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Relationship between specific binding of ^{125}I-ω-conotoxin GVIA and GTP binding protein: effects of the GTP analogues, mastoparan and AlF_4^-

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Abstract

We investigated whether the specific binding or labeling of ^{125}I-ω-CgTX on crude membranes from chick whole brain was affected when endogenous GTP binding protein (G protein) was activated by GTP analogues, mastoparan (MP) and aluminum fluoride (AlF_4^-; $AlCl_3$ + NaF). Both GTPγS and Gpp(NH)p attenuated the inhibitory effect of selective N-type Ca channel inhibitors such as aminoglycoside antibiotics (AGs) or dynorphine (1-13)(Dyn) on specific ^{125}I-ω-CgTX binding in a dose-dependent manner. On the other hand, the inhibitory effects of the divalent metal cations Cd^{2+}, Co^{2+}, Mg^{2+} and Mn^{2+} on such binding were not attenuated by GTPγS. MP and AlF_4^- also attenuated the inhibitory effect of Neo on this binding similar to GTPγS. The attenuating effect of MP was enhanced by the presence of Mg^{2+} in a dose-dependent manner. However, GTP analogues, MP and AlF_4^-, did not affect binding or labeling without AGs or Dyn. GTPγS, MP and AlF_4^- also attenuated the specific labeling of a 215-kDa band in crude membranes with ^{125}I-ω-CgTX using the cross-linker DSS (non-reduced condition) in the presence of Neo. These results indicate that there are direct or indirect relationships between N-type Ca channels and G proteins via binding sites for AGs or MP.

Keywords: N-type Ca channel; GTP binding protein; Omega-conotoxin GVIA binding; Omega-conotoxin GVIA labeling

1. Introduction

Voltage-sensitive calcium channels (Ca channels) are widely distributed in excitable cells and play a fundamental role in regulating many intracellular processes [1–3]. Electrophysiological and pharmacological studies have revealed the presence of four types of Ca channels in neuronal tissue: i.e., L-, N-, P- and T-type Ca channels [4–6].

Omega-conotoxin GVIA (ω-CgTX), which was isolated from the venom of the marine snail *Conus geographus* [7], specifically blocks calcium currents through neuron-specific Ca channels [8–12]. This toxin appears to act on neuronal N-type Ca channels [9,11,13,14].

The characteristics of specific ^{125}I-ω-CgTX binding to chick and rat brain membranes have also been investigated [15–20]. The molecular masses of ω-CgTX binding sites have been determined by photoaffinity labeling in rat brain membranes [21–23] and by chemical cross-linking in chick brain membranes [23,24]. We previously reported that bands of 135 kDa (reduced condition) and 215 or 216 kDa (non-re-

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duced condition) are selectively labeled with ^{125}I-ω-CgTX using the cross-linker disuccinimidyl suberate (DSS) or dithio bis[succinimidyl propionate] in chick whole brain [17,20,25].

A family of membrane-associated guanine nucleotide-binding regulatory proteins (G proteins) is essential for mediating signal transduction between cell-surface receptors and effectors such as adenylate cyclase, phospholipase A_2, phospholipase C and ion channels [26–29]. A wide variety of neurotransmitters and hormones inhibit neuronal Ca^{2+} currents, and it is thought that this is a general mechanism for controlling the release of neurotransmitters at presynaptic terminals [30]. N-type Ca channels also serve as a target for transmitter-induced inhibition [9,31]. The receptor-mediated inhibition of N-type Ca channels requires the activation of G proteins [32,33]. These reports suggest that if N-type Ca channels are directly or indirectly related to G proteins, the specific binding of ^{125}I-ω-CgTX may be affected by the activation of G proteins.

Stumpo et al. [34] reported that the inhibition curves of the aminoglycoside antibiotic (AG) neomycin sulfate (Neo) on ^{125}I-ω-CgTX-binding to hippocampal membranes from rat brain were shifted to the right by GTP analogues or fluoride, and indicated that the inhibition of ^{125}I-ω-CgTX-binding by Neo may be mediated by a G protein.

In this study, to clarify the relationship between the two on crude membranes from chick whole brain, we examined the effects of G protein activators [35–38] such as GTP analogues [35,36], mastoparan (MP) [37] and aluminum fluoride AlF_4^-; $AlCl_3$ + NaF [38] on specific ^{125}I-ω-CgTX binding or ^{125}I-ω-CgTX labeling in the absence or presence of selective inhibitors of N-type Ca channels (such as AGs and dynorphine (1-13) (Dyn) [20]).

A preliminary report of part of this work has appeared previously in abstract form [39].

2. Materials and methods

2.1. Preparation of crude membranes

Crude membranes were prepared from whole brain of 5-day-old chicks as described previously [20], except that the final precipitate was suspended with 10 mM 4 (2-hydroxyethyl)-1-piperazine ethane sulfonic acid (Hepes)-NaOH (pH 7.4 at 4°C).

2.2. Assay of ^{125}I-ω-CgTX binding

Specific binding of ^{125}I-ω-CgTX to crude membranes was assayed by the method of Ichida et al. [20], except that 30 mM Hepes-NaOH (pH 7.4 at 30°C) was used in the assay medium, the concentration of the crude membranes in the assay medium was about 25 μg of protein/tube unless otherwise indicated, and the reaction for specific ^{125}I-ω-CgTX binding was carried out for 90 min at 30°C.

