3. Results

3.1. Increase in $[Ca^{2+}]_i$ by spinal astrocytes through NK-1 receptor activation

In the presence of extracellular Ca^{2+}, SP evoked an increase in the $[Ca^{2+}]_i$ in a dose-dependent manner at a concentration range of 1–100 nM as shown in Fig. 1A–C. The Ca^{2+} response rapidly peaked after treatment with SP, and then gradually returned toward the basal level within several minutes. The extent of the SP-induced increase in $[Ca^{2+}]_i$ was calculated using the differences between the fura-2 fluorescence ratio (340/380) of the resting level observed before SP treatment and the peak level obtained after SP treatment (Fig. 1G). Next, we investigated which subtypes of NK receptors were involved in the increase of $[Ca^{2+}]_i$ in cells treated with 100 nM of SP. The SP-induced increase in $[Ca^{2+}]_i$ was completely suppressed by pretreatment with CP-96346 (10 μM), a selective antagonist of the NK-1 receptor (Fig. 1D and H). In contrast, neither GR94800 (10 μM), a selective NK-2 antagonist, nor SB222200 (10 μM), a selective NK3 antagonist, affected the SP-

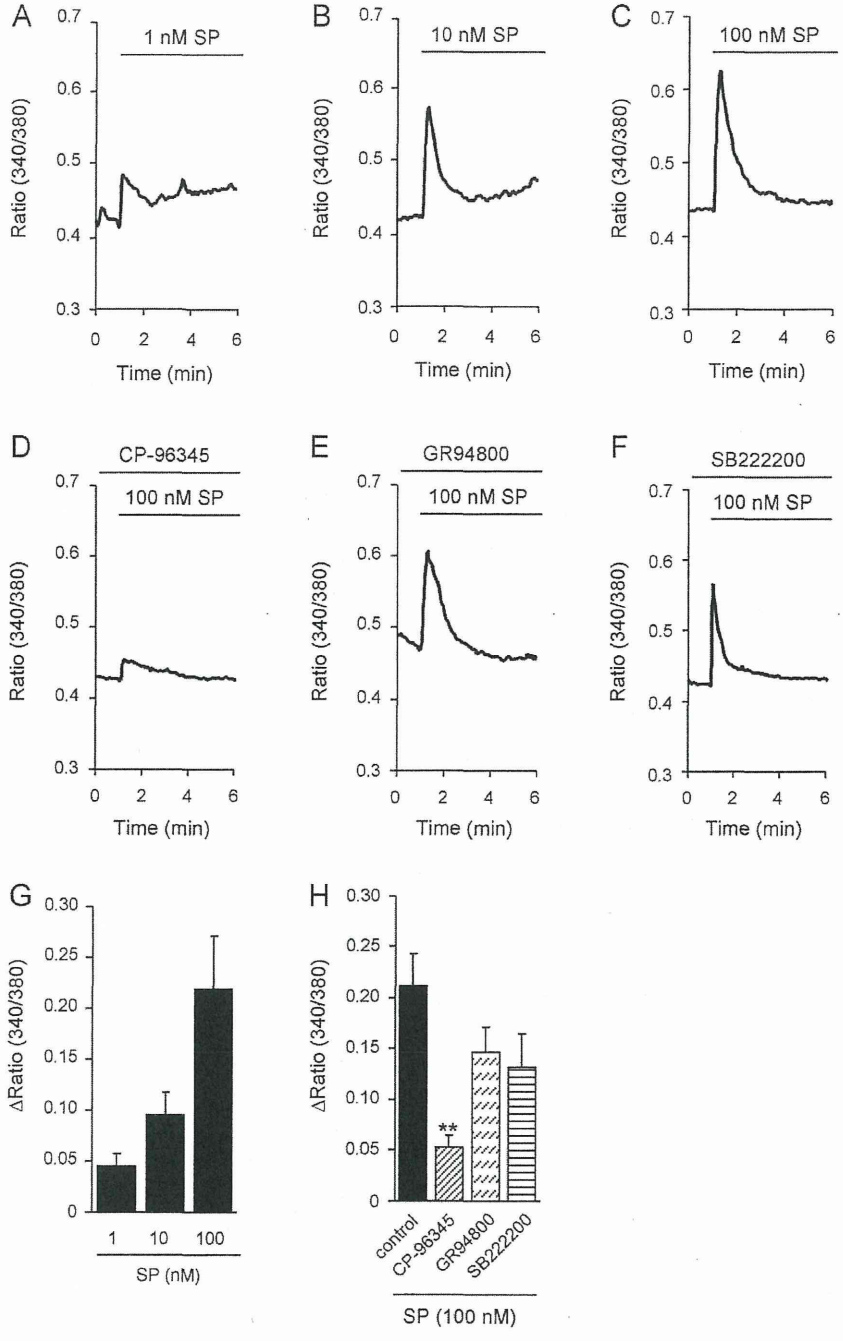

Fig. 1. Mobilization of $[Ca^{2+}]_i$ in spinal astrocytes stimulated with SP. The trace in each graph (A–F) shows the representative mean $[Ca^{2+}]_i$ in randomly selected cells. The fura 2-loaded cells were treated with 1–100 nM of SP in Hanks' buffer, respectively (A–C). After the cells were pretreated with 10 μM of CP96345 (D), GR94800 (E) or SB222200 (F) for 20 min, then cells were stimulated with 100 nM of SP. The extent of the increase in $[Ca^{2+}]_i$ induced by SP was quantified by determining the differences between the ratio (340/380) of the basal and the peak level obtained after SP treatment (G and H). 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 SP alone.

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

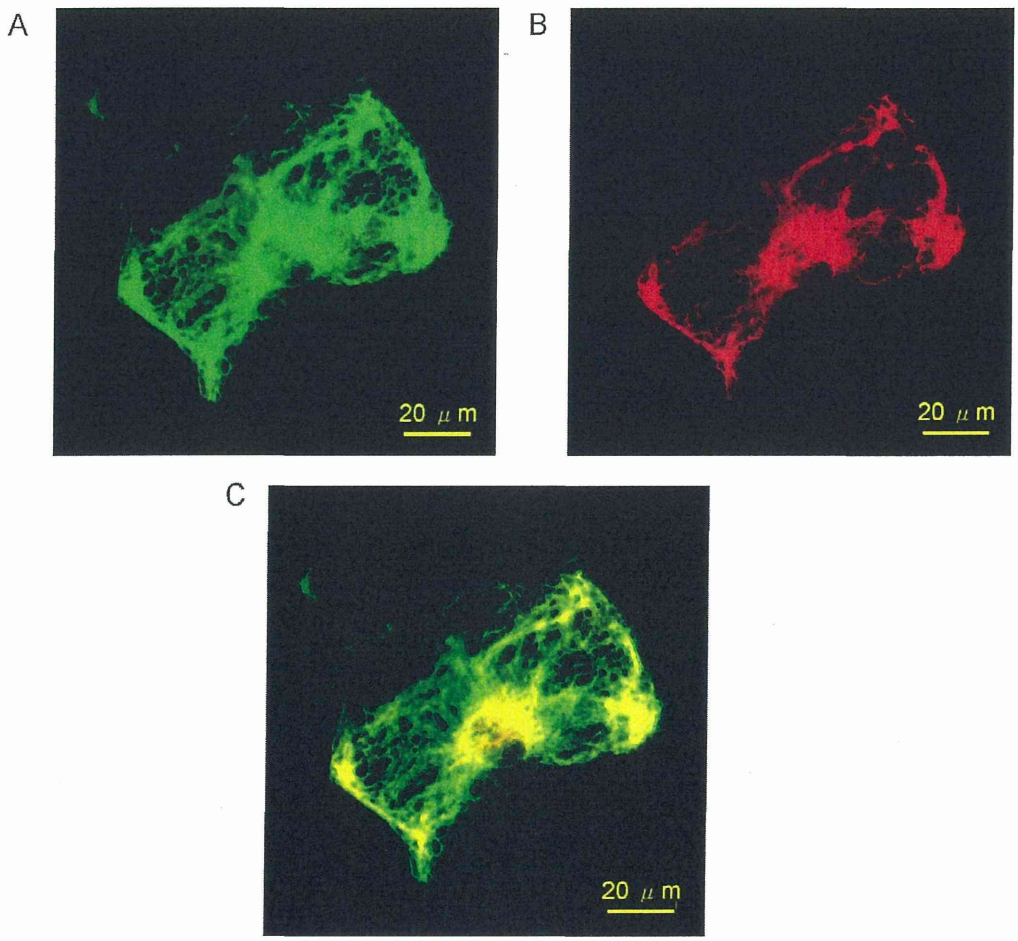

Fig. 2. Spinal astrocytes express the NK-1 receptor. Immunofluorescent analysis of GFAP (green; A) and the NK-1 receptor (red; B) expression in cultured spinal astrocytes. The expression of the NK-1 receptor was found in GFAP-labeled cells (C).

