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Effects of GTP analogues and activation of endogenous protein kinases on photoaffinity labeling with [^{3}H](+)PN200-110 of crude membranes from rat heart and brain

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The effects of GTP analogues and conditions in which various endogenous protein kinases were activated on photoaffinity labeling with [^{3}H](+)PN200-110 (PN) of crude membranes from rat cardiac muscle and whole brain were investigated. Photoaffinity labeling with 20 nM [^{3}H](+)PN of these crude membranes was decreased by 100 μM GTP-γ-S, but not by 100 μM GTP or 100 μM GDP-β-S. Similar results were obtained on the effects of GTP and its analogues on the specific binding of 20 nM [^{3}H](+)PN to these crude membranes under the same conditions. Activation of endogenous protein kinases in these crude membranes did not influence the photoaffinity labeling with [^{3}H](+)PN. These results suggested the binding sites, or DPH-sensitive, or L-type, calcium channels in eurde membranes from rat cardiac muscle and whole brain are directly or indirectly modulated by endogenous GTP-binding protein, but not by various endogenous protein kinases in these crude membranes.

Introduction

Voltage-dependent calcium channels play a fundamental role in the regulation of various intracellular processes in excitable cells [1–3]. 1,4-Dihydropyridine (DHP)-sensitive, or L-type, calcium channels mediate long-lasting calcium currents that are inhibited by DHP derivatives such as PN200-110 (PN), phenylalkylamines such as verapamil (Ver), and benzothiazepines such as diltiazem (Dil) through interaction with three different receptor sites [3–5].

Recently, we reported the characteristics of photoaffinity labeling by DHP derivatives, especially (+)PN, of crude membranes from rat skeletal, cardiac, ileal, and uterine muscles and whole brain, and showed that photoaffinity labeling with [^{3}H](+)PN by UV irradiation is a useful method for investigating the characteristics of the voltage-dependent L-type calcium channels, the function of which is affected by DHP derivatives [6,7].

On the other hand, there is much suggestive evidence that the functions of DHP-sensitive, or L-type, calcium channels in skeletal, cardiac or neuronal cells are directly or indirectly regulated by various protein kinases (cAMP-dependent protein kinase (PK-A), protein kinase C (PK-C), cGMP-dependent protein kinase (PK-G) [8–15]) and GTP-binding proteins [16–19]. If endogenous GTP-binding protein and/or protein kinases regulate(s) the function of DHP-sensitive, or L-type, calcium channel in these crude membranes, the photoaffinity labeling with [^{3}H](+)PN of these crude membranes should be affected by the binding of GTP analogues to GTP-binding proteins and/or by phosphorylations with various kinds of protein kinases in these crude membranes.

In this work, we examined the effects of GTP analogues and conditions in which various types of endogenous PKs were activated on the photoaffinity labeling with [^{3}H](+)PN of crude membranes from rat cardiac muscle and whole brain.

Abbreviations: CaM, calmodulin; DHP, 1,4-dihydropyridine; Dil, diltiazem; GDP-β-S, guanosine 5'-*O*-2-thiodiphosphate; GTP-γ-S, guanosine 5'-*O*-3-thiotriphosphate; 4αPDD, 4α-phorbol 12,13-didecanoate; PKs, protein kinases; PK-A, cAMP-dependent protein kinase; PK-C, protein kinase C; calcium/calmodulin-dependent protein kinase; PK-G, cGMP-dependent protein kinase; PMA, phorbol 12-myristate 13-acetate; PN, PN200-110; PS, phosphatidylserine; SDS-PAGE; SDS-polyacrylamide gel electrophoresis; Ver, verapamil.

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Materials and Methods

Preparation of crude membranes. Crude membranes were prepared from cardiac muscle and whole brain of male Wistar rats weighing 150–200 g as described previously [6], and were used at final concentrations of 6–8 and 8–9 mg protein/ml, respectively.