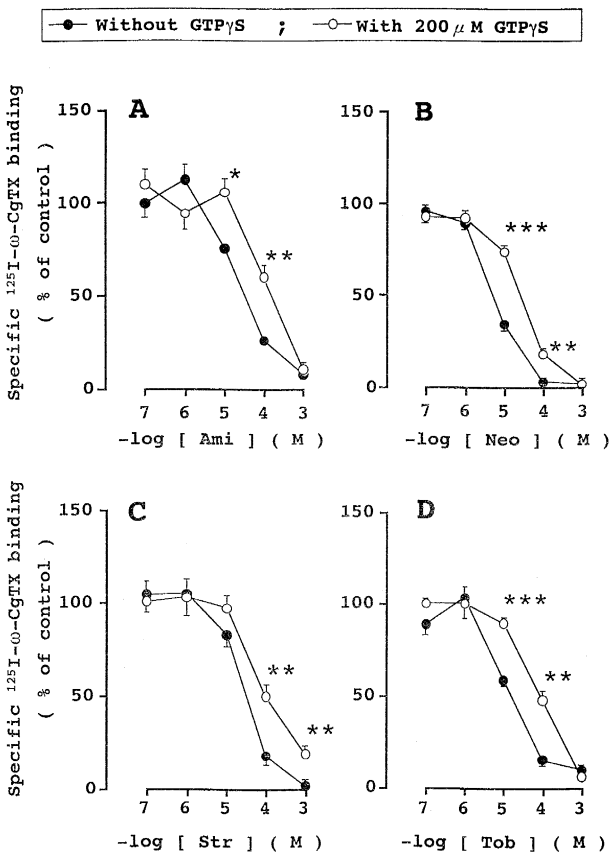

Fig. 1. Effect of GTPγS on specific ^{125}I-ω-CgTX binding as a function of the concentration of AGs. Panels A, B, C and D show the effects of Ami, Neo, Str and Tob, respectively, in the absence (filled circle) and presence (empty circles) of 200 μM GTPγS. Points represent the means of 5–8 preparations from separate animals. Bars indicate standard errors. $^{*}P < 0.05$, $^{**}P < 0.02$ and $^{***}P < 0.01$ vs. binding in the absence of GTPγS.

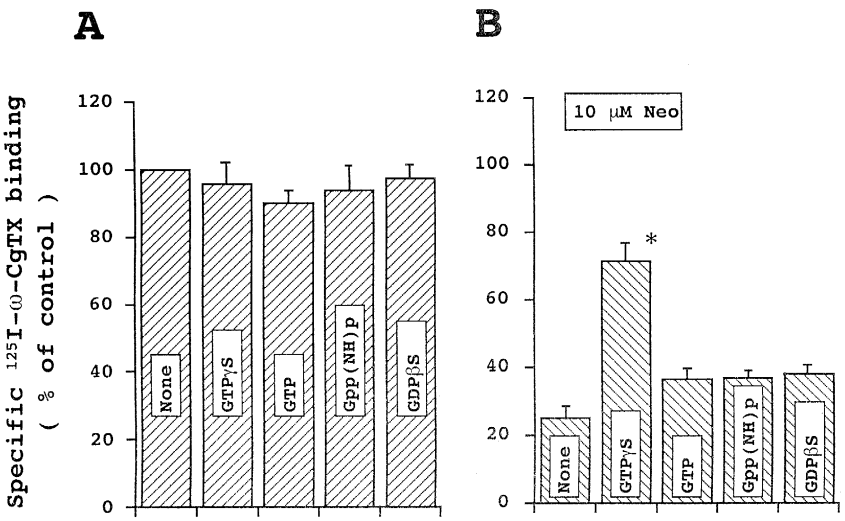

Fig. 2. Effects of GTP analogues (200 μM) on specific ^{125}I-ω-CgTX binding in the absence (panel A) and presence (panel B) of 10 μM Neo. Columns represent the means for 6–8 preparations from separate animals. Bars indicate standard errors. * $P < 0.05$ vs. control.

For experiments on the effects of GTP analogues, MP and AlF_4^- in the absence and presence of AGs or Dyn, these agents were added to the assay medium before the binding reaction was started by adding the crude membranes.

The specific binding of ^{125}I-ω-CgTX was defined as the difference between the radioactivities bound in the absence (total binding) and presence (non-specific binding) of unlabeled ω-CgTX at 1000-fold the concentration of ^{125}I-ω-CgTX.

2.3. Reaction for cross-linking

Specific binding of ^{125}I-ω-CgTX was assayed as described in Section 2.2 with the following modification: 80-μl aliquots of the crude membranes (about 300 μg protein/tube; the concentration of the crude membranes was adjusted to about 3.75 mg protein/ml) were added to the assay medium before the binding reaction was started.

After the specific binding of ^{125}I-ω-CgTX to the crude membranes was carried out for 90 min at 30°C, cross-linking of ^{125}I-ω-CgTX with DSS to its binding sites was assayed by the method of Ichida et al. [20]. The concentration of DSS was 500 μM, and the incubation time for the cross-linking reaction was 1.5 min at 22°C. After the cross-linking reaction was stopped by the addition of 1.2 ml of 100 mM Tris-HCl (pH 8.5 at 22°C), centrifugation, SDS-PAGE under non-reduced conditions and autoradiography were carried out as reported previously [20].

2.4. Other methods

Protein was measured by the method of Lowry et al. [40] with BSA as a standard.

Statistical analyses were performed using the paired or unpaired Student's *t*-test and multiple groups were evaluated by a one-way analysis of variance. A *P*-

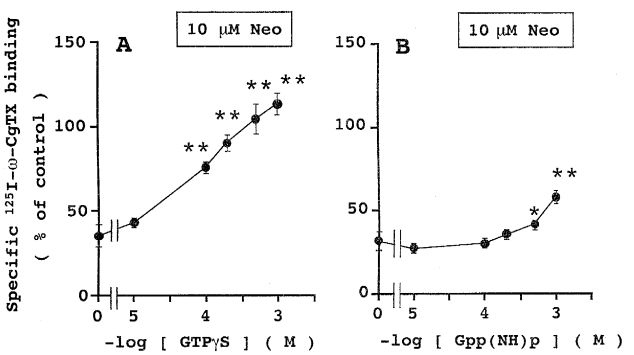

Fig. 3. Specific ^{125}I-ω-CgTX binding in the presence of 10 μM Neo as a function of the concentration of GTPγS (panel A) or Gpp(NH)p (panel B). Points represent the means of 5 preparations from separate animals. Bars indicate standard errors. * $P < 0.05$, ** $P < 0.01$ vs. binding in the absence of GTPγS (panel A) or Gpp(NH)p (panel B).

value of less than 0.05 was considered statistically significant.

