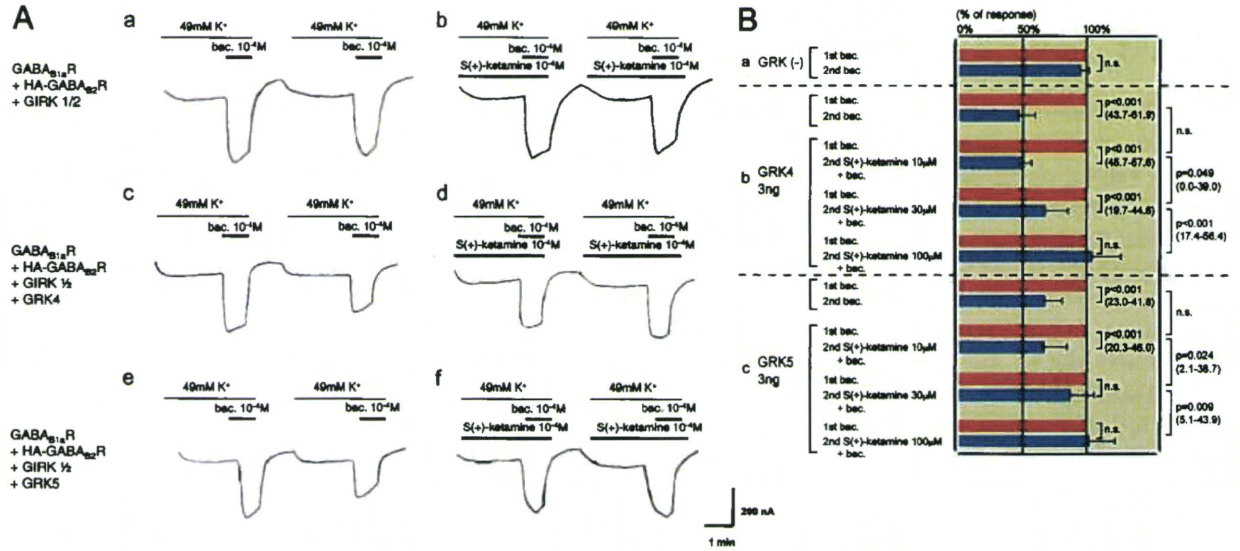

Fig. 1. Effects of *S*(+)-ketamine on the desensitization of γ-aminobutyric acid type B receptor ($GABA_BR$)–mediated G protein–activated inwardly rectifying K^+ channel (GIRK) currents in *Xenopus* oocytes. (*A*) Typical tracing of GIRK currents induced by the first and second application of baclofen (bac) (100 μM) for 1 min in a time lag of 4 min in oocytes coexpressing $GABA_{B1a}$ receptor subunit ($GB_{1a}R$), hemagglutinin (HA)–$GABA_{B2}$ subunit (GB_2R), and GIRK1/2 without (*a*) or with (*b*) *S*(+)-ketamine (100 μM) before (2 min) and during (1 min) application of a second preapplication of bac. Typical tracing of GIRK currents induced by the first and second application of bac (100 μM) for 1 min in a time lag of 4 min in oocytes coexpressing $GB_{1a}R$, HA-GB_2R, GIRK1/2, and G protein–coupled receptor kinase (GRK) 4 or 5 without (*c* and *e*) or with (*d* and *f*) *S*(+)-ketamine (100 μM) before (2 min) and during (1 min) application of a second preapplication of bac 49 mM k^+: 49 mM K^+ (high potassium) solution. (*B*) Summary of the effects of *S*(+)-ketamine on $GABA_BR$ desensitization. Each bar represents the mean ± SD of the peak GIRK currents induced by second application, expressed as percentage to each current induced by first application of bac in oocytes. (*a*) A group coexpressing $GB_{1a}R$, HA-GB_2R, and GIRK1/2, n = 8, (*b*) groups coexpressing $GB_{1a}R$, HA-GB_2R, GIRK1/2, and GRK 4 (n = 10 for each group), (*c*) groups coexpressing $GB_{1a}R$, HA-GB_2R, GIRK1/2, and GRK5 (n = 10 for each group). Statistical results are represented as *P* values (95% confidence interval for the differences in the two conditions). ns = not significant.

Statistical Analysis

Data are expressed as mean ± SD. For comparisons of the peak GIRK currents induced by second application of baclofen with those by first application of baclofen in *Xenopus* oocytes coexpressing $GB_{1a}R$, HA-GB_2R, and GIRK1/2 with or without GRK 4 or 5, two-tailed paired *t* tests were performed and the 95% confidence intervals (CIs) are depicted. The effects of *S*(+)-ketamine on the percentages of GIRK currents induced by second application of baclofen to each current induced by first application of baclofen were compared using one-way ANOVA, followed by the Tukey test. For comparison of FRET efficiency in BHK cells coexpressing $GB_{1a}R$, GB_2R-Venus, and GRKs-Cerulean, with or without *S*(+)-ketamine application before and during baclofen stimulation, two-tailed unpaired *t* tests were performed. Statistical significance was accepted at $P < 0.05$. All analyses were performed using computer software (IBM SPSS Statistics 18; IBM Corp, Armonk, NY).

Results

S(+)-Ketamine Inhibits the Desensitization of $GABA_B$ Receptor-Mediated Signaling by GRK 4 or 5 in Xenopus *Oocytes*

It was previously reported that baclofen elicited a GIRK conductance in *Xenopus* oocytes coexpressing heterodimeric $GABA_BR$ ($GB_{1a}R$ and HA-tagged GB_2R [HA-GB_2R]) with GIRKs 1 and 2 (GIRK1/2).[7] In addition, $GABA_BR$ desensitization was observed after repeated application of baclofen at 100 μM, which was a submaximum concentration to elicit inward K^+ current through GIRK1/2 to oocytes, coexpressing GRK 4 or 5 but not 2, 3, or 6.[7]

As previously demonstrated,[7] no desensitization was observed after repeated application of baclofen at 100 μM (for 1 min, each application) to oocytes coexpressing the $GB_{1a}R$ and HA-GB_2R with GIRK1/2 (fig. 1, A and B). When either GRK 4 (3 ng) or 5 (3 ng) cRNA was coinjected with heterodimeric $GABA_BR$ and GIRK1/2 cRNA, the amplitude of first baclofen-induced K^+ currents was almost the same as that in oocytes coexpressing $GABA_BR$ and GIRK1/2 without GRKs, whereas that of the second K^+ currents induced by baclofen was attenuated to 47.2 ± 12.7% (n = 8) in oocytes coexpressing GRK4 and to 67.6 ± 13.1% (n = 8) in oocytes coexpressing GRK5. This indicates that GRK 4 or 5 induced $GABA_BR$ desensitization (fig. 1, A and B). *S*(+)-Ketamine (100–300 μM) by itself had no effects on both the 49-mM K^+- and baclofen-induced K^+ currents in oocytes expressing $GABA_BR$ and GIRK1/2 without GRKs (fig. 1A and data not shown).