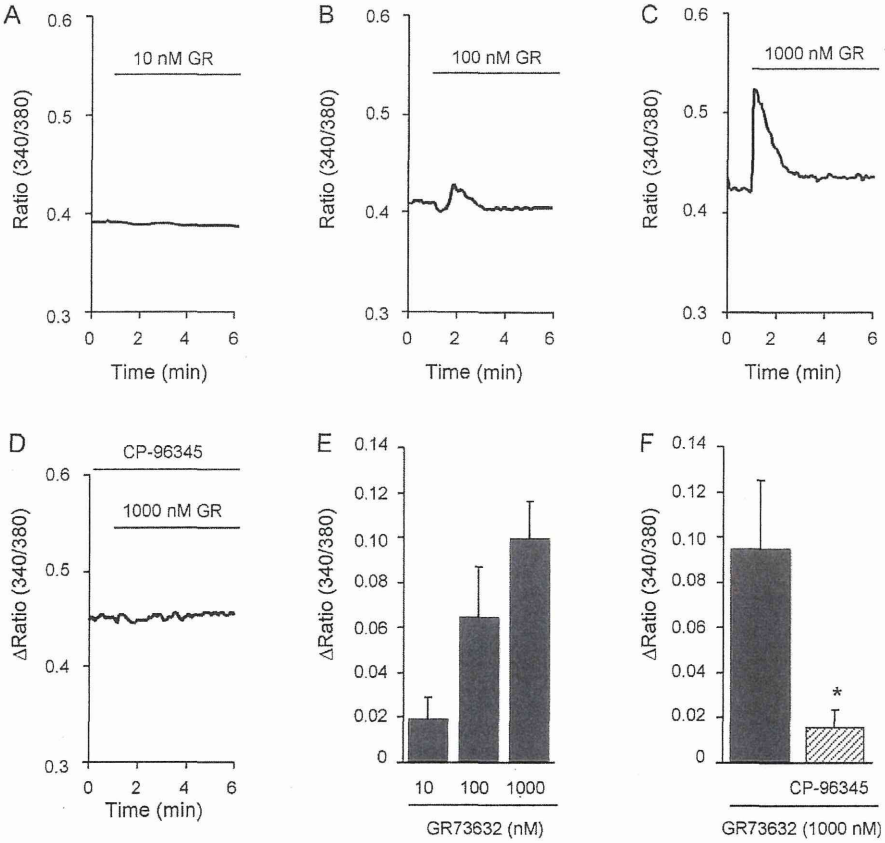

Fig. 3. Mobilization of $[Ca^{2+}]_i$ in spinal astrocytes stimulated with GR73632. The trace in each graph (A–D) shows the representative mean $[Ca^{2+}]_i$ in randomly selected cells. The fura-2-loaded cells were treated with 10–1000 nM of GR73632 (GR) in Hanks' buffer (A–C). After the cells were pretreated 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