2.5. Materials

^{125}I-ω-CgTX (81.4 TBq/m mol) was purchased from Amersham or New England Nuclear Co. DSS was purchased from Pierce Co (Rockford, IL). Dynorphine (1-13), mastoparan and ω-CgTX were from Peptide Institute, Inc. (Osaka, Japan). Amikacin (Ami), neomycin (Neo), streptomycin (Str) and tobramycin (Tob) were from Sigma (St. Louis, MO), Nakalai (Kyoto, Japan) or Wako (Osaka, Japan). Aluminum chloride, cadmium chloride, cobalt dichloride, magnesium chloride, manganese chloride and sodium fluoride were from Wako (Osaka, Japan). GDP and GTP were from Yamasa (Tokyo, Japan). Guanosine 5′-O-2-thiodiphosphate (GDP-β-S), 5′-guanylimidodiphosphate [Gpp(NH)p] and guanosine 5′-O-3-thiotriphosphate (GTPγS) were from Boehringer Mannheim (Mannheim, Germany). U-50488H was a gift from Dr. A. Kawabata (Kinki University).

3. Results

3.1. Effects of GTP analogues

GTPγS (200 μM) attenuated the inhibitory effects of AGs (Ami, Neo, Str and Tob) at various concentrations on the specific binding of ^{125}I-ω-CgTX, as shown in Fig. 1A–D. When the percent inhibition by an AG was more than about 30%, the attenuating effect of GTPγS was particularly significant. The attenuating effect of GTPγS was observed, but those of other GTP analogues (200 μM) such as GTP, Gpp(NH)p and GDP was not significantly observed (Fig. 2B). On the other hand, GTP analogues (200 μM) had no significant effect in the absence of 10 μM Neo (Fig. 2A). The attenuating effect of GTPγS or Gpp(NH)p was dose-dependent (Fig. 3A). Note that Gpp(NH)p at a concentration of more than 400 μM significantly attenuated the inhibitory effect of 10 μM Neo (Fig. 3B), and the attenuating effect of Gpp(NH)p was weaker than that of GTPγS.

Fig. 4B shows the autoradiographic pattern for the effect of GTP analogues (500 μM) on the inhibitory

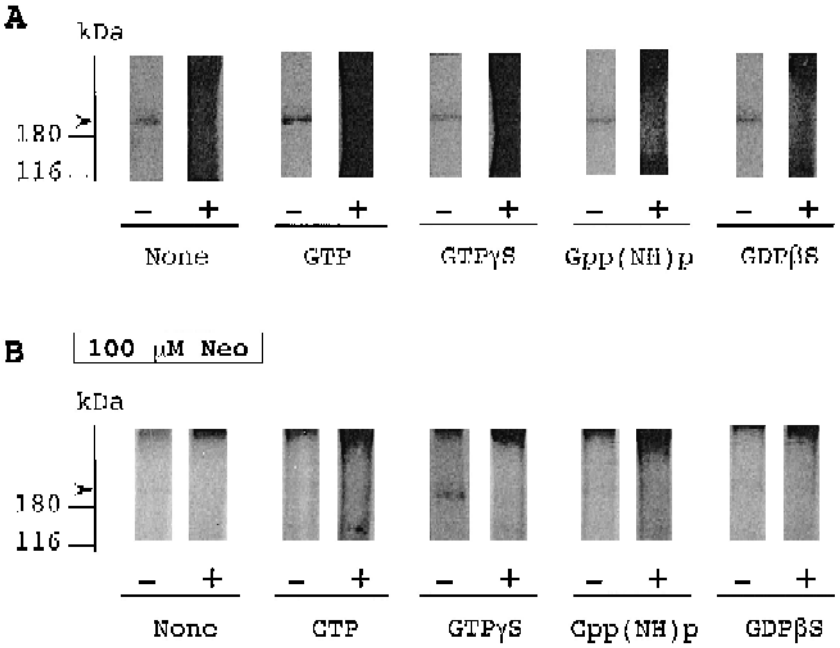

Fig. 4. Effects of GTP analogues on ^{125}I-ω-CgTX labeling in the absence (panel A) or presence (panel B) of 100 μM Neo. GTP and GTP analogues were used at a final concentration of 500 μM. The symbols (−) and (+) indicate the absence and presence of unlabeled ^{125}I-ω-CgTX, at 1000-fold the concentration of ^{125}I-ω-CgTX, in the reaction medium, respectively. The arrowheads in panels A and B indicate the 215-kDa band.

effect of 100 μM Neo on specific ^{125}I-ω-CgTX labeling with the cross-linker DSS. GTPγS only attenuated the inhibitory effect of 100 μM Neo on specific ^{125}I-ω-CgTX labeling to the 215-kDa band. In the absence of Neo, however, none of the GTP analogues affected labeling to the 215-kDa band (Fig. 4A). The concentrations of GTP analogues and Neo used in this labeling experiment were different from those used in the binding experiment because 10 μM Neo and/or 200 μM GTPγS did not have any effect on labeling (data not shown). After further experiments, it became clear that the reason for this difference involved a difference in the protein concentrations of the crude membranes; i.e., binding used 25 μg of protein/tube and labeling used 300 μg of protein/tube (see Section 2). As shown in Fig. 5A, the attenuating effect of 200 μM GTPγS in the presence of 10 μM Neo on binding disappeared at a protein concentration higher than 75 μg/tube. To demonstrate inhibitory and attenuating effects on labeling, higher concentrations of Neo and GTPγS, such as 100 and 500 μM, were needed (Fig. 5B).