When *S*(+)-ketamine at a concentration of 10, 30, or 100 μM was applied before (2 min) and during the second application of baclofen (1 min) to oocytes coexpressing heterodimeric $GABA_BR$ and GIRK1/2 with GRK 4 or 5, the attenuation of the second baclofen-induced K^+ currents was

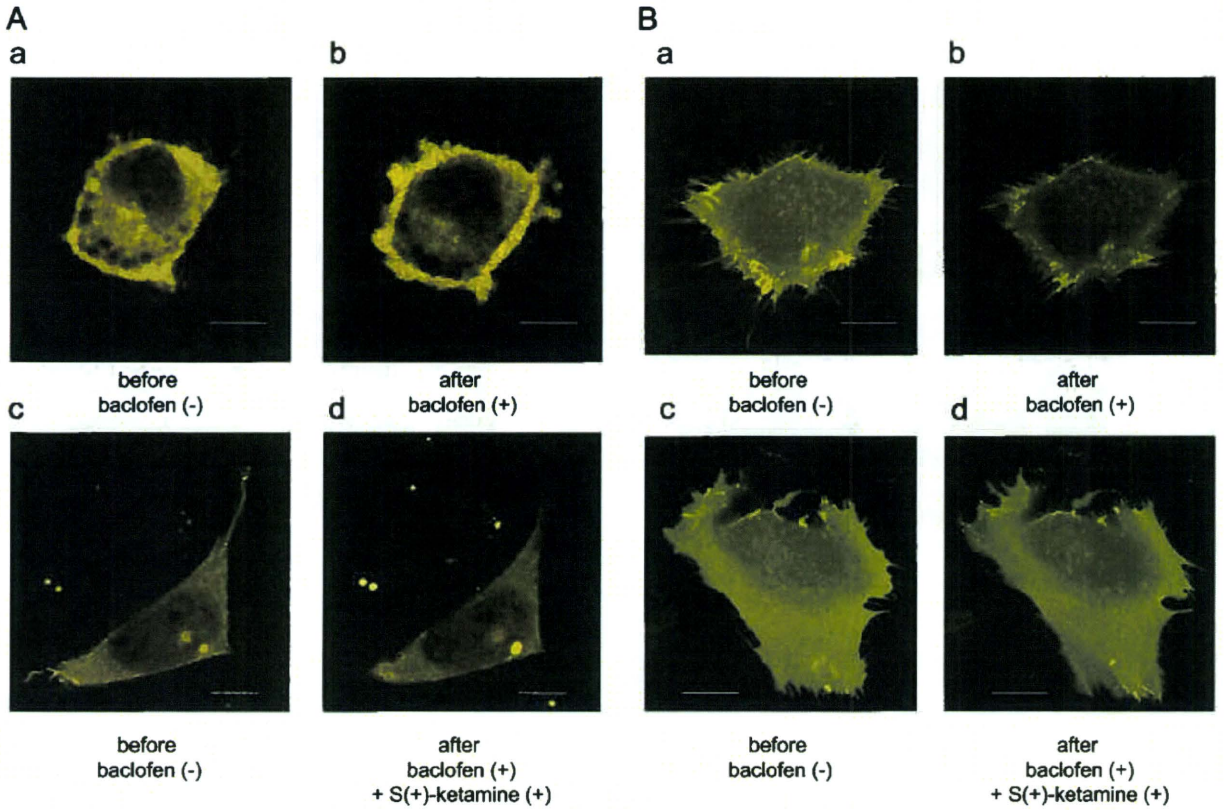

Fig. 2. Confocal imaging showing the effects of *S*(+)-ketamine on the translocation of G protein–coupled receptor kinase (GRK) 4–Venus or GRK5-Venus to the plasma membranes in baby hamster kidney (BHK) cells coexpressing the γ-aminobutyric acid (GABA)$_{B1a}$ receptor subunit (GB$_{1a}$R), hemagglutinin (HA)–GABA$_{B2}$ subunit (GB$_2$R), and GRKs-Venus. Each bar represents 10 μm. (*A*) Visualization of GRK4-Venus in the cells before (*a* and *c*) and after stimulation of baclofen (100 μM) for 5 min with (*d*) or without (*b*) previous application of *S*(+)-ketamine (100 μM) for 5 min in BHK cells coexpressing GB$_{1a}$R, HA-GB$_2$R, and GRK4-Venus. (*B*) Visualization of GRK5-Venus in BHK cells before (*a* and *c*) and after stimulation of baclofen for 5 min with (*d*) or without (*b*) previous application of *S*(+)-ketamine for 5 min in BHK cells coexpressing GB$_{1a}$R, HA-GB$_2$R, and GRK5-Venus.

significantly restored in a concentration-dependent manner (fig. 1, A and B). The amplitude of K^+ currents induced by the second application of baclofen with 10-, 30-, or 100-μM *S*(+)-ketamine was 48.3 ± 8.4%, 67.9 ± 17.4%, and 104.8 ± 22.7% in oocytes coexpressing GRK4 (n = 10 each) and 66.8 ± 17.9%, 87.2 ± 18.7%, and 102.4 ± 20.6% in oocytes coexpressing GRK5 (n = 10 each) of those induced by the first application of baclofen, respectively (fig. 1, A and B). When typical GIRK currents were not obtained by first application of baclofen, such data were excluded. Overall, approximately 67–83% of recording data in each group of oocytes were obtained for statistical analyses.

