Activation of Gα-proteins by Membrane Depolarization Traced by in Situ Photoaffinity Labeling of Gα-proteins with \([\alpha^{32}\text{P}]\text{GTP-azidoanilide}\)*

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Evidence for depolarization-induced activation of G-proteins in membranes of rat brain synaptoneurosomes has been previously reported (Cohen-Armon, M., and Sokolovsky, M. (1991) J. Biol. Chem. 266, 2595–2605; Cohen-Armon, M., and Sokolovsky, M. (1993) J. Biol. Chem. 268, 9824–9838). In the present work we identify the activated G-proteins as Gα-proteins by tracing their depolarization-induced in situ photoaffinity labeling with \([\alpha^{32}\text{P}]\text{GTP-azidoanilide}\) (GTPAA). Labeled GTPAA was introduced into transiently permeabilized rat brainstem synaptoneurosomes. The resealed synaptoneurosomes, while being UV-irradiated, were depolarized. Relative to synaptoneurosomes at resting potential, the covalent binding of \([\alpha^{32}\text{P}]\text{GTPAA}\) to Gα1- and Gα3-proteins, but not to Gα2- isoforms, was enhanced by 5- to 7-fold in depolarized synaptoneurosomes, thereby implying an accelerated exchange of GDP for \([\alpha^{32}\text{P}]\text{GTP}\). Their depolarization-induced photoaffinity labeling was independent of stimulation of Gα-protein-coupled receptors and could be reversed by membrane repolarization, thus excluding induction by transmitter releasers. It was, however, dependent on depolarization-induced activation of the voltage-gated sodium channels (VGSC), regardless of Na⁺ current. The α subunit of VGSC was cross-linked and co-immunoprecipitated with Gα1-α-proteins in depolarized brain-stem and cortical synaptoneurosomes. VGSC α subunit most efficiently cross-linked with guanosine 5′-O-2-thiodiphosphate-bound rather than to guanosine 5′-O-(3-thiotriphosphate)-bound Gα1-α-proteins in isolated synaptoneurosomal membranes. These findings support a possible involvement of VGSC in depolarization-induced activation of Gα-proteins.

GTP-binding trimeric proteins have been implicated in signal transduction from receptors in the cell membrane to intracellular effectors and ion channels in a variety of cells (1–5). The mechanism involves signal-induced G-protein activation initiated by an exchange of GDP for GTP on the α subunit of the protein (5–7). Subsequent GTPase activity of the Go subunit converts the activated G-proteins into their inactive, GDP-bound state (7). Activation of G-proteins has been induced experimentally by stimulation of G-protein-coupled receptors in the cell membrane (2, 3, 8, 9). Evidence indicating activation of G-proteins in response to membrane depolarization were previously observed in brain stem synaptoneurosomes (10–12).

Gα-proteins are widely distributed in the central nervous system (15–17). Three subtypes showing marked homology but exhibiting functional differences have been identified (13, 14, 18). The Gα1 subtype appears to be involved in the coupling of muscarinic receptors to Ca²⁺ channels, and the Gα2 subtype mediates inhibition of Ca²⁺ current activated by somatostatin receptors (19). The function of the Gα3 subtype is not clear (14). Phospholipase C activation mediated by activation of Gα-proteins has been demonstrated in the cell-free system (20), and Gα-protein-mediated activation of protein kinase C has been observed in Chinese hamster ovary (CHO) cells (21).

In the present study, in situ photoaffinity labeling with \([\alpha^{32}\text{P}]\text{GTPAA}\) indicated a depolarization-induced accelerated exchange of GDP for \([\alpha^{32}\text{P}]\text{GTPAA}\) in Gα1- and Gα3-proteins, implying a depolarization-induced activation of these Gα-proteins. \([\alpha^{32}\text{P}]\text{-GTPAA}\) was introduced into transiently permeabilized synaptoneurosomes as described before (10). Unlike the endogenously bound guanine nucleotides, \([\alpha^{32}\text{P}]\text{GTPAA}\), covalently bound to Gα-proteins by photoaffinity labeling, was not displaced during SDS-polyacrylamide gel electrophoresis, providing a possible tool for identification of in situ activated Gα-proteins.