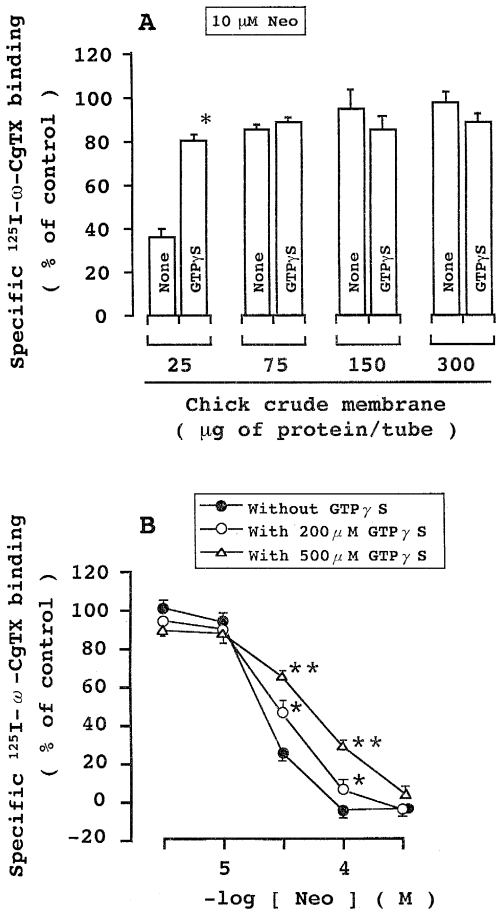

Fig. 5. Panel A shows the effect of 200 μM GTPγS on specific ^{125}I-ω-CgTX binding in the presence of 10 μM Neo as a function of the concentration of protein in the tube. Columns represent the means for 4 preparations from separate animals. Bars indicate standard errors. $^{*}P < 0.05$ vs. binding in the absence of GTPγS. Panel B shows the effects of 200 and 500 μM GTPγS on specific ^{125}I-ω-CgTX binding as a function of the concentration of Neo. The concentration of protein from chick crude membranes is 300 μg of protein/tube. In both panels A and B, points represent the means of 4–6 preparations from separate animals. Bars indicate standard errors. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. binding in the absence of GTPγS.

Thirty μM MP or 30 μM MP plus 1 mM $MgCl_2$ significantly attenuated the inhibitory effect of Neo on specific ^{125}I-ω-CgTX binding, as shown in Fig. 8, and this attenuating effect of MP was dose-dependent (Fig. 9A). The attenuating effect of MP was enhanced in the presence of 1 mM $MgCl_2$, and this enhancement by $MgCl_2$ was also dose-dependent (Fig. 9B).

The inhibitory effect of Dyn at various concentrations on specific ^{125}I-ω-CgTX binding was also significantly attenuated by 200 μM GTPγS (Fig. 6A). We previously reported that AGs and Dyn were selective blockers of specific ^{125}I-ω-CgTX binding [20]; therefore, the attenuating effect for Dyn instead of AG was examined. It is possible that the attenuating effect of GTPγS is mediated by κ-opiate receptors, although we previously reported that the inhibitory effect of Dyn was not due to an opiate-related mechanism [20]. To assess this possibility, the effect of U-50488H, a κ-opiate receptor agonist, was examined, as shown in Fig. 6B. U-50488H at concentrations of 3×10^{-6}–10^{-4} M did not affect specific ^{125}I-ω-CgTX binding in the absence or presence of 200 μM GTPγS.

To investigate the selectivity of the attenuating effect of GTPγS, the inhibitory effects of various divalent metal ions were examined in the absence and presence of GTPγS (Fig. 7A–D). GTPγS (200 μM) did not attenuate the inhibitory effects of various concentrations of the divalent metal ions Cd^{2+}, Co^{2+}, Mg^{2+} and Mn^{2+}.

3.2. Effect of MP

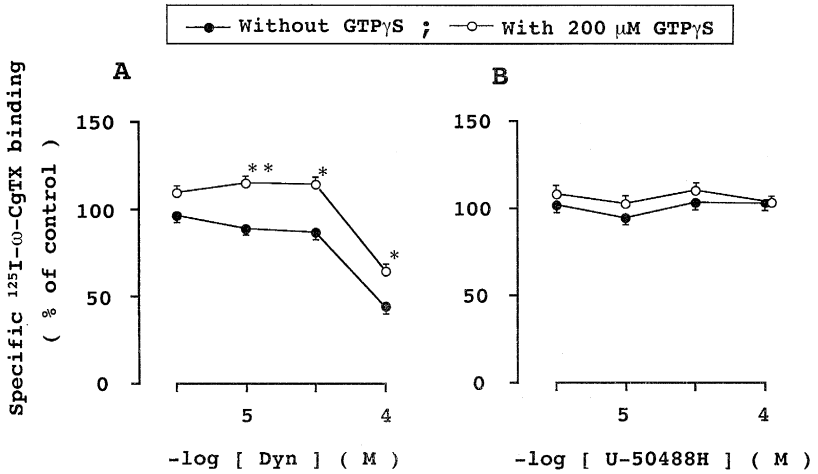

Fig. 6. Effect of 200 μM GTPγS on specific ^{125}I-ω-CgTX binding as a function of the concentration of Dyn (panel A) or U-50488H (panel B). Points represent the means of 5–8 preparations from separate animals. Bars indicate standard errors. * $P < 0.05$, ** $P < 0.01$ vs. binding in the absence of GTPγS.