Translocation of Venus-Fused GRK 4 or 5 to the Plasma Membranes after Activation of GABA$_B$R Is Inhibited in the Presence of S(+)-Ketamine

To determine the effects of *S*(+)-ketamine on the translocation of GRK 4 or 5 in response to baclofen in BHK cells, we cotransfected GRK4-Venus or GRK5-Venus cDNA with GB$_{1a}$R and HA-GB$_2$R cDNAs and determined the intracellular distribution and translocation properties of GRK4-Venus or GRK5-Venus. We then applied baclofen with or without *S*(+)-ketamine application to living BHK cells. As shown in figure 2, A and B, GRK4-Venus or GRK5-Venus was diffusely distributed in the cytosol without agonist stimulation in BHK cells but was translocated to the plasma membranes gradually in 5 min after application of baclofen (100 μM). When *S*(+)-ketamine (100 μM) was applied to such cells 2.5 min before and during application of baclofen, the translocation of GRK4-Venus or GRK5-Venus to the plasma membranes was almost inhibited (fig. 2, A and B). Treatment of *S*(+)-ketamine (100 and 300 μM) alone for 10 min did not affect translocation properties of both GRK4-Venus and GRK5-Venus in BHK cells coexpressing heterodimeric GABA$_B$R with GRK4-Venus or GRK5-Venus (data not shown).

FRET and Acceptor Photobleaching Analysis of BHK Cells Coexpressing GRK 4 or 5 with Heterodimeric GABA$_B$R

Previously, we showed that functional GABA$_B$R formed heterodimers with GB$_{1a}$R and GB$_2$R by analysis with FRET and

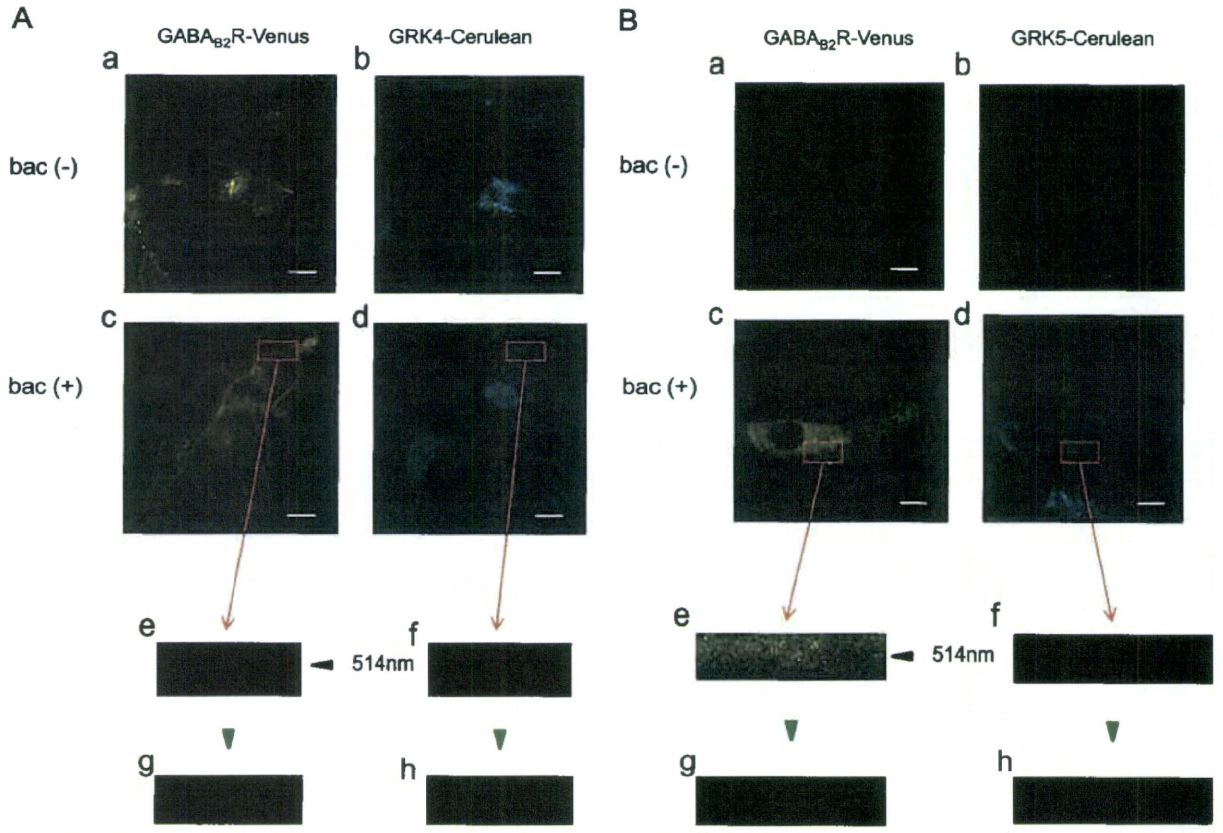

Fig. 3. Confocal imaging and fluorescence resonance energy transfer (FRET) analysis showing the protein complex formation of the γ-aminobutyric acid $(GABA)_{B2}$ subunit (GB_2R) with G protein–coupled receptor kinase (GRK) in baby hamster kidney (BHK) cells coexpressing the $GABA_{B1a}$ receptor subunit ($GB_{1a}R$), GB_2R-Venus, and GRKs-Cerulean. Each bar represents 10 μm. (*A*) Visualization of GB_2R-Venus and GRK4-Cerulean in nonstimulated (*a* and *b*) and baclofen (bac)-stimulated (100 μM, 5 min) BHK cells (*c* and *d*). Fluorescence changes by acceptor photobleaching (1-min application of 514-nm wavelength) in bac-stimulated BHK cells (*e–h*). (*B*) Visualization of GB_2R-Venus and GRK5-Cerulean in nonstimulated (*a* and *b*) and bac-stimulated (100 μM, 5 min) BHK cells (*c* and *d*). Fluorescence changes by acceptor photobleaching in bac-stimulated BHK cells (*e–h*).