In view of findings indicating a reciprocal influence of depolarization-induced activation of VGSC and uncoupling of G-proteins from muscarinic receptors (12, 24, 25), we examined the possibility that VGSC can be involved in depolarization-induced activation of Gα3-proteins. Our results indicated that depolarization-induced activation of Gα-proteins can be prevented by preventing VGSC activation. In addition, in depolarized brain-stem and cortical synaptoneurosomes, the α subunit of VGSC cross-linked most efficiently with Gα3-proteins. In isolated synaptoneurosomal membranes, VGSC-α subunit cross-linked most efficiently with GDPβS-bound rather than GTPγS-bound Gα3-proteins. These findings suggest repeated

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7431
interactions between VGSC-α subunit and Gα-proteins as long as membrane depolarization lasts.

MATERIALS AND METHODS

Reagents—ATP (grade I), GDP/S, GTP-γS, tetrodotoxin, dithiotreitol, carbamylcholine, atropine, serotonin, sperine, propranolol, naloxone, and yohimbine were all purchased from Sigma. 4-Azidoaniline hydrochloride was supplied by Fluka (Switzerland). d-2-amino-5-phosphovaleric acid was purchased from Cambridge Research Biochemicals. DFI-205-429 DFI R enantiomer; see Footnote 1) was kindly supplied by Dr. E. Rissi and Dr. D. Romer of Sandoz Ltd., Pharmaceutical Division, Preclinical Research (Basel, Switzerland). N,N′-1,4-Phenylenedimaleimide (PDM) was purchased from Aldrich. Pertussis toxin (PTX) and the A-protomer of PTX, cholera toxin (CTX), and the A-protomer of CTX were purchased from List Biological Laboratories, Campbell, CA. Guanosine-5′-triphosphate tetra(triethylammonium) salt, [α-32P]GTP (500 Ci/mmol), and antibodies against peptide derived from the amino-terminal domain of Gαi-proteins (CG2) were purchased from NEN Life Science Products. [3H]Acetylcholine ([3H]AcCh) (96 Ci/mmol, 98% pure) and [phenyl-3H]tetraphenylphosphonium bromide ([3H]TPP) (33.6 Ci/mmol) were purchased from Amersham Pharmacia Biotech. Phosphorus-32-[32P]H3PO4 (400–800 mCi/mmol) was purchased from ICN Pharmaceuticals, Inc. [14C]Methyl-4-piperidylbenzilate was prepared by GTP-binding proteins were photolabeled by [α-32P]GTP. For estimation of the nonspecific binding of [α-32P]GTPA, synaptic membranes were incubated in the presence of [α-32P]GTPA and GTPyS (200 μM). The nonspecific photofinity labeling of membrane proteins with [α-32P]GTPA was estimated by measuring the accumulation of [3H]TPP in ressealed synaptoneurosomes, samples of nonpermeabilized synaptoneurosomes were subjected to photofinity labeling under the above conditions. Photofinity labeling of membrane proteins with [α-32P]GTPA in nonpermeabilized synaptoneurosomes was negligible. Membrane proteins were separated by SDS-PAGE and autoradiographed. [32P]GTPA-labeled proteins were quantified by densitometry using a laser densitometer (LKB Bromma Ultrascan).

Estimation of Membrane Potential in Terms of [3H]TPP Accumulation—Because [3H]TPP permeates freely across cell membranes, it follows that at equilibrium the transmembrane concentration gradient of [3H]TPP is proportional to membrane potential, according to the Nerst equation (33). Changes in membrane potential were therefore estimated by measuring the accumulation of [3H]TPP in the synaptoneurosomes according to the method of Cheng et al. (33). Synaptoneurosomes (approximately 4 mg of protein/ml in 40-μl samples) were incubated with approximately 7 × 10^-9 M [3H]TPP at 25 °C for 20 min. The samples were then passed through Whatman GF/C filters, and the filters were counted for tritium using a scintillation mixture (Hydrosol H) described in detail previously (11).

PTX-catalyzed ADP-ribosylation of Ga-proteins in Brain-stem Synaptoneurosomes—Synaptoneurosomes in Ca^2+-free Krebs-Henseleit buffer, pH 7.4, were incubated with 200 ng/ml PTX for 2 h at 37 °C and 95% O_2, 5% CO_2 as described before (11).