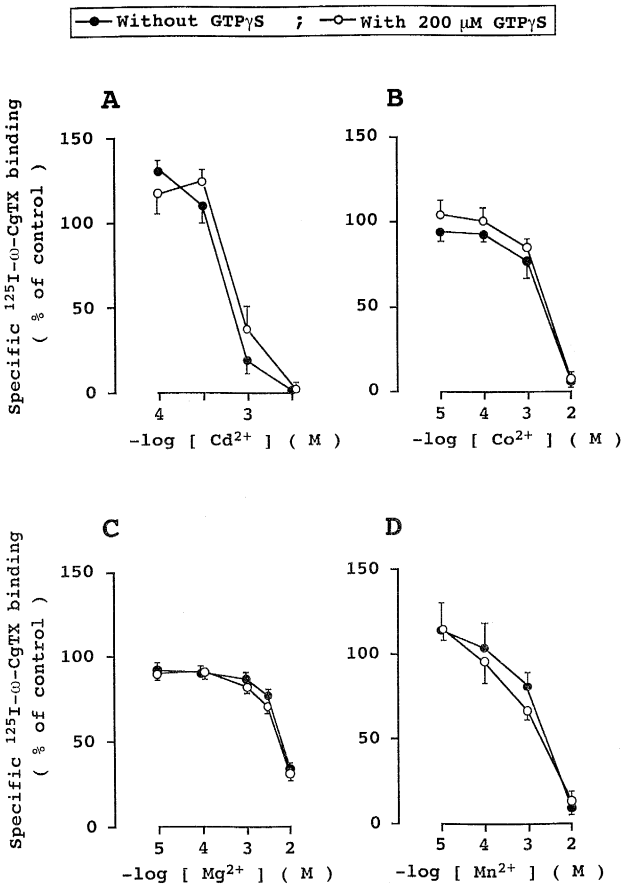

Fig. 7. Effect of 200 μM GTPγS on specific ^{125}I-ω-CgTX binding as a function of the concentration of Cd^{2+} (panel A), Co^{2+} (panel B), Mg^{2+} (panel C) or Mn^{2+} (panel D). Points represent the means of 4–6 preparations from separate animals. Bars indicate standard errors.

The inhibitory effect of 100 μM Neo on specific ^{125}I-ω-CgTX labeling to the 215-kDa band with DSS was attenuated by 300 μM MP or 300 μM MP plus 1 mM $MgCl_2$ in the presence of 100 μM Neo (Fig. 10). The result shown in Fig. 10 was consistent with that for the attenuating effect of MP or MP plus $MgCl_2$ on specific ^{125}I-ω-CgTX binding, although different concentrations of MP were used.

The inhibitory effect of Dyn at various concentra-

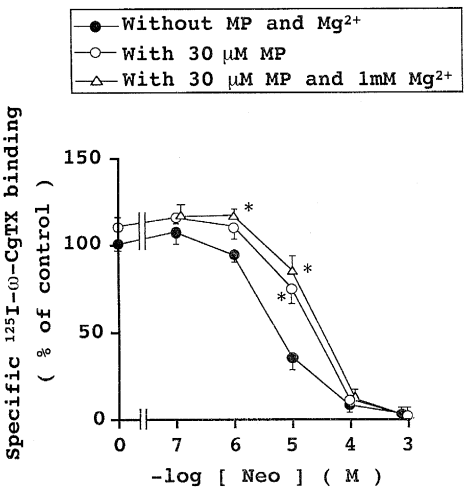

Fig. 8. Effect of 30 μM MP on specific ^{125}I-ω-CgTX binding in the absence and presence of 1 mM Mg^{2+} as a function of the concentration of Neo. Points represent the means of 5–7 preparations from separate animals. Bars indicate standard errors. * $P <$ 0.05 vs. binding in the absence of MP and Mg^{2+}.

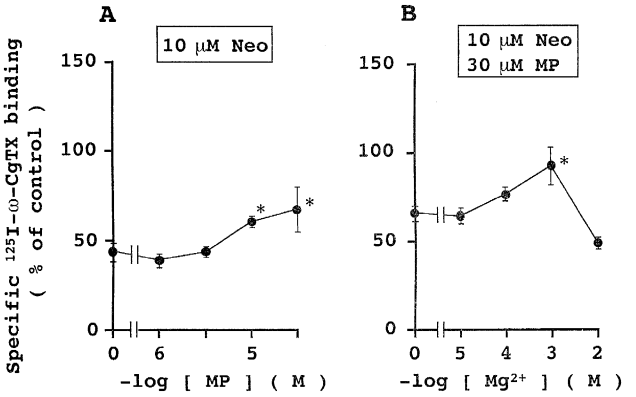

Fig. 9. Panel A shows specific ^{125}I-ω-CgTX binding in the presence of 10 μM Neo as a function of the concentration of MP. Panel B shows specific ^{125}I-ω-CgTX binding in the presence of 10 μM Neo plus 30 μM MP as a function of the concentration of Mg^{2+}. In both panels A and B, points represent the means of 3–5 preparations from separate animals. Bars indicate standard errors. * $P < 0.05$ vs. binding in the absence of MP (panel A) or Mg^{2+} (panel B).