acceptor photobleaching in BHK cells coexpressing $GB_{1a}R$-Venus and GB_2R-Cerulean.[7,20] We also showed that GRK 4 or 5, but not GRK 2, 3, or 6, formed protein complexes with the GB_2R subunit after $GABA_BR$ activation in the cells coexpressing Venus-fused $GB_{1a}R$ or GB_2R and Cerulean-fused GRKs.[7] We examined the effects of *S*(+)-ketamine on the formation of protein complexes of GRK 4 or 5 with GB_2R in BHK cells coexpressing $GB_{1a}R$, GB_2R-Venus, and GRK4-Cerulean (fig. 3A) or GRK5-Cerulean (fig. 3B). The fluorescence from GB_2R-Venus was mostly localized on the plasma membranes, whereas that from GRK4-Cerulean or GRK5-Cerulean was localized in the cytosol and to some extent on the plasma membranes (fig. 3A, a and b, and 3B, a and b). When cells were stimulated with baclofen (100 μM) for 5 min, the fluorescence of GRK4-Ceulean or GRK5-Cerulean and GB_2R-Venus was detected on and around the plasma membranes (fig. 3A, c and d, and 3B, c and d). Photobleaching analysis demonstrated that Venus fluorescence was reduced by application of a 514-nm wavelength at 100% intensity of the argon laser power to the indicated area (fig. 3A, e–h, and 3B, e–h). This application did not affect the fluorescent intensity of Venus and Cerulean in the unbleached area (data not shown). Acceptor photobleaching showed increased Cerulean fluorescence (donor) with decreased Venus fluorescence (acceptor) (fig. 3A, e–h, and 3B, e–h).

To determine the effects of *S*(+)-ketamine on the protein complex formation of GRK4-Cerulean or GRK5-Cerulean with GB_2-Venus plus $GB_{1a}R$, we applied *S*(+)-ketamine (100 μM) to the cells 5 min before application of baclofen (100 μM) and then simultaneously treated the cells for 5 min with baclofen and *S*(+)-ketamine. The fluorescence from GRK4-Cerulean or GRK5-Cerulean was detected diffusely in the cytosol and on the plasma membranes, whereas the fluorescence from GB_2R-Venus was mostly detected on the plasma membranes. Acceptor photobleaching demonstrated the reduction of the fluorescence from GB_2R-Venus; however, the fluorescence from GRK4-Cerulean or GRK5-Cerulean hardly changed (fig. 4, A and B; and fig. 5), which indicates that GRK4-Cerulean or GRK5-Cerulean and

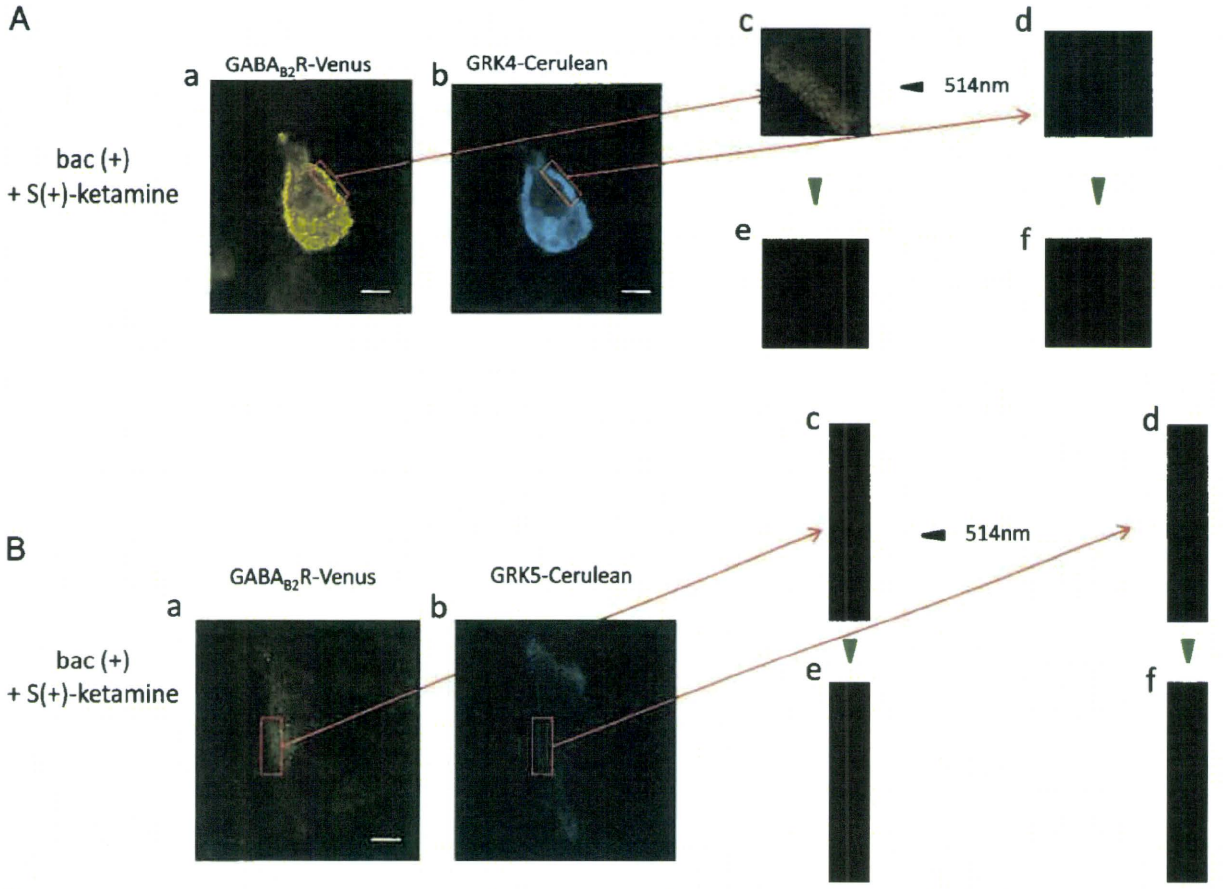

Fig. 4. Confocal imaging and fluorescence resonance energy transfer (FRET) analysis showing the effects of *S*(+)-ketamine on the interaction of γ-aminobutyric acid (GABA)$_{B2}$ subunit (GB$_2$R) with G protein–coupled receptor kinase (GRK) in baby hamster kidney (BHK) cells coexpressing GABA$_{B1a}$ receptor subunit (GB$_{1a}$R), GB$_2$R-Venus, and GRKs-Cerulean. Each bar represents 10 μm. (*A*) Visualization of GB$_2$R-Venus and GRK4-Cerulean in a BHK cell treated by *S*(+)-ketamine (100 μM) before (5 min) and during (5 min) baclofen (bac) stimulation (*a* and *b*). Fluorescence changes by acceptor photobleaching in bac-stimulated BHK cells (*c–f*). (*B*) Visualization of GB$_2$R-Venus and GRK5-Cerulean in a BHK cell pretreated with *S*(+)-ketamine (100 μM) before (5 min) and during (5 min) bac stimulation (*a* and *b*). Fluorescence changes by acceptor photobleaching in bac-stimulated BHK cells (*c–f*).