Photoaffinity labeling. For experiments of the effects of GTP analogues, the conditions used were as described previously [7], with the following modifications: (a) The reaction medium for the binding of [^{3}H](+)PN contained 2 mM $MgCl_2$. (b) 100 μM guanosine 5'-*O*-3-thiotriphosphate (GTP-γ-S), 100 μM guanosine 5'-*O*-2-thiodiphosphate (GDP-β-S) or 100 μM GTP was added to the reaction medium containing 2 mM $MgCl_2$, unless otherwise indicated. Control medium contained water instead of GTP and GTP analogues. After the binding of [^{3}H](+)PN, the procedures for photoaffinity labeling, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography were carried out as reported previously [7]. These crude membranes were used at final protein concentration of 1.25–1.65 mg/ml.

For experiments on photoaffinity labeling of crude membranes from rat cardiac muscle and whole brain in conditions in which various endogenous PKs were activated, the crude membranes (final concentration, 1.25–1.65 mg protein/ml) were first incubated for 10 min at 30°C under modifications of the conditions described by Benovic et al. [20] as follows. (a) For activation of PK-A: standard medium (pH 7.4 at 30°C) (50 mM Tris-Cl (pH 7.4 at 30°C) containing 0.5 mM phenylmethylsulfonyl fluoride, 1.5 mM EDTA, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid, 5 μM Na_3VO_4, 20 mM NaCl, 5 mM $MgCl_2$, 5 mM NaF and 0.5 mM ATP) supplemented with 0.2 mM cAMP. (b) For activation of PK-C: standard medium (pH 7.4 at 30°C) supplemented with 2 mM $CaCl_2$, 66.7 μg/ml phosphatidylserine (PS), 20 μM phorbol 12-myristate 13-acetate (PMA) or 20 μM 4α-phorbol 12,13-didecanoate (4αPDD). (c) For activation

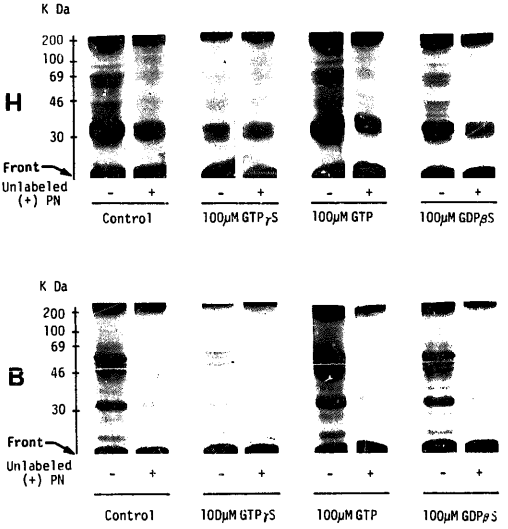

Fig. 1. Effects of GTP and GTP analogues on photoaffinity labeling with [^{3}H](+)PN of crude membranes from rat cardiac muscle (H) and whole brain (B). The reactions for specific binding of [^{3}H](+)PN in the absence (Control) and presence 100 μM GTP-γ-S, 100 μM GTP and 100 μM GDP-β-S were carried out by addition of these crude membranes, and then samples in an ice-bath were irradiated with a mercury arc lamp. After UV-irradiation, samples were subjected to SDS-PAGE (10% acrylamide) and then to fluorography, as described in Materials and Methods. In some cases, unlabeled (+)PN was added in 1000-fold excess of 20 nM [^{3}H](+)PN; these cases are indicated with the symbol +.

of calcium/calmodulin-dependent protein kinases (PK-CaM): standard medium (pH 7.4 at 30°C) supplemented with 2 mM $CaCl_2$ and 40 μg/ml calmodulin (CaM). (d) For activation of PK-G: standard medium (pH 7.4 at 30°C) supplemented with 0.2 mM cGMP. Control media contained standard medium with water or vehicle instead of above supplements. Then, the binding of [^{3}H](+)PN to crude membranes from rat cardiac muscle and whole brain was assayed. For this, the crude membranes were incubated with 20 nM [^{3}H](+)PN with or without unlabeled 20 μM (+)PN for 50 min at 30°C. The mixtures were placed in an ice-bath for about 10 min and irradiated for 20 in with UV-light from a 500 W mercury arc lamp (Eikow), as reported previously [7]. Subsequent procedures, such as irradiation, SDS-PAGE and fluorography, were carried out as reported previously [7], unless otherwise indicated.