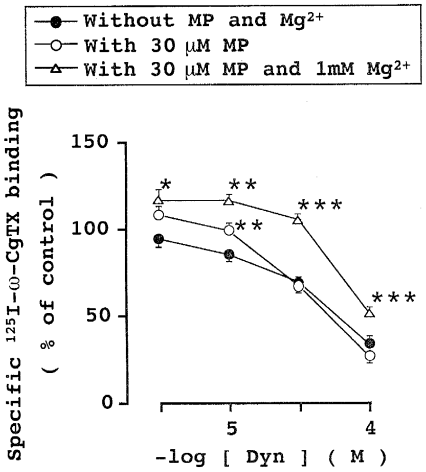

Fig. 11. Effect of 30 μM MP on specific ^{125}I-ω-CgTX binding in the absence and presence of 1 mM Mg^{2+} as a function of the concentration of Dyn. Points represent the means of 5–8 preparations from separate animals. Bars indicate standard errors. * $P < 0.05$, ** $P < 0.02$ and *** $P < 0.01$ vs. binding in the absence of MP and Mg^{2+}.

tions on specific ^{125}I-ω-CgTX binding was also significantly attenuated by 30 μM MP plus 1 mM $MgCl_2$, as shown in Fig. 11. MP shifted the inhibition curve of Dyn not only rightwards, but also upwards. The curve of the attenuating effect of MP on the inhibitory effect of Dyn was comparable to that of Neo (Fig. 6A).

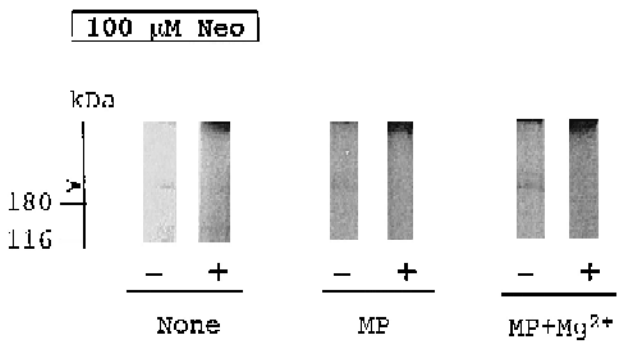

Fig. 10. Effects of 300 μM MP or 300 μM MP plus 1 mM Mg^{2+} on ^{125}I-ω-CgTX labeling in the presence of 100 μM Neo. The symbols (–) and (+) indicate the absence and presence of unlabeled ^{125}I-ω-CgTX, at 1000-fold the concentration of ^{125}I-ω-CgTX, in the reaction medium, respectively. The arrowheads in panels A and B indicate the 215-kDa band.

3.3. Effect of AlF_4^-

AlF_4^- (100 μM $AlCl_3$ plus 50 mM NaF) significantly attenuated the inhibitory effect of Neo on specific ^{125}I-ω-CgTX binding, as shown in Fig. 12,

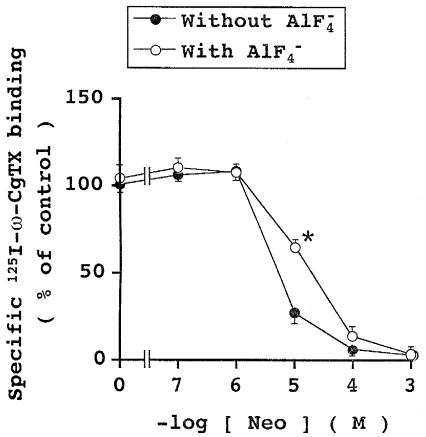

Fig. 12. Effect of AlF_4^- (100 μM $AlCl_3$ plus 50 mM NaF) on specific ^{125}I-ω-CgTX binding as a function of the concentration of Neo. Points represent the means of 5–7 preparations from separate animals. Bars indicate standard errors. * $P < 0.01$ vs. binding in the absence of AlF_4^-.

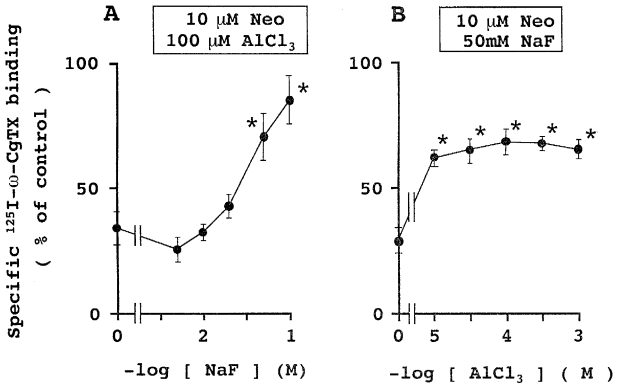

Fig. 13. Panel A shows specific ^{125}I-ω-CgTX binding in the presence of 10 μM Neo plus 100 μM $AlCl_3$ as a function of the concentration of NaF. Panel B shows specific ^{125}I-ω-CgTX binding in the presence of 10 μM Neo plus 50 mM NaF as a function of the concentration of $AlCl_3$. In both panels A and B, points represent the means of 3–5 preparations from separate animals. Bars indicate standard errors. * $P < 0.01$ vs. binding in the absence of NaF (panel A) or $AlCl_3$ (panel B), respectively.

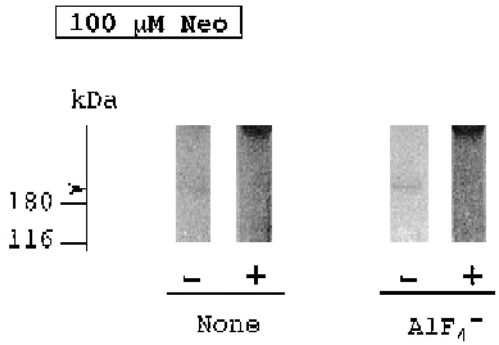

Fig. 15. Effect of AlF_4^- (100 μM $AlCl_3$ plus 100 mM NaF) on ^{125}I-ω-CgTX labeling in the presence of 100 μM Neo. The symbols (−) and (+) indicate the absence and presence of unlabeled ^{125}I-ω-CgTX, at 1000-fold the concentration of ^{125}I-ω-CgTX, in the reaction medium, respectively. The arrowheads in panels A and B indicate the 215-kDa band.

and this attenuating effect by AlF_4^- was dose-dependent for NaF in the presence of 100 μM $AlCl_3$ (Fig. 13A). However, the inhibitory effect of 10 μM Neo was not changed by 10–1000 μM $AlCl_3$ in the presence of 50 mM NaF (Fig. 13B). The attenuating effect of AlF_4^- (100 μM $AlCl_3$ plus 50 mM NaF) was also significantly increased by 1 mM GDP (Fig. 14).