GB$_2$R-Venus do not form baclofen-induced protein complexes in the presence of *S*(+)-ketamine.

Coimmunoprecipitation and Western Blot Analysis of GRK 4 or 5 Using BHK Cells Coexpressing FLAG-GRKs, HA-GB$_2$R, and GB$_{1a}$R

Previously, it was shown that FLAG-GRK 4 or 5, but not GRK 2, 3, or 6, formed protein complexes with HA-GB$_2$R after baclofen stimulation (100 μM, 5 min) in BHK cells determined with coimmunoprecipitation and Western blot analysis.[7] We investigated whether *S*(+)-ketamine has an effect on the protein complex formation of GRK 4 or 5 with GB$_2$R induced by baclofen. Western blot analysis was performed with proteins extracted from BHK cells coexpressing FLAG-GRK4 or FLAG-GRK5, GB$_{1a}$R, and HA-GB$_2$R after immunoprecipitation with anti-HA. In the precipitate using anti-HA from the BHK cells coexpressing FLAG-GRK4 or FLAG-GRK5, HA-GB$_2$R, and GB$_{1a}$R, the band intensity of the immune complex determined with anti-HA was similar in nonstimulated and baclofen-stimulated (100 μM, 5 min) BHK cells (fig. 6A). On the other hand, the immune complex determined with anti-FLAG was stronger in baclofen-stimulated cells than that in nonstimulated cells (fig. 6B).

To determine the effect of *S*(+)-ketamine on the protein complex formation of FLAG-GRK4 or FLAG-GRK5 with GB$_2$R, we treated *S*(+)-ketamine (100 μM) to the cells coexpressing FLAG-GRK4 or FLAG-GRK5, HA-GB$_2$R, and GB$_{1a}$R 5 min before and during the stimulation of baclofen (5 min, 100 μM). In the precipitate using anti-HA from the cells coexpressing either FLAG-GRK4 or FLAG-GRK5 with HA-GB$_2$R and GB$_{1a}$R, the intensity of the immune complex with anti-HA was similar among nonstimulated and baclofen-stimulated cells with or without *S*(+)-ketamine treatment (fig. 6A). On the other hand, the intensity of the immune complex determined with anti-FLAG was less in baclofen-stimulated cells with *S*(+)-ketamine treatment than in baclofen-stimulated cells without *S*(+)-ketamine treatment; and the intensity in baclofen-stimulated cells with

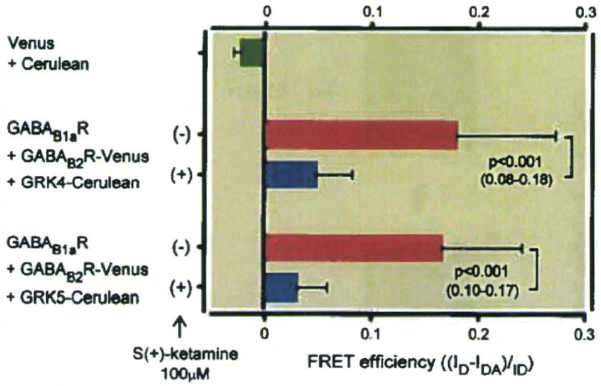

Fig. 5. Comparison of fluorescence resonance energy transfer (FRET) efficiency in baby hamster kidney (BHK) cells expressing γ-aminobutyric acid $(GABA)_{B1a}$ receptor subunit ($GB_{1a}R$), $GABA_{B2}$ subunit (GB_2R)–Venus, and G protein–coupled receptor (GRK) 4–Cerulean or GRK5-Cerulean, with or without previous stimulation of *S*(+)-ketamine (n = 8 for each group). The FRET efficiency was calculated from emission spectra. Each bar represents the mean ± SD. Statistical results are represented as *P* values (95% confidence interval for the differences in the two conditions). I_D = peak of donor emission in presence of sensitized acceptor; I_{DA} = peak of donor emission in presence of acceptor.

S(+)-ketamine was almost similar to that in nonstimulated cells (fig. 6B). In the total lysate, the intensity of the immune complex determined with anti-FLAG was similar among nonstimulated and baclofen-stimulated cells with or without *S*(+)-ketamine treatment (fig. 6C). *S*(+)-Ketamine treatment alone (100 μM) did not affect the intensity of the immune complex determined with anti-HA (HA-$GABA_{B2}R$) and that determined with anti-FLAG (FLAG-GRK4 and FLAG-GRK5) (data not shown).

Discussion

Previously, it was demonstrated that the desensitization of $GABA_BR$-mediated responses was associated with the formation of protein complexes of the GB_2R subunit with GRK 4 or 5 on the plasma membranes, which may cause signal disconnection from the receptors to downstream transducers, such as G proteins.[7] In the current study, the same desensitization was observed by the second application of baclofen in *Xenopus* oocytes coexpressing heterodimeric $GABA_BR$ and GIRKs in the presence of GRK 4 or 5. We demonstrated that pretreatment of *S*(+)-ketamine significantly suppressed such desensitization. Furthermore, our results showed that the translocation of GRK4-Venus or GRK5-Venus to the plasma membranes after stimulation of baclofen was inhibited by pretreatment of *S*(+)-ketamine in BHK cells. In addition, FRET analysis showed that *S*(+)-ketamine inhibited the protein complex formation of GB_2R-Venus with GRK4-Cerulean or GRK5-Cerulean in the cells. Such an inhibitory effect of protein complex formation by *S*(+)-ketamine was also confirmed by coimmunoprecipitation and Western blot analysis in cells coexpressing HA-GB_2R, $GB_{1a}R$, and FLAG-