Assay of binding of [^{3}H](+)PN. The specific binding of [^{3}H](+)PN to crude membranes from rat cardiac muscle and whole brain was assayed by a modification of the method reported previously [6]. For comparison with results on the effects of GTP analogues on the photoaffinity labeling with [^{3}H](+)PN, the following conditions were different from those in a previous study [6]: (a) The final concentration of [^{3}H](+)PN used was 20 nM, not 5 nM. (b) The reaction medium for the binding of [^{3}H](+)PN was supplemented with 2 mM $MgCl_2$. Control medium contained water instead of GTP or GTP analogues. The specific binding of [^{3}H](+)PN was defined as the difference between the radioactivities bound in the presence and absence of unlabeled 20 μM (+)PN. The crude membranes were used at a final concentration of 0.35–0.50 mg protein/ml.

Other methods. Protein was measured by the method of Lowry et al. [21] with bovine serum albumin as a standard.

Materials. (+)[5-*Methyl*-^{3}H]PN200-110 (70–87 Ci/mmol) were purchased from Amersham or New England Nuclear. 4αPDD, phenylmethylsulfonyl fluoride, PMA and PS were from Sigma Chemicals (St. Louis, MO). cAMP, cGMP and GTP were from Yamasa (Tokyo). GDP-β-S and GTP-γ-S were from Boehringer Mannheim (Germany). CaM was from Calbiochem (San Diego, CA). (+)PN was a gift from Sandoz AG (Basel, through Drs. D. Romer and H. Weber). All assays using DHP derivatives were performed under a safety light.

Results

The effects of GTP-γ-S, GDP-β-S and GTP on the photoaffinity labeling with [^{3}H](+)PN of crude membranes from rat cardiac muscle and whole brain are shown in Fig. 1. In these crude membranes, 100 μM GTP-γ-S decreased the photoaffinity labeling to the various bands in the absence of a large amount of unlabeled (+)PN, but 100 μM GDP-β-S and 100 μM GTP had no affect in the absence or presence of a large amount of unlabeled (+)PN. The photoaffinity labeling of these crude membranes in the presence of a large amount of unlabeled (+)PN was barely decreased by 100 μM GTP-γ-S. Under conditions in which GTP was not hydrolyzed during the [^{3}H](+)PN binding reaction, but even then we also found that 100

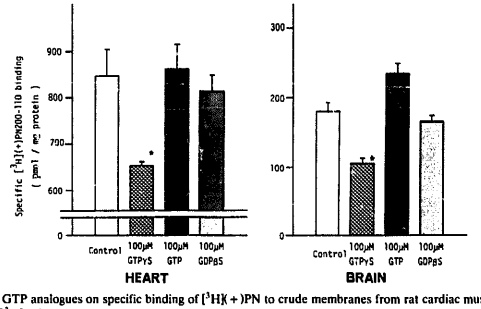

Fig. 2. Effects of GTP and GTP analogues on specific binding of [^{3}H](+)PN to crude membranes from rat cardiac muscle and whole brain. The specific binding of 20 nM [^{3}H](+)PN in the absence (Control) and presence of 100 μM GTP-γ-S, 100 μM GTP and 100 μM GDP-β-S were determined as described in Materials and Methods. For determination of nonspecific binding of [^{3}H](+)PN, unlabeled (+)PN was added in 1000-fold excess of 20 nM [^{3}H](+)PN. Columns are means for four or five separate experiments. Bars indicate standard errors. * $P < 0.05$: significantly different from the control value.