The inhibitory effect of 100 μM Neo on specific ^{125}I-ω-CgTX labeling to the 215-kDa band with DSS was attenuated by AlF_4^- (100 μM $AlCl_3$ plus 100 mM NaF), as shown in Fig. 15. This result was consistent with that for the attenuating effect of AlF_4^- on specific ^{125}I-ω-CgTX binding, although different concentrations of NaF were used.

The inhibitory effect of Dyn at various concentrations on specific ^{125}I-ω-CgTX binding was also significantly attenuated by AlF_4^- (100 μM $AlCl_3$ plus 50 mM NaF), as shown in Fig. 16. AlF_4^- shifted the inhibition curve for Dyn not only rightward, but also

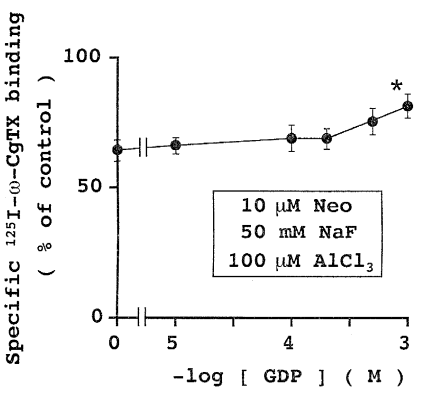

Fig. 14. Effect of AlF_4^- (100 μM $AlCl_3$ plus 50 mM NaF) on specific ^{125}I-ω-CgTX binding in the presence of 10 μM Neo as a function of the concentration of GDP. Points represent the means of 5 preparations from separate animals. Bars indicate standard errors. * $P < 0.05$ vs. binding in the absence of GDP.

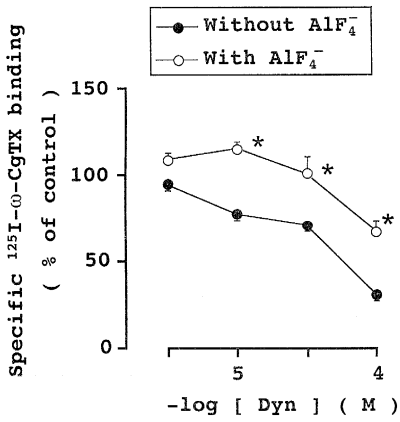

Fig. 16. Effect of AlF_4^- (100 μM $AlCl_3$ plus 50 mM NaF) on specific ^{125}I-ω-CgTX binding as a function of the concentration of Dyn. Points represent the means of 5–7 preparations from separate animals. Bars indicate standard errors. * $P < 0.05$, ** $P < 0.01$ vs. binding in the absence of AlF_4^-.

upward. The curve for the attenuating effect of AlF_4^- on the inhibitory effect of Dyn was comparable to that of Neo (Fig. 6A and Fig. 11).

4. Discussion

N-type Ca channels are known to be a target for the neurotransmitter-induced inhibition of neuronal calcium currents [9,31]. In this case, the activation of G proteins is required for the neurotransmitter-mediated inhibition of N-type Ca channels [32,33]. It has been reported that the venom of a marine snail, ω-CgTX, selectively blocks calcium current in neural N-type Ca channels [8–14]. Based on these experiments, it is reasonable to consider that specific ^{125}I-ω-CgTX binding on crude membranes from the brain may be affected under the activation of G protein. In this study, we investigated whether specific ^{125}I-ω-CgTX binding on crude membranes from chick whole brain was affected when G protein in the crude membranes was activated by GTPγS, MP or AlF_4^- [35–38].

GTPγS (200 μM) attenuated the inhibitory effect of various AGs; their IC_{50} values increased from 5–30 μM to 25–150 μM. This result is comparable to that of Stumpo et al. [34], although they investigated only the effect of Neo, and its IC_{50} value was lower than that in our study.

Gpp(NH)p also attenuated the inhibitory effect of Neo in a dose-dependent manner (Fig. 3B), but this effect was very weak compared to that of GTPγS (Fig. 3A). GTPγS is known to have several irreversible effects in the hormone-activated adenylate cyclase system which are not observed with Gpp(NH)p [41]. Citri and Schramm [41] also reported that Gpp(NH)p, but not $G_{GTP\gamma S}$, competed with G_{GDP} for β-adrenergic receptor, so that G_{GPPNHP} could be converted back to the inactive form G_{GDP}, but $G_{GTP\gamma S}$ could not. Therefore, the difference in the attenuating effect between GTPγS and Gpp(NH)p may be due to the difference in the characteristics between G protein combined with GTPγS and Gpp(NH)p.

GTPγS (200 μM) also had an attenuating effect on inhibition by Dyn, suggesting that the attenuating effect of GTPγS was not specific for inhibition by AGs, although the attenuation curve for GTPγS on the inhibitory effect of Dyn was different from that for AGs. It seems likely based on these results that while the inhibitory effect of Dyn is also mediated by G protein, the mechanism is not the same as that of Neo because the curves for the attenuating effects of GTPγS, MP and AlF_4^- on the inhibitory effect of Dyn were different from those for Neo.