Binding of [3H]AcCh to Muscarinic Receptors in Resealed Synaptoneurosomes—To determine the effect of membrane depolarization on the binding of [3H]AcCh to high-affinity muscarinic receptors, Ca^2+-free Krebs-Henseleit buffer containing either 4.7 or 50 mM K^+ (resting potential or depolarization, respectively) in the presence of an inhibitor of acetylcholinesterase (34) was exchanged for K^+ in the high [K^+] buffer. Under these experimental conditions the specific binding of [3H]AcCh to high-affinity muscarinic receptors can be measured (34). Nonspecific [3H]AcCh binding was measured in the presence of 1 μM atropine. The binding assay has been described in detail previously (10, 25, 34). To prevent interference by AcCh release, [3H]AcCh was added to synaptoneurosomes preincubated for 30 min at room temperature in the absence of acetylcholinesterase inhibitors (34). [3H]AcCh binding to muscarinic receptors was measured in Ca^2+-free Krebs-Henseleit buffer (described above), with a Ca^2+:[Mg^2+] ratio of 1:100 (35).

Two-dimensional Gel Electrophoresis—Samples containing 200 μg of protein were applied to isoelectric focusing gels (1st dimension) containing 1.8% ampholines at pH 5–8 and 0.2% ampholines at pH 3–10. Isoelectric focusing followed by SDS-PAGE (2nd dimension) was performed according to Ferro-Luzzi and Nikaido (36). In the 2nd dimension gels containing the electrophoresed proteins were applied on 8 or 10% polyacrylamide slab gels (37), subjected to SDS-PAGE at room temperature, and electroblotted.

Immunolabeling—Proteins in the SDS-gels were electroblotted (Western blots) onto nitrocellulose paper overnight at 10 °C and with a constant current of 150 mA, as described by Towbin et al (38). Nitrocellulose strips were exposed to specific antibodies detecting amino-
terminal domains of Gαo-proteins (residues 22–35 (AS 6) (13, 14), and residues 2–16 (GC/2) (39)) and to antibodies detecting carboxyl-terminal domains of Gαi1-proteins, Gαi2-proteins (AS/7) (40), and Gαs-proteins (As 348) (27). In addition, polyclonal antibodies against carboxyl-terminal domains of Gαo1 and Gαo2 subtypes were used (AS 201 and AS 248, respectively) (14). Protein bands with bound antibodies were labeled by binding of peroxidase-conjugated second antibody (38, 41).

**Cross-linking of Membrane Proteins by PDM** (4)—The permeable cross-linker PDM (20–30 μM) was added to synaptoneurosomes at resting potential and during high [K⁺]-induced membrane depolarization. PDM dissolved in N,N′-dimethylformamide (Merck) was added to synaptoneurosomes incubated in Ca²⁺-free Krebs-Henseleit containing 4.7 or 50 mM [K⁺]. Cross-linking was stopped by the addition of 8 mM 2-mercaptoethanol (4).

**Immunoprecipitation of the α-Subunit of VGSC by anti-Gαo (AS 6)—**Membranes were solubilized with 4% (w/v) SDS for 10 min at room temperature, and then buffer was added containing 1% (w/v) Nonidet P-40, 1% (w/v) desoxycholate, 150 mM NaCl, 1 mM dithiotreitol, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 units/ml aprotinin, 10 mM Tris-Cl (pH 7.4). Solubilized membranes were immunoreacted with antisera against Gαo-proteins (AS 6) (2 h, 4 °C) and then incubated overnight at 4 °C with protein A-Sepharose beads (12.5% (w/v)). The Sepharose beads were then pelleted and washed with high ionic strength solution, thereby eliminating nonspecifically bound proteins. The immunoprecipitated proteins bound to the A-Sepharose beads were eluted in SDS-PAGE sample buffer, boiled for 1 min, then separated on SDS-PAGE (5% acrylamide) and electroblotted (Western blot). The immunoprecipitated proteins immunoreacted with antibodies against

**Fig. 1. Effect of membrane depolarization on the specific in situ photoaffinity labeling of Gαo-proteins with [α32P]GTPAA in membranes prepared from transiently permeabilized synaptoneurosomes.** [α32P]GTPAA was introduced into permeabilized synaptoneurosomes. Membrane proteins were separated by two-dimensional gel electrophoresis (pH 4–8 in the 1st dimension, 10% polyacrylamide in the second dimension). Autoradiograms of photoaffinity-labeled proteins in synaptoneurosomes exposed to 50 mM [K⁺]-Krebs-Henseleit buffer (depolarization) (A) or 4.7 mM [K⁺] (resting potential) (C) are presented. Western-blotted [α32P]GTPAA-labeled proteins were immunolabeled by antibodies directed against the amino-terminal domain in Gαo-proteins (GC/2). Immunolabeled Gαo-proteins in membranes isolated from depolarized synaptoneurosomes (B) and from synaptoneurosomes at resting potential (D) were detected. The 32P-labeling of autoradiographed Gαo-proteins was quantified by densitometry (E). The upper and lower traces present changes in the relative optical density produced by autoradiographed labeled Gαo-proteins in A and C, respectively. Data are from a typical experiment, one of five performed. Each sample contained 200 μg of protein.
Depolarization-induced Activation of G\(_{\alpha}\)-proteins