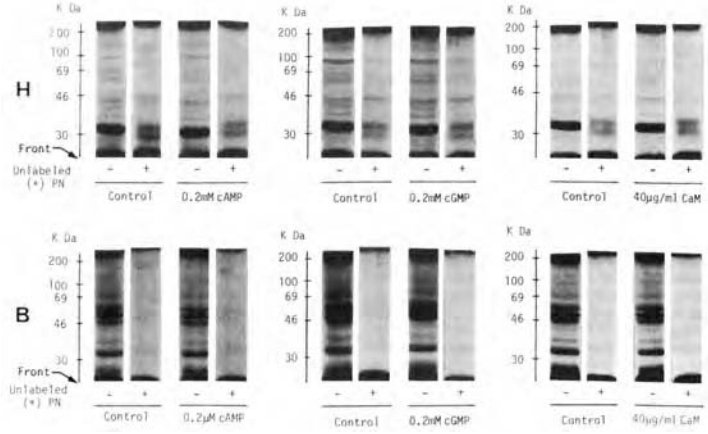

Fig. 3. Photoaffinity labeling with [^{3}H](+)PN of crude membranes from rat cardiac muscle (H) and whole brain (B) under conditions in which PK-A, -G and -CaM, respectively, were activated. Conditions were as described in Materials and Methods. In some cases, unlabeled (+)PN was added in 1000-fold excess of 20 nM [^{3}H](+)PN; these cases are indicated with the symbol +.

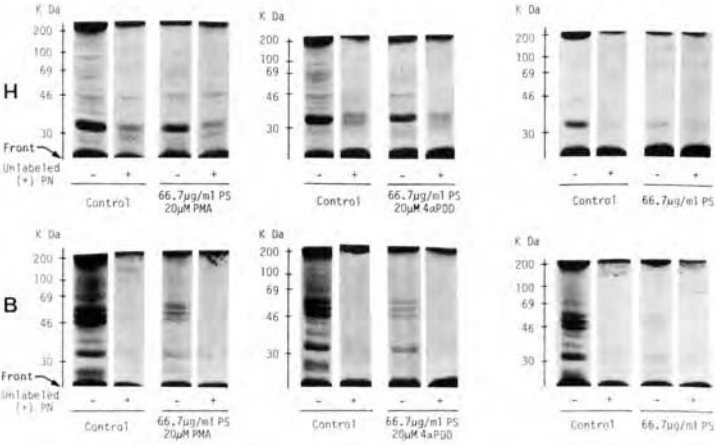

Fig. 4. Photoaffinity labeling with [^{3}H](+)PN of crude membranes from rat cardiac muscle (H) and whole brain (B) under conditions in which PK-C were activated and in which 4αPDD plus PS or PS alone was presented. Experiments were carried out as described in Materials and Methods. In some cases, unlabeled (+)PN was added in 1000-fold excess of 20 nM [^{3}H](+)PN; these cases are indicated with the symbol +.

μM GTP did not affect the photoaffinity labeling with [^{3}H](+)PN of these crude membranes (data not shown). Therefore, we next examined whether these GTP analogues had similar effects on the specific binding of [^{3}H](+)PN to crude membranes from rat cardiac muscle and whole brain under the same conditions as for photoaffinity labeling with [^{3}H](+)PN. As shown in Fig. 2, the specific binding of [^{3}H](+)PN to crude membranes from rat cardiac muscle and whole brain were decreased by 100 μM GTP-γ-S but not by 100 μM GDP-β-S or 100 μM GTP.

To determine the effects of endogenous PKs on the photoaffinity labeling with [^{3}H](+)PN of crude membranes from rat cardiac muscle and whole brain, we measured the photoaffinity labeling under conditions in which PK-A, -G, -C or -CaM in these crude membranes were activated. The photoaffinity labeling was not affected by activations of PK-A, -G and -CaM (Fig. 3), but was reduced under conditions in which PK-C was activated (Fig. 4). However, as shown in Fig. 4, the photoaffinity labeling of these crude membranes was also reduced by treatment with 4αPDD or PS alone. Moreover, treatment with PMA and 4αPDD with a lower concentration (1 μg/ml) of PS (at which concentration of PS alone did not influence the photoaffinity labeling with [^{3}H](+)PN) did not reduce the photoaffinity labeling (Fig. 5).