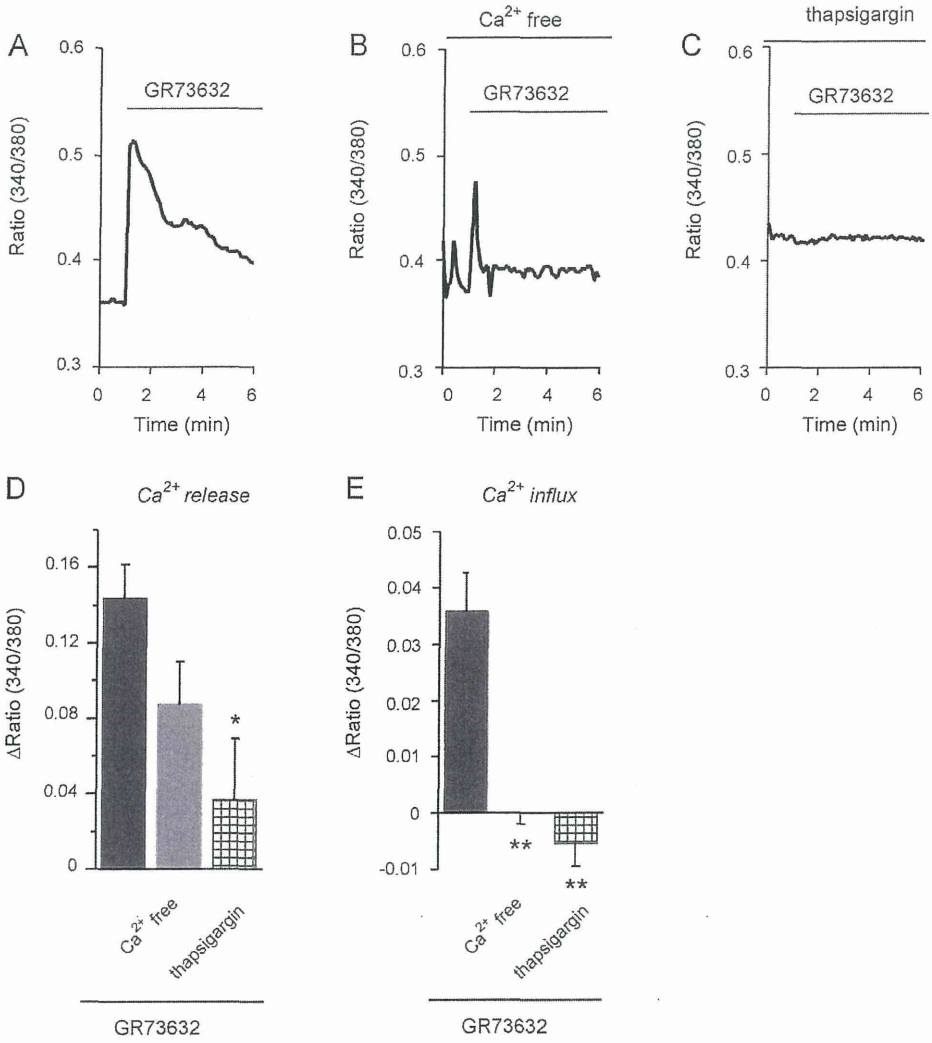

Fig. 4. Influence of both Ca^{2+} store and extracellular Ca^{2+} on the GR73632-induced increase in $[Ca^{2+}]_i$ in spinal astrocytes. The trace in each graph (A–C) shows the representative mean $[Ca^{2+}]_i$ in randomly selected cells. The fura-2-loaded cells were treated with 1000 nM of GR73632 in 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.