**RESULTS**

**Depolarization-induced Photoaffinity Labeling of G\(_{\alpha}\)-proteins with \([\alpha^{32}\text{P}]\)GTPAA—**[\(\alpha^{32}\text{P}]\)GTPAA was introduced into transiently permeabilized synaptoneuroses (see “Materials and Methods”). The “loaded” synaptoneuroses were UV-irradiated at resting potential or during membrane depolarization in Ca\(^{2+}\)-free Krebs-Henseleit buffer. Photoaffinity-labeled proteins were separated by two-dimensional SDS-gel electrophoresis and electroblotted.

[\(\alpha^{32}\text{P}]\)GTPAA-labeled G\(_{\alpha}\)-proteins were immunoidentified in membranes of synaptoneuroses UV-irradiated at either resting potential or during membrane depolarization (5–10 min). Their photoaffinity labeling in depolarized synaptoneuroses was 5- to 7-fold higher than that measured in unstimulated synaptoneuroses (Fig. 1). The intensity of \([\alpha^{32}\text{P}]\)GTPAA-labeling was not observed in synaptoneuroses permeabilized in the presence of \([\alpha^{32}\text{P}]\)GTPAA and GDP/S (100 \(\mu\text{M}\)), i.e. under conditions preventing exchange of GDP for GTP in G\(_{\alpha}\)-proteins (1–3). The isoelectric pH of the photolabeled G\(_{\alpha}\)-proteins in depolarized synaptoneuroses was shifted by approximately 0.5 pH unit toward a more acidic pH relative to that in synaptoneuroses at resting potential (Fig. 1). These results are consistent with an accelerated exchange of GDP for GTP for [\(\alpha^{32}\text{P}]\)GTPAA in G\(_{\alpha}\)-proteins (5, 23) in depolarized synaptoneuroses.

A further identification of the photolabeled G\(_{\alpha}\)-proteins has been enabled by immunolabeling with antibodies directed against the carboxyl-terminal domains of two G\(_{\alpha}\)-protein subtypes: G\(_{\alpha}\)-subunit and its isofrom, G\(_{\alpha}\)-subunit, the most abundant G\(_{\alpha}\)-proteins in the brain, and G\(_{\alpha}\)-protein (see “Materials and Methods”). Immunolabeling indicated a depolarization-induced shift in the isoelectric pH of G\(_{\alpha}\)-proteins toward a more acidic pH (Fig. 2), which could result from a depolarization-induced exchange of GDP for [\(\alpha^{32}\text{P}]\)GTPAA. The isoelectric pH of these G\(_{\alpha}\)-protein subtypes was not significantly altered by membrane depolarization (Fig. 3).

**Depolarization-induced Activation of G\(_{\alpha}\)-Proteins Was Not Mediated by Stimulation of G-protein-coupled Receptors—**The enhanced photoaffinity labeling of G\(_{\alpha}\)-proteins with [\(\alpha^{32}\text{P}]\)GTPAA in depolarized synaptoneuroses may suggest that, apart from being activated by stimulation of G\(_{\alpha}\)-protein-coupled receptors, G\(_{\alpha}\)-proteins were activated by membrane depolarization. However, this effect could conceivably be attributable to a depolarization-induced release of transmitters (42) evoking receptor stimulation as well.

To exclude a possible effect of transmitters released by membrane depolarization on the exchange of GDP for GTPAA in G\(_{\alpha}\)-proteins, all experiments were conducted under conditions preventing transmitter release (see “Materials and Methods”). Moreover, depolarization-induced activation of G\(_{\alpha}\)-proteins was examined in synaptoneuroses treated with antagonists of G\(_{\alpha}\)-proteins-coupled and abundantly spread receptors in the central nervous system. Resealed synaptoneuroses preloaded with \([\alpha^{32}\text{P}]\)GTPAA were depolarized and photoabeled in the presence of antagonists of muscarinic, NMDA-glutamate, dopaminergic, serotonergic, or adrenergic receptors in concentrations that inhibit 90% of agonist binding to these receptors. However, the increase in the photoaffinity labeling of G\(_{\alpha}\)-proteins with \([\alpha^{32}\text{P}]\)GTPAA in the depolarized synaptoneuroses was preserved despite their treatment with antagonists (Fig. 4).