Discussion

Many investigators have suggested that GTP-binding protein and phosphorylation by various kinds of endogenous PKs may regulate the function of L-type calcium channels in skeletal, cardiac and neural cells [8–19]. However, nothing is known about whether GTP analogues and/or phosphorylation by various endogenous PKs affects the photoaffinity labeling by radiolabeled DHP derivatives of crude membranes from excitable organs. Therefore, to examine the relationships between DHP-sensitive, or L-type, calcium channels and GTP-binding proteins and /or various endogenous PKs, in this work, we investigated the effects of GTP analogues and activations of various PKs on the photo-

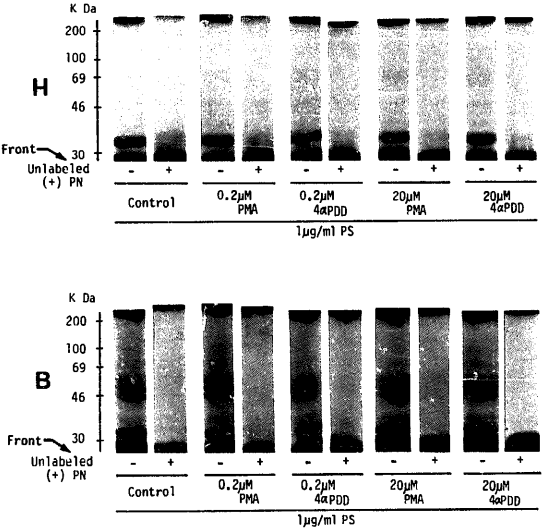

Fig. 5. Photoaffinity labeling with [^{3}H](+)PN of crude membranes from rat cardiac muscle (H) and whole brain (B) under conditions in which PMA or 4αPDD with a lower concentration (1 μg/ml) of PS was presented. The experiments were carried out as described in Materials and Methods. In some cases, unlabeled (+)PN was added in 1000-fold excess of 20 nM [^{3}H](+)PN; these cases are indicated with the symbol +.

affinity labeling with [^{3}H](+)PN of crude membranes from rat cardiac muscle and whole brain.

Results showed that the photoaffinity labeling of these crude membranes was inhibited by GTP-γ-S, but not by GTP. This finding does not support with the assumption that a GTP-binding protein participates in the function of DHP-sensitive, or L-type calcium channels. The concentration of [^{3}H](+)PN used for studies on photoaffinity labeling was much higher than that for those on specific binding of [^{3}H](+)PN. So, we also investigated the effects of GTP analogues on the specific binding of [^{3}H](+)PN to crude membranes from rat cardiac muscle and whole brain. We found that the effects of GTP, GTP-γ-S and GDP-β-S on the specific binding of 20 nM [^{3}H](+)PN were similar those on photoaffinity labeling of these crude membranes. It is unknown why GTP-γ-S inhibited the specific binding and the photoaffinity labeling with [^{3}H](+)PN of these crude membranes, whereas GTP did not. The possibility that GTP was hydrolyzed by endogenous enzymes in these crude membranes was excluded by our finding that GTP also did not inhibit the photoaffinity labeling of these crude membranes under conditions in which the reaction medium contained 500 μM ATP or a system for recycling GTP (data not shown).

We also found that photoaffinity labeling with [^{3}H]-(+)PN of crude membranes from rat cardiac muscle and whole brain was not influenced under conditions in which various endogenous PKs in these crude membranes were activated (Figs. 3–5). However, these findings do not exclude the possibility that the direct or indirect phosphorylation of DHP-sensitive, or L-type, calcium channels in these crude membranes by various endogenous PKs does not contribute to modulation of their function for the following reasons: (a) Photoaffinity labeling with [^{3}H](+)PN of these crude membranes was not affected by conditions in which various endogenous PKs in these crude membranes were activated, but some function(s) of L-type calcium channels in the crude membranes other than that related to binding sites of DHP derivatives may have been affected by phosphorylations with various endogenous PKs. (b) The possibility that conditions in which various endogenous PKs were activated may not be the best for moduration of photoaffinity labeling with [^{3}H](+)PN cannot be excluded.

In this study, we found that the GTP analogue GTP-γ-S decreased photoaffinity labeling with [^{3}H]-(+)PN of crude membranes from rat cardiac muscle and whole brain. Therefore, this finding suggested that DHP-sensitive, or L-type, calcium channels in these crude membranes are directly or indirectly regulated by GTP binding proteins, as proposed previously from electrophysiological studies [16–19].

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