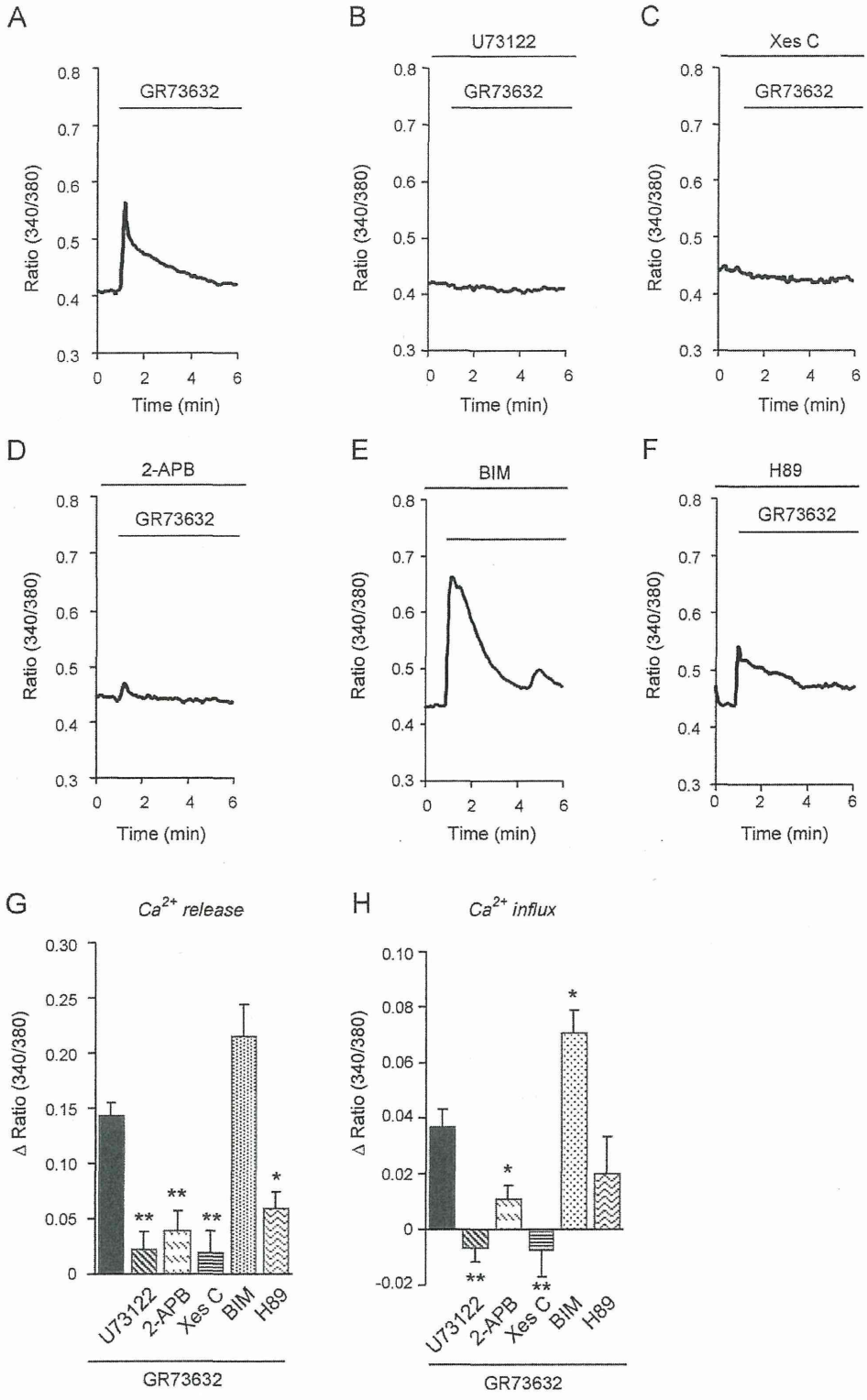

Fig. 5. Effects of inhibitors of several intracellular signaling molecules on the GR73632-induced increase in $[Ca^{2+}]_i$ in spinal astrocytes. The trace in each graph (A–F) shows the representative mean of $[Ca^{2+}]_i$ in randomly selected cells. The fura-2-loaded cells were treated with 1000 nM 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.