Furthermore, the possible effect of transmitters on photoaffinity labeling of G\(_{\alpha}\)-proteins with \([\alpha^{32}\text{P}]\)GTPAA was examined in isolated synaptoneurosomal membranes rather than in synaptoneuroses, thereby eliminating possible effects induced by changes in membrane potential. Photoaffinity-labeled proteins in these preparations were specifically immunolabeled by anti-G\(_{\alpha}\)-antibodies (AS 6) (Fig. 5, A and B). In isolated
membranes the isoelectric pH of photoaffinity-labeled Go-proteins shifted toward a more acidic pH as compared with that of photoaffinity-labeled Go-proteins in synaptoneurosomes at resting potential (compare Figs. 1 and 2 with Fig. 5A). In comparison to the photoaffinity labeling of Go-proteins in control membranes, a modest additional increase in their labeling with [*32P]*GTPAA and a slight additional shift in their isoelectric pH were observed in membranes treated with agonists of muscarinic or serotoninergic receptors (Fig. 5B). Photoaffinity labeling of Go-proteins was similarly mildly enhanced by glutamate or melatonin (not shown). The [*32P]*photoaffinity labeling of Go-proteins was quantified by densitometry (Fig. 5C).

The substantial increase in photoaffinity labeling of Go-proteins during membrane depolarization, as compared with the slight effect of receptors stimulation on their photoaffinity labeling (compare Figs. 1E and 5C), further supports the assumption that depolarization-induced activation of Go-proteins is not mediated by receptors stimulation.

**Effect of Depolarization on PTX-catalyzed ADP-ribosylation of Go-proteins**—Because Go-proteins are better substrates for PTX-catalyzed ADP-ribosylation when coupled to Gβγ (43), their ADP-ribosylation is inhibited by activation of Go-proteins (1, 5). PTX-catalyzed ADP-ribosylation of Go-proteins in depolarized synaptoneurosomes should therefore be inhibited, whereas Go-proteins are activated by membrane depolarization. In contrast, Go-proteins are better substrates for CTX-catalyzed ADP-ribosylation when GDP is replaced by GTP, i.e. under conditions activating Go-proteins (44). A possible activation of Go-proteins by membrane depolarization would therefore accelerate their ADP-ribosylation.

We compared the effect of membrane depolarization in [Ca**2+**]-free Krebs-Henseleit buffer on ADP-ribosylation of Go-proteins *in situ* with its effect on ADP-ribosylation of Go-proteins by comparing their complementary [*32P]*ADP-ribosylation carried in membranes prepared from PTX- or CTX-pretreated synaptoneurosomes. [*32P]*ADP-ribosylated Go-proteins were immunoprecipitated by antibody AS 6 (13, 14).

![Depolarization](image)

**FIG. 3.** Effect of membrane depolarization on the isoelectric pH of Go-proteins. Proteins in transiently permeabilized synaptoneurosomes were exposed to photoaffinity labeling with [*32P]*GTPAA. Proteins in membranes prepared from resting potential and depolarized synaptoneurosomes were separated by two-dimensional gel electrophoresis (see Fig. 1) and electroblotted (Western blot). Blotted proteins (40–41 kDa) that were immuno-labeled by antibodies directed against a common carboxyl-terminal domain in Go, and Go-proteins (AS7) are presented (*n* = 3). Each sample contained 200 µg of protein.

[**32P**]ADP-ribosylated Go-proteins were immunolabeled with antibody AS348 (27). (Go-proteins are present as four splice variants appearing as two bands, each including Go isoforms differing in only one amino acid (27). Membrane depolarization inhibited PTX-catalyzed ADP-ribosylation of Go-proteins (Fig. 6A). This is in accordance with a depolarization-induced activation of Go-proteins (43). In contrast, CTX-catalyzed ADP-ribosylation of Go-proteins was not affected by membrane depolarization (Fig. 6B).