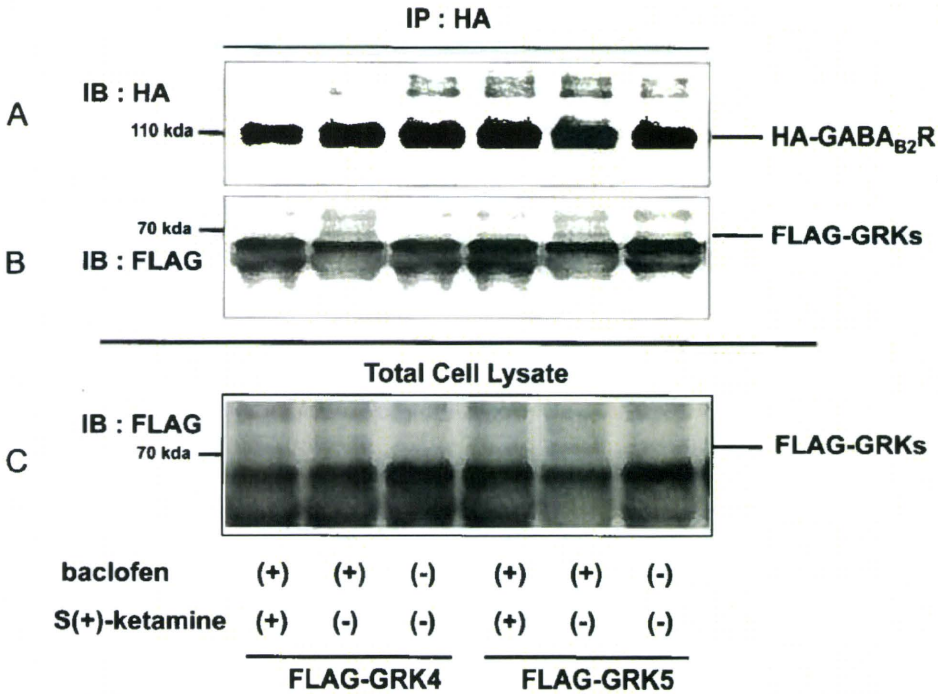

Fig. 6. Immunoprecipitation and Western blot analysis of hemagglutinin (HA)–γ-aminobutyric acid $(GABA)_{B2}$ subunit (GB_2R) and N-DYKDDDDK-C (FLAG)–G protein–coupled receptor (GRK) proteins extracted from nonstimulated cells, baclofen-stimulated cells (100 μM, 5 min), or baclofen-stimulated cells (100 μM, 5 min) with previous stimulation of *S*(+)-ketamine (100 μM, 5 min), coexpressing $GABA_{B1a}$ receptor subunit ($GB_{1a}R$), HA-GB_2R, and FLAG-GRKs. Western blot of anti–HA immunoprecipitates from FLAG-GRK4– or FLAG-GRK5–expressing cells determined with anti-HA (*A*) and anti-FLAG (*B*) and with anti-FLAG in the total lysate (*C*).

GRK4 or FLAG-GRK5. Collectively, these results suggest that *S*(+)-ketamine could suppress the GRK 4– or 5–induced $GABA_BR$ desensitization, at least in part, by interfering with the protein complex formation of GRK 4 or 5 with the GB_2R subunit.

The selective $GABA_BR$ agonist baclofen is widely used as a spasmolytic drug. ITB therapy, proposed by Penn and Kroin[26] in 1984, is a method for the treatment of spasticity and rigidity of spinal and cerebral origin, approved by the Food and Drug Administration in 1992.[1] Recently, it was reported that ITB therapy is also effective in the management of various forms of chronic pain, with or without spasticity.[1–5] There is no doubt that ITB therapy will play a greater part in the management of chronic pain[1]; however, long-term management of ITB therapy has been reported to occasionally result in the development of tolerance to baclofen in both clinical[6] and animal[27] studies. Several reports have shown that intrathecal administration of morphine in place of baclofen for some period (the so-called baclofen holiday)[28] or a shift in treatment to continuous intrathecal morphine administration[29] was effective for pain management in patients who had developed tolerance against ITB therapy. However, the preventive measures for the development of baclofen tolerance have not been established yet.

Baclofen tolerance is the condition in that gradually increased doses of baclofen are required to keep the therapeutic effects stable. Many processes underlie baclofen tolerance *in vivo*, including adaptations in neural circuitry (*e.g.*, descending excitatory pathways) and changes in neurotransmitter signaling pathways surrounding the $GABA_BR$ neuron. In addition, cellular responses mediated by $GABA_BR$ are attributed to the development of baclofen tolerance. In the rat model, ITB down-regulated the number of $GABA_BR$ binding sites in the spinal cord.[30] Desensitization of $GABA_BR$-mediated signaling is one of the mechanisms of development of baclofen tolerance. The desensitization of $GABA_BR$ was induced after protein complex formation of GB_2R with GRK 4 or 5.[7,8] Ketamine is an agent that has widely been used as an analgesic for postoperative pain,[18] chronic noncancer pain,[31] and cancer pain.[32] Although it has been commonly acknowledged that ketamine shows an analgesic effect by blocking the *N*-methyl-D-aspartate receptors in the central nervous system, many other prospective targets are reported (*e.g.*, muscarinic acetylcholine receptors,[33] opioid receptors,[34] substance P receptors,[35] and voltage-dependent Na^+ and K^+ channels).[36] In animal studies, intrathecal[13] or subcutaneous[14] administration of ketamine attenuated the development of tolerance to morphine. The precise mechanisms of such phenomena were not understood; however, tolerance of opioids to μ-opioid receptors could be attributed by receptor desensitization, in which GRKs 2 and 3 were involved.[15–17] One possibility is that ketamine would inhibit μ-opioid receptor–mediated desensitization by modulation of GRK 2 or 3. Likewise, we expected, and suggested, that *S*(+)-ketamine would attenuate the development of tolerance to baclofen to the sites where GRK 4 or 5 is involved in $GABA_BR$-mediated desensitization.[7,8] It is not known how *S*(+)-ketamine interferes the baclofen-induced protein complex formation of GB_2R with GRK 4 or 5. Because there are no *N*-methyl-D-aspartate, muscarinic, opioid, substance P receptors, and no voltage-dependent Na^+ and K^+ channels, expressed in our experimental system, we could say that we find another intracellular target site for ketamine that is independent of the previously reported receptors and ion channel modulation. Taken together, we showed, for the first time to our knowledge, that desensitization of $GABA_BR$-mediated signaling was significantly attenuated by pretreatment of *S*(+)-ketamine, suggesting that S(+)-ketamine suppresses baclofen-induced $GABA_BR$ desensitization, possibly followed by greater antinociceptive effects when used in ITB therapy for long-term pain management.