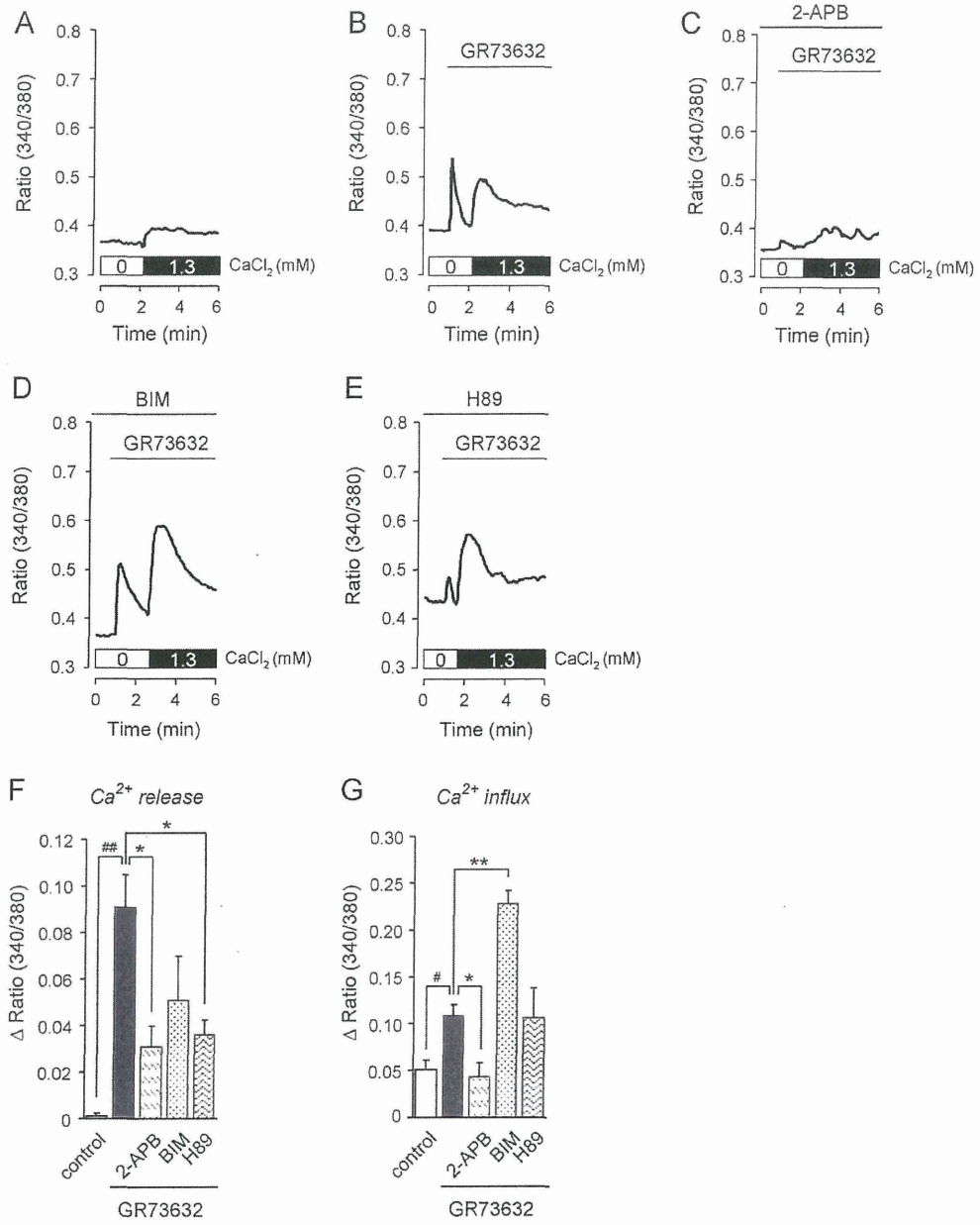

Fig. 6. Effects of 2-APB, BIM and H89 on both Ca^{2+} release and extracellular Ca^{2+} influx induced by GR73632 in spinal astrocytes. The trace in each graph (A–E) shows the representative mean $[Ca^{2+}]_i$ in randomly selected cells. The fura-2-loaded cells were either untreated (A) or were treated 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.