**Blocking the activation of VGSC Prevented the Depolarization-induced Photoaffinity Labeling of Go-proteins with [*32P]*GTPAA—**Depolarization-induced photoaffinity labeling of Go-proteins with [*32P]*GTPAA was prevented by the R enantiomer of the cardioselective and antiarrhythmic drug DPI 201–106 (10 µM), which prevents activation of VGSC (45) (Fig. 7). [*32P]*GTPAA was introduced into transiently permeabilized control synaptoneurosomes and synaptoneurosomes treated with the R enantiomer of DPI. After rescaling, synaptoneurosomes were UV-irradiated at either resting potential or depolarization in Ca**2+**-free buffered Krebs-Henseleit. Photolabeled membrane proteins were separated by two-dimensional SDS-PAGE and electroblotted. [*32P]*GTPAA-photolabeled membrane proteins were immunolabeled with common anti Go-proteins antibodies (GC/2) (Figs. 7, A and B). The en-
Depolarization-induced Activation of $G_o$-proteins

Fig. 5. A, immunolabeling of $G_o$-proteins with antibodies directed against a common domain of $G_{o1}$-proteins (AS 6). Proteins in isolated membranes were subjected to photoaffinity labeling with [$\alpha$-32P]GTPAA and were separated by two-dimensional SDS-PAGE (pH 4–8 in the first dimension, 10% acrylamide in the second dimension) and electroblotted (Western blots). Blotted proteins labeled with Ponceto and $G_o$-proteins immunolabeled by AS 6 are presented. B, autoradiograms of the Western-blotted $G_o$-proteins, photoaffinity labeled with [$\alpha$-32P]GTPAA. Isolated synaptoneurosemal membranes were incubated with 10 $\mu$m GTP/GDP and 1 $\mu$Ci of [$\alpha$-32P]GTPAA in the absence (control) or in the presence of carbamylcholine (100 $\mu$m) or serotonin (100 $\mu$m). Photoaffinity labeling of $G_o$-proteins with [$\alpha$-32P]GTPAA in these membrane preparations was quantified by densitometry (C). Each sample contained 200 $\mu$g protein ($n = 3$).

Enhanced photoaffinity labeling of $G_o$-proteins with [$\alpha$-32P]GTPAA in depolarized synaptoneurosemes, as well as the shift in their isoelectric pH (see Figs. 1 and 2), were eliminated following pretreatment of the synaptoneurosemes with the $R$-enantiomer of DPI, i.e. when activation of VGSC had been prevented (Figs. 7, A and B). In contrast, the neurotoxin batrachotoxin (46) and the $S$-enantiomer of DPI (45), both of which prolong the activation of VGSC (45–47) although they share a common binding site with DPI $R$-enantiomer on the $\alpha$-subunit of VGSC (45), did not interfere with the depolarization-induced photoaffinity labeling of $G_o$-proteins or with the coupling of G-proteins to muscarinic receptors (12). These findings may suggest that voltage-induced activation of VGSC is essential for depolarization-induced activation of $G_o$-proteins. Blocking of the voltage-dependent Na$^+$ current by tetrodotoxin (48) did not affect the depolarization-induced photoaffinity labeling of $G_o$-proteins, ruling out a possible involvement of Na$^+$-current in the activation of $G_o$-proteins. This is in consistence with previous findings (10, 12).

The effect of DPI $R$-enantiomer on photoaffinity labeling of $G_o$-proteins with [$\alpha$-32P]GTPAA in depolarized synaptoneurosemes (Figs. 7, A and B) was accompanied by prevention of the previously described high to low affinity conversion in muscarinic receptors of depolarized synaptoneurosemes (10) (Fig. 7, C and D). Both effects of DPI $R$-enantiomer may be attributable to the prevention of depolarization-induced activation of $G_o$-proteins (10, 12).

Co-immunoprecipitation of the $\alpha$-Subunit of VGSC Cross-linked with $G_o$-proteins—We examined the possibility that VGSC and $G_o$-proteins interact with each other. Membrane proteins of unstimulated and depolarized brain-stem and cortical synaptoneurosemes were cross-linked by the permeable cross-linker, PDM (4), which reacts with SH sulphydryls, producing uncleavable products (4) (Fig. 8A). As a result, a fraction of $G_o$-proteins co-immunoprecipitated with the $\alpha$-subunit of VGSC (see Figs. 8, B and C). The cross-linked product (approximately 300 kDa) was immunoprecipitated by a common anti-$G_o$-protein antibody (AS 6) and immunoreacted specifically with antibodies against a common epitope in the $\alpha$ subunit of all VGSC subtypes (SP19) as well as with antibody directed against the carboxyl terminus of $G_{o1}$ and $G_{o3}$-proteins (AS 248) (Fig. 8C).