We previously suggested that the inhibitory effect of Dyn on specific ^{125}I-ω-CgTX binding to crude membranes was not due to a mechanism involving κ-opiate receptors [20]. In this study, we also observed that the κ-agonist U50488H at concentrations of 3×10^{-5} M to 10^{-4} M did not affect specific ^{125}I-ω-CgTX binding in the absence or presence of 200 μM GTPγS. These findings suggest that the effects of Dyn and GTPγS do not involve a κ-opiate-related mechanism.

The inhibitory effects of Cd^{2+}, Co^{2+}, Mg^{2+} or Mn^{2+} on specific ^{125}I-ω-CgTX binding were not attenuated by 200 μM GTPγS (Fig. 7A–D). Therefore, these results indicate that the inhibitory effect of these divalent metal ions on binding was not mediated by G protein, suggesting that the attenuating effect of GTPγS was selective for the inhibitory effect of specific blockers such as AGs and Dyn [20] on ^{125}I-ω-CgTX binding.

MP, a toxin from wasp venom, is a potent secretagogue. Higashijima et al. [37] suggested that MP strongly promotes nucleotide exchange by G protein to mimic agonist-liganded receptors. Bigay et al. [38] reported that AlF_4^-, which acts as the α-subunit of G protein, mimics γ-phosphate of GTP on G protein in retinal membranes, since there are striking structural similarities between AlF_4^- and PO_4^{3-}. Therefore, we used MP and AlF_4^- as activators of G protein for specific ^{125}I-ω-CgTX binding.

MP attenuated the inhibitory effect of Neo similar to GTPγS. This attenuating effect of MP was enhanced by the presence of Mg^{2+} in a dose-dependent manner, which again suggests that MP promotes nucleotide exchange on G protein, since it has been reported that receptor-catalyzed and non-receptor-catalyzed nucleotide exchange on G protein require micromolar levels and 10–100 mM Mg^{2+}, respectively [26]. In our work, however, the attenuating effect of 30 μM MP on the inhibitory effect of 10 μM Neo was significantly enhanced by 1 mM Mg^{2+}, but inhibited by 10 mM Mg^{2+}, as shown in Fig. 9. Since a Mg^{2+} concentration of more than 3 mM

significantly inhibited specific ^{125}I-ω-CgTX binding in the absence of MP and Neo, as shown in Fig. 7, the enhancement of the attenuating effect of MP by 10 mM Mg^{2+} disappeared, even if 10 mM Mg^{2+} accelerated the activation of G protein by MP.

We observed that AlF_4^- attenuated the inhibitory effect of Neo or Dyn, and these results were similar to those with GTPγS or MP. As suggested by Bigay et al. [38], GDP enhances the attenuating effect of AlF_4^- on the inhibitory effect of Neo or Dyn. However, in our study, the enhancing effect of GDP in the presence of AlF_4^- was very small. The discrepancy between these results remains unresolved.

Ichida et al. suggested that the labeling of the 135-kDa (reduced condition) or 215-kDa (non-reduced condition) band with ^{125}I-ω-CgTX using the cross-linker DSS involved specific binding sites of ^{125}I-ω-CgTX, perhaps including an N-type Ca channel subunit in the crude membranes from chick whole brain [20]. We previously reported that AGs and Dyn selectively inhibited specific ^{125}I-ω-CgTX binding, and also decreased labeling with ^{125}I-ω-CgTX using DSS [20]. Therefore, we examined whether the labeling of the 215-kDa band with ^{125}I-ω-CgTX using DSS was affected by GTPγS in the absence or presence of Neo. First, the effect of Neo and/or GTPγS on this labeling was examined under the same conditions as for specific ^{125}I-ω-CgTX binding, but no attenuating effect of GTPγS on the inhibitory effect of Neo was observed. The protein concentration of the crude membranes used in this labeling was about 300 μg of protein/tub, which is about 4 times higher than that for specific ^{125}I-ω-CgTX binding (25 μg of protein/tube; see Section 2) [20]. Therefore, we again attempted labeling of ^{125}I-ω-CgTX after determining the optimum concentrations of GTPγS and Neo for affecting specific ^{125}I-ω-CgTX binding under a high concentration of protein (300 μg of protein/tube), as shown in Fig. 5B. GTPγS (500 μM) attenuated the inhibitory effect of 100 μM Neo on specific ^{125}I-ω-CgTX labeling to the 215-kDa band. MP (300 μM) in the presence of 1 mM Mg^{2+} and AlF_4^- (100 mM $AlCl_3$ + 100 mM NaF) also had an attenuating effect on such labeling in the presence of 100 μM Neo (Figs. 10 and 15). These results support the notion that labeling of the 215-kDa band by ^{125}I-ω-CgTX involved specific binding sites for ^{125}I-ω-CgTX, and perhaps neuronal N-type Ca channels as reported previously [20]. In addition, the amount of protein in the binding system appears to influence the inhibitory effect of Neo or the attenuating effects of GTPγS, MP and AlF_4^-, although the mechanism(s) is not yet clear.

These results indicate that there are direct or indirect relationships between N-type Ca channels and the binding sites of the selective antagonists AGs and Neo that involve a G protein-related mechanism.

Recently, it was reported that ^{125}I-ω-CgTX sensitive N type Ca channels interact with the proteins that regulate neuronal exocytosis pathway(s) [42,43], suggesting that N type Ca channels have relation to a mechanism of neurotransmitter release. If N type Ca channels have relation to receptors of neurotransmitters and to G protein in the synaptic plasma membranes of presynapse area, neurotransmitters over-released may be controlled with a mechanism by which Ca influx through N type Ca channels are inhibited by receptors of neurotransmitters following G proteins, although we have no data to prove this idea. Our finding may be relate to the mechanism of neurotransmitter release.

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