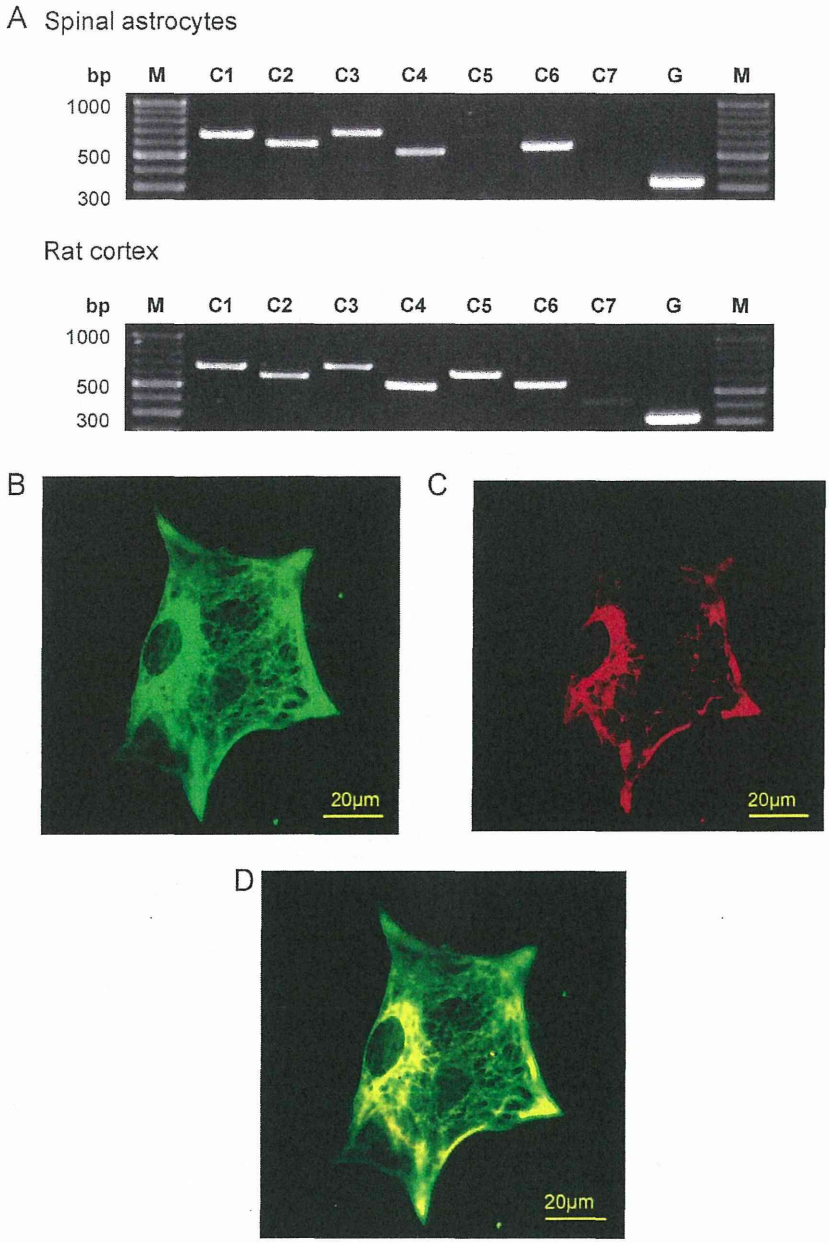

Fig. 7. TRPC3 is expressed in cultured spinal astrocytes. (A) RT-PCR analysis of TRPC channels mRNA expression in cultured rat spinal astrocytes. Each lane represents the cDNA fragments of TRPC1–7 (C1–C7) and GAPDH (G) amplified from the RNA of cultured spinal astrocytes (the upper panel) or rat cortex (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