Cross-linking of $G_o$-proteins with the $\alpha$ subunit of VGSC was much more efficient when conducted during depolarization than at resting potential (Fig. 8C). Cross-linking occurred within seconds (Fig. 8A), whereas VGSC inactivation follows their short activation, lasting for ms (49). Thus, $G_o$-proteins were, apparently, cross-linked to inactivated VGSC in depolarized synaptoneurosemes. $G_o$-proteins were also most efficiently cross-linked with the $\alpha$ subunit of VGSC in synaptoneurosemes pretreated with batrachotoxin in the presence or absence of the Na$^+$ current blocker tetrodotoxin, i.e. when activation of VGSC had been prolonged (46, 47) (not shown). When cross-linking was conducted in isolated synaptoneurosemal membranes, the $\alpha$ subunit of VGSC was cross-linked most efficiently with $G_o$-proteins bound to GDP/GTP $\gamma$S rather than to GTP $\gamma$S (Fig. 8C). In accordance, the addition of muscarinic agonists did not induce cross-linking of $G_o$-proteins with the VGSC $\alpha$ subunit (not shown). This may exclude the possibility that VGSC act as effectors of $G_o$-proteins (5–7).

Without cross-linking, $G_o$-proteins and the $\alpha$ subunit of VGSC did not co-immunoprecipitate (Fig. 8C). The VGSC $\alpha$ subunit was not cross-linked with the $\beta_2\gamma$ subunit of G-proteins in depolarized synaptoneurosemes (Figs. 8, B and C).

Because reportedly VGSC inactivation does not interfere with their depolarization-induced activation (49, 50) different parts in the VGSC $\alpha$ subunit undergo conformational changes that are responsible for the depolarization-induced activation and their successive fast inactivation (50–52), our findings may conclude that an interaction between $G_o$-proteins and the $\alpha$ subunit of VGSC is possible once VGSC have been activated. Regarding the GTPase activity of $G_o$-proteins, these findings also suggest that $G_o$-proteins may repeatedly interact with VGSC $\alpha$ subunits as long as membrane depolarization lasts.

Effect of Membrane Depolarization on Phosphorylation of $G_o$-proteins—The isoelectric pH of $G_o$-proteins photolabeled with [$\alpha$-32P]GTPAA was lower by approximately 0.5 pH units in depolarized synaptoneurosemes than in synaptoneurosemes at
Depolarization-induced Activation of $G_o$-proteins

**Fig. 6.** Effect of membrane depolarization on the ADP-ribosylation of $G_{o1}$- and $G_{o3}$-proteins. A, synaptoneurosomes were pretreated in the presence of atropine (1 mM) with PTX (200 ng/ml, 37 °C, 2 h, 95% O$_2$/5% CO$_2$) (lanes 1 and 2) during depolarization (lane 1) and at resting potential (lane 2). PTX-catalyzed $[^{32}P]$ADP-ribosylation of $G_{o1}$-proteins was subsequently performed in membranes isolated from the PTX-pretreated synaptoneurosomes (lanes 1 and 2) and from control synaptoneurosomes preincubated without PTX during depolarization (lane 3) and at resting potential (lane 4). $[^{32}P]$ADP-ribosylated $G_{o1}$-proteins were immunoprecipitated by antibodies AS 6, subjected to SDS-PAGE, and blotted (Western blot). Their autoradiograms are presented in the upper frame. Immunolabeling of the immunoprecipitated $G_{o1}$-proteins is presented in the lower frame. $G_{o1}$-proteins were immunodetected in the immunoprecipitates by antibody AS 248. The extent of $[^{32}P]$ADP-labeling of $G_{o1}$-proteins was quantified by densitometry ($n > 5$). B, synaptoneurosomes were pretreated in the presence of propranolol (1 mM) with CTX (10 µg/ml, 37 °C, 2 h, 95% O$_2$/5% CO$_2$) during depolarization (lane 1) or at resting potential (lane 2). $[^{32}P]$ADP-ribosylation of $G_{o3}$-proteins was subsequently performed in CTX-pretreated (lanes 1 and 2) and in untreated synaptoneurosomes (lane 3, depolarized during preincubation; lanes 4 and 5, preincubated at resting potential. In lane 4, synaptoneurosomes were pretreated with CTX in the presence of isoproterenol (10 µM) in the absence of propranolol). $[^{32}P]$ADP-ribosylation of $G_{o3}$-proteins was catalyzed by CTX-A protomer in the presence of Gpp(NH)p (100 µM). Membrane proteins were separated by SDS-PAGE and electroblotted (Western blot). $G_{o3}$-proteins were immunolabeled by antibodies AS 348. Autoradiograms of the $[^{32}P]$ADP-ribosylated proteins are presented in the upper frame. Immunolabeling of $[^{32}P]$ADP-labeled $G_{o3}$-proteins is presented in the lower frame. The extent of $[^{32}P]$ADP labeling was quantified by densitometry (O.D., absorbance) ($n = 3$).