Clinically, our results propose the possibility that combination intrathecal administration of *S*(+)-ketamine with ITB therapy provides high-quality pain relief without tolerance of ITB to patients experiencing chronic pain. Intrathecal ketamine has been administered in an animal model and to humans, but the safety of preservative-free ketamine through the intrathecal route remains controversial.[37–40] Although some reports have shown no neurotoxic damage after intrathecal administration of preservative-free ketamine using pig[37] and rabbit[38] models, recent animal studies have shown the severe neurotoxicity of intrathecal administration of ketamine with canine[39] and rabbit.[40] Pathologic findings also demonstrated subpial spinal cord vacuolar myelopathy after intrathecal ketamine in a terminally ill cancer patient who received continuous-infusion intrathecal ketamine for 3 weeks.[41] Furthermore, the continuous intrathecal administration of *S*(+)-ketamine, in combination with morphine, bupivacaine, and clonidine, resulted in adequate pain relief in a patient experiencing intractable neuropathic cancer pain; however, postmortem observation of the spinal cord and nerve roots revealed severe histologic abnormalities, including central chromatolysis, nerve cell shrinkage, neuronophagia, microglial up-regulation, and gliosis.[42] A recent report[43] indicates that the neurotoxicity of *S*(+)-ketamine is produced by blockade of *N*-methyl-D-aspartate receptors on the inhibitory neurons, resulting in an exicitotoxic injury through hyperactivation of muscarinic M_3 receptors and non–*N*-methyl-D-aspartate glutamate receptors in the cerebral cortex. Yaksh *et al.*[39] recently reported the detailed toxicology profile of an *N*-methyl-D-aspartate antagonist, including ketamine, delivered through long-term (28-day) intrathecal infusion in the canine model and suggested needs for reevaluation of the use of these agents in long-term spinal delivery. Clinical and pathologic results from an animal or clinical study with intrathecal administration of a combination of baclofen and ketamine have not been reported. Thus, carefully designed studies with an animal model and a clinical trial should be required to know how ketamine (*i.e.*, timing of administration, concentration, duration of adminis-

tration, and ratio of doses of ketamine and baclofen) is safely administered without pathophysiologic findings and how it might suppress the development of baclofen-induced tolerance clinically.

In conclusion, we demonstrated that S(+)-ketamine suppressed the baclofen-induced desensitization of $GABA_BR$-mediated signaling, at least in part, through inhibition of protein complex formation of the GB_2R subunit and GRK 4 or 5. If the safety of intrathecal administration of S(+)-ketamine is established, it could be a candidate for preventing the development of tolerance against ITB therapy in long-term spasticity and pain management.

The authors thank Kohtaro Taniyama, M.D., Ph.D., Department of Technology, Nagasaki Institute of Applied Science, Nagasaki, Japan, for helpful discussion, and Shinichi Haruta and Ai Ohnishi, Medical Students, Nagasaki University School of Medicine, Nagasaki, Japan, for their skilled technical assistance.