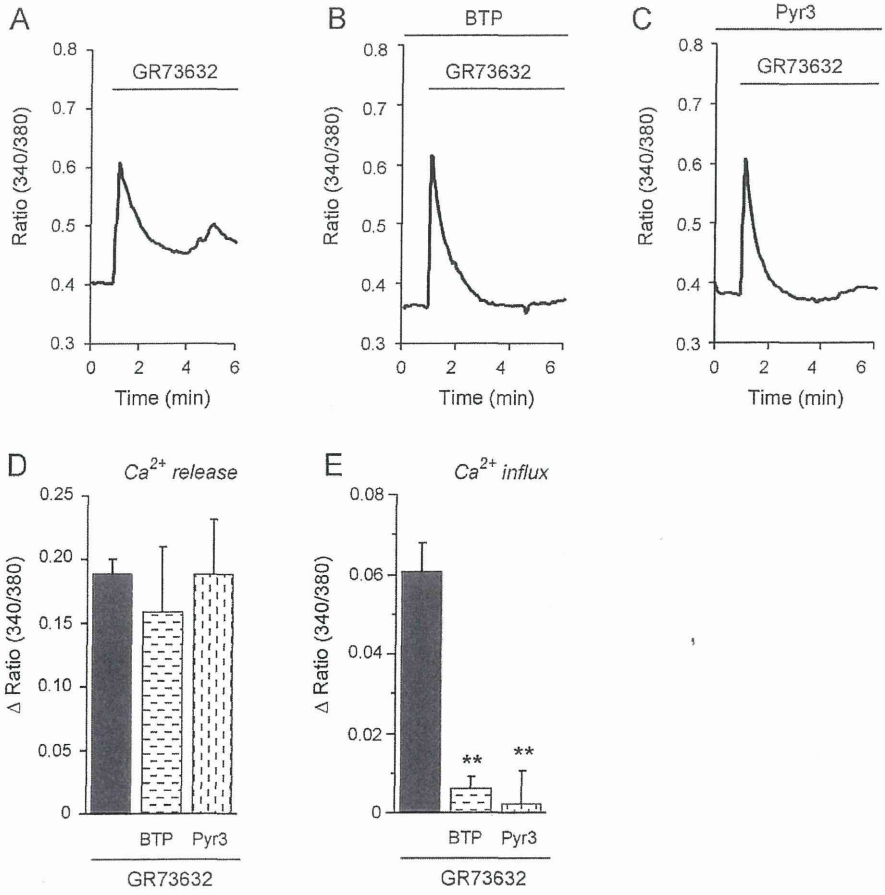

Fig. 8. Effects of inhibitors of TRPC channels on the GR73632-induced increase in $[Ca^{2+}]_i$ by spinal astrocytes. The trace in each graph (A–C) shows the representative mean $[Ca^{2+}]_i$ in randomly selected cells. The fura-2-loaded cells were treated with 1000 nM of GR73632 in Hanks' buffer (A–C). 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+}

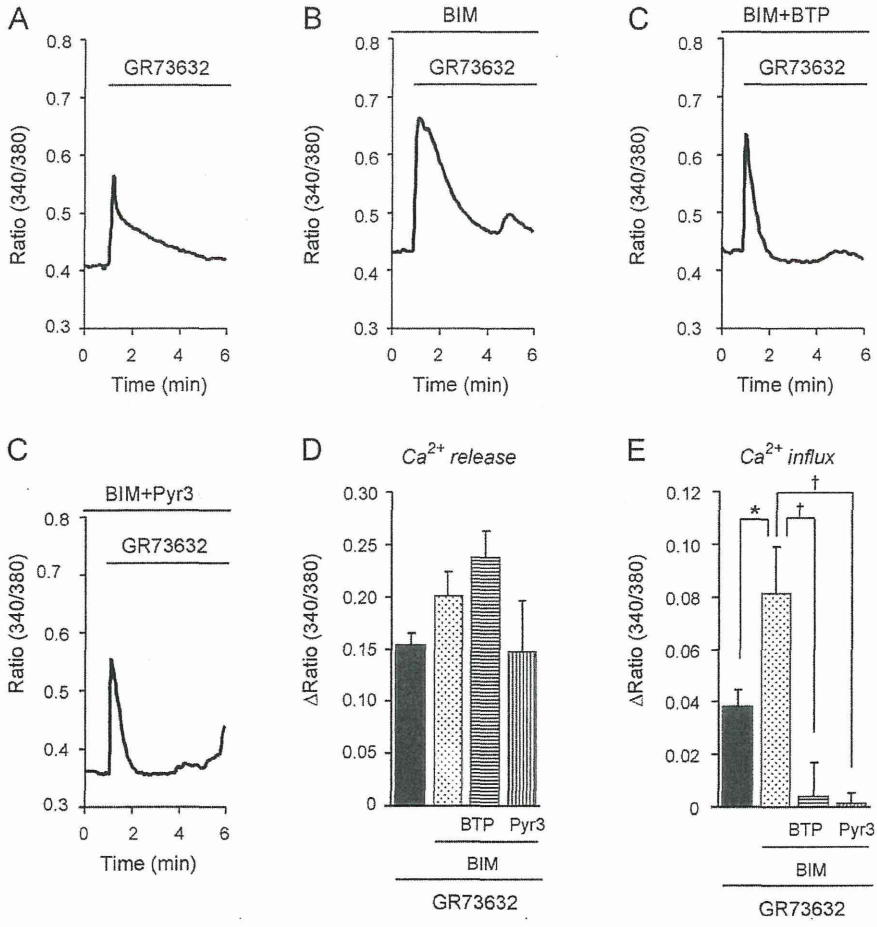

Fig. 9. Involvement of PKC in the GR73632-induced increase in $[Ca^{2+}]_i$ through TRPC channels in spinal astrocytes. The trace in each graph (A–D) shows the representative mean $[Ca^{2+}]_i$ in randomly selected cells. The fura-2-loaded cells were treated with 1000 nM of GR73632 (GR) in Hanks' buffer (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.

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