References

1. Slonimski M, Abram SE, Zuniga RE: Intrathecal baclofen in pain management. Reg Anesth Pain Med 2004; 29:269-76
2. Zuniga RE, Schlicht CR, Abram SE: Intrathecal baclofen is analgesic in patients with chronic pain. Anesthesiology 2000; 92:876-80
3. Taira T, Hori T: Intrathecal baclofen in the treatment of post-stroke central pain, dystonia, and persistent vegetative state. Acta Neurochir Suppl 2007; 97:227-9
4. Van Hilten BJ, Van de Beek WJT, Hoff JI, Voormolen JHC, Delhaas EM: Intrathecal baclofen for the treatment of dystonia in patients with reflex sympathetic dystrophy. N Engl J Med 2000; 343:625-30
5. Zuniga RE, Perera S, Abram SE: Intrathecal baclofen: A useful agent in the treatment of well-established complex regional pain syndrome. Reg Anesth Pain Med 2002; 27:90-3
6. Nielsen JF, Hansen HJ, Sunde N, Christensen JJ: Evidence of tolerance to baclofen in treatment of severe spasticity with intrathecal baclofen. Clin Neurol Neurosurg 2002; 104:142-5
7. Kanaide M, Uezono Y, Matsumoto M, Hojo M, Ando Y, Sudo Y, Sumikawa K, Taniyama K: Desensitization of $GABA_B$ receptor signaling by formation of protein complexes of $GABA_{B2}$ subunit with GRK4 or GRK5. J Cell Physiol 2007; 210:237-45
8. Perroy J, Adam L, Qanbar R, Chenier S, Bouvier M: Phosphorylation-independent desensitization of $GABA_B$ receptor by GRK4. EMBO J 2003; 22:3816-24
9. Pitcher JA, Freedman NJ, Lefkowitz RJ: G protein-coupled receptor kinases. Annu Rev Biochem 1998; 67:653-92
10. Wallace M, Yaksh TL: Long-term spinal analgesic delivery: A review of the preclinical and clinical literature. Reg Anesth Pain Med 2000; 25:117-57
11. Kedlaya D, Reynolds L, Waldman S: Epidural and intrathecal analgesia for cancer pain. Best Pract Res Clin Anaesthesiol 2002; 16:651-65
12. Kosson D, Klinowiecka A, Kosson P, Bonney I, Carr D, Mayzner-Zawadzka E, Lipkowski A: Intrathecal antinociceptive interaction between the NMDA antagonist ketamine and the opioids, morphine and biphalin. Eur J Pain 2008; 12: 611-6
13. Miyamoto H, Saito Y, Kirihara Y, Hara K, Sakura S, Kosaka Y: Spinal coadministration of ketamine reduces the development of tolerance to visceral as well as somatic antinociception during spinal morphine infusion. Anesth Analg 2000; 90:136-41
14. Shimoyama N, Shimoyama M, Inturrisi CE, Elliott K: Ketamine attenuates and reverses morphine tolerance in rodents. Anesthesiology 1996; 85:1357-66
15. Zhang J, Ferguson SSG, Barak LS, Bodduluri SR, Laporte SA, Law PY, Caron MG: Role for G protein-coupled receptor kinase in agonist-specific regulation of μ-opioid receptor responsiveness. Proc Natl Acad Sci U S A 1998; 95:7157-62
16. Terman GW, Jin W, Cheong YP, Lowe J, Caron MG, Lefkowits RJ, Chavkin C: G-protein receptor kinase 3 (GRK3) influences opioid analgesic tolerance but not opioid withdrawal. Br J Pharmacol 2004; 141:55-64
17. Kovoor A, Nappey V, Kieffer BL, Chavkin C: μ And δ opioid receptors are differentially desensitized by the coexpression of β-adrenergic receptor kinase 2 and β-arrestin 2 in *Xenopus* oocyte. J Biol Chem 1997; 272:27605-11
18. Craven R: Ketamine. Anesthesia 2007; 62:48-53
19. Ivanina T, Varon D, Peleg S, Rishal I, Porozov Y, Dessauer CW, Keren-Raifman T, Dascal N: $G\alpha_{i1}$ and $G\alpha_{i3}$ differentially interact with, and regulate, the G protein-activated K^+ channel. J Biol Chem 2004; 279:17260-8
20. Uezono Y, Kanaide M, Kaibara M, Barzilai R, Dascal N, Sumikawa K, Taniyama K: Coupling of $GABA_B$ receptor $GABA_{B2}$ subunit to G proteins: Evidence from *Xenopus* oocyte and baby hamster kidney cell expression system. Am J Physiol Cell Physiol 2006; 290:C200-7
21. Uezono Y, Bradley J, Min C, McCarty NA, Quick M, Riordan JR, Chavkin C, Zinn K, Lester HA, Davidson N: Receptors that couple to 2 classes of G proteins increase cAMP and activate CFTR expressed in *Xenopus* oocytes. Receptor Channels 1993; 1:233-41
22. Uezono Y, Akihara M, Kaibara M, Kawano C, Shibuya I, Ueda Y, Yanagihara N, Toyohira Y, Yamashita H, Taniyama K, Izumi F: Activation of inwardly rectifying K^+ channels by GABA-B receptors expressed in *Xenopus* oocytes. Neuroreport 1998; 9:583-7
23. Villemure JF, Adam L, Bevan NJ, Gearing K, Chenier S, Bouvier M: Subcellular distribution of $GABA_B$ receptor homo- and hetero-dimers. Biochem J 2005; 388:47-55
24. Miyawaki A, Tsien RY: Monitoring protein conformations and interactions by fluorescence resonance energy transfer between mutants of green fluorescent protein. Methods Enzymol 2000; 327:472-500
25. Riven I, Kalmanzon E, Segev L, Reuveny E: Conformational rearrangements associated with the gating of the G protein-coupled potassium channel revealed by FRET microscopy. Neuron 2003; 38:225-35
26. Penn RD, Kroin JS: Intrathecal baclofen alleviates spinal cord spasticity. Lancet 1984; 12:1078
27. Hefferan MP, Fuchigami T, Marsala M: Development of baclofen tolerance in a rat model of chronic spasticity and rigidity. Neurosci Lett 2006; 403:195-200
28. Vidal J, Gregori P, Guevara D, Portell E, Valles M: Efficacy of intrathecal morphine in the treatment of baclofen tolerance in a patient on intrathecal baclofen therapy (ITB). Spinal Cord 2004; 42:50-1
29. Soni BM, Mani RM, Oo T, Vaidyanathan S: Treatment of spasticity in a spinal cord-injured patient with intrathecal morphine due to intrathecal baclofen tolerance: A case report and review of literature. Spinal Cord 2003; 41:586-9
30. Kroin JS, Bianchi GD, Penn RD: Intrathecal baclofen down-regulates GABAB receptors in the rat substantia gelatinosa. J Neurosurg 1993; 79:544-9
31. Bell RF: Ketamine for chronic non-cancer pain. Pain 2009; 141:210-4
32. Okon T: Ketamine: An introduction for the pain and palliative medicine physician. Pain Physician 2007; 10:493-500
33. Durieux ME: Inhibition by ketamine of muscarinic acetylcholine receptor function. Anesth Analg 1995; 81:57-62
34. Sarton E, Teppema LJ, Olivier C, Nieuwenhuijs D, Matthes

HWD, Kieffer BL, Dahan A: The involvement of the μ-opioid receptor in ketamine-induced respiratory depression and antinociception. Anesth Analg 2001; 93:1495–500

35. Okamoto T, Minami K, Uezono Y, Ogata J, Shiraishi M, Shigematsu A, Ueta Y: The inhibitory effects of ketamine and pentobarbital on substance P receptors expressed in *Xenopus* oocytes. Anesth Analg 2003; 97:104–10
36. Schnoebel R, Wolff M, Peters SC, Brau ME, Scholz A, Hempelmann G, Olschewski H, Olshewski A: Ketamine impairs excitability in superficial dorsal horn neurons by blocking sodium and voltage-gated potassium currents. Br J Pharmacol 2005; 146:826–33
37. Errando CL, Sifre C, Moliner S, Valia JC, Gimeno O, Minguez A, Boils P: Subarachnoid ketamine in swine—pathological findings after repeated doses: Acute toxicity study. Reg Anesth Pain Med 1999; 24:146–52
38. Malinovsky JM, Lepage JY, Cozian A, Mussini JM, Pinaudt M, Souron R: Is ketamine or its preservative responsible for neurotoxicity in the rabbit? ANESTHESIOLOGY 1993; 78:109–15
39. Yaksh TL, Tozier N, Horais KA, Malkmus S, Rathbun M, LaFranco L, Eisenach J: Toxicology profile of N-methyl-D-aspartate antagonists delivered by intrathecal infusion in the canine model. ANESTHESIOLOGY 2008; 108:938–49
40. Vranken JH, Troost D, de Hssn P, Pennings FA, van der Vegt MH, Dijlgraaf MGW, Hollmann MW: Severe toxic damage to the rabbit spinal cord after intrathecal administration of preservative-free S(+)-ketamine. ANESTHESIOLOGY 2006; 105: 813–8
41. Karpinski N, Dunn J, Hansen L, Masliah E: Subpial vacuolar myelopathy after intrathecal ketamine: Report of a case. Pain 1997; 73:103–5
42. Vranken JH, Troost D, Wegener JT, Kruis MR, van der Vegt MH: Neuropathological findings after continuous intrathecal administration of S(+)-ketamine for the management of neuropathis cancer pain. Pain 2005; 117:231–5
43. Farber NB, Kim SH, Dikranian K, Jiang XP, Heinkel C: Receptor mechanisms and circuitry underlying NMDA antagonist neurotoxicity. Mol Psychiatry 2002; 7:32–43