**DISCUSSION**

The experiments described in this study provide evidence for a depolarization-induced activation of $G_{o1}$-protein and its isoform $G_{o3}$-protein in membranes of rat brain-stem synaptoneurosomes. The voltage-induced effect was clearly demonstrated by comparing their in situ photoaffinity labeling with $[o^{32}P]$GTPAA in membranes of depolarized synaptoneurosomes with that in synaptoneurosomes at resting potential (Figs. 1, 2, and 4). The increased photoaffinity labeling of $G_{o1}$ and $G_{o3}$ subtypes in depolarized synaptoneurosomes and the shift in their isoelectric pH toward a more acidic pH (Figs. 1 and 2) are attributable to an accelerated exchange of GDP for GTPAA. This is also consistent with the depolarization-induced inhibition of PTX-catalyzed ADP-ribosylation of $G_{o1}$-proteins (Fig. 6). The depolarization-induced exchange of GDP for $[o^{32}P]$GTPAA in $G_{o3}$-proteins was reversed by repolarization and was independent of transmitters release and stimulation of muscarinic, NMDA-glutamate receptors, dopaminergic, or serotonegic receptors (2, 3, 8, 9) (Figs. 4 and 5). Thus, it is apparently not a consequence of depolarization-induced release of these transmitters into the synaptic cleft (42).

The shift in the isoelectric pH of photoaffinity-labeled $G_{o1}$- and $G_{o3}$-proteins in depolarized membranes (Fig. 2) could also be associated with $G_{o3}$-protein phosphorylation. This seems unlikely, however, in view of the negligible effect of membrane depolarization on their labeled in situ phosphorylation under resting potential (Figs. 1 and 2). This depolarization-induced shift in their pI may be attributable, as mentioned above, to a depolarization-induced acceleration in the exchange of GDP for covalently bound GTPAA. Alternatively or in addition it might indicate a depolarization-induced phosphorylation of $G_{o3}$-proteins. This possibility was examined by in situ labeled phosphorylation of membrane proteins in depolarized synaptoneurosomes.

Synaptoneurosomes were incubated for 30 min with $[^{32}P]$phosphorus (10 µCi) and then resuspended in either 4.7 or 50 mM [K+] in Ca$^{2+}$-free Krebs-Henseleit buffer. Membrane proteins were separated by SDS-PAGE, electroblotted (Western blot), and examined by autoradiography and immunolabeling (GC/2). Only a small fraction of the immunolabeled $G_{o3}$-proteins was $^{32}$P-phosphorylated (Fig. 9). Although high [K+] induced membrane depolarization increased their photoaffinity labeling with $[o^{32}P]$GTPAA by approximately 5–7-fold (Figs. 1 and 2), it did not significantly alter their $^{32}$P-phosphorylation (Fig. 9), suggesting that the effect of membrane depolarization is not attributable to phosphorylation of these proteins.
the experimental conditions employed (Fig. 9).

Membrane depolarization or changes in the electric field of the membrane would induce charge redistribution in the phospholipid matrix, which may modify the anchorage of \( \Gamma \)o-proteins to phospholipids (53–59). Recent reports have indicated that modifications in the binding of \( \Gamma \)o-proteins to myristate and palmitate in the phospholipid matrix affect their interaction with the \( \Gamma \)bg subunit, (53, 57, 60) as well as with \( \Gamma \)o-protein-coupled receptors (61). Changes in palmitylation and myristoylation of \( \Gamma \)o-proteins apparently affect the stability of GDP binding (55, 57, 61, 62) and thereby may affect \( \Gamma \)o-protein activation (62). Depolarization-induced modifications of the anchorage of \( \Gamma \)o-protein subunits in the phospholipid matrix might occur via modification of labile thioester bonds between cysteine residues in \( \Gamma \)o-proteins or GAP-43 (63) and palmitate (56, 57, 64–68). Voltage-induced modifications in the phospholipid matrix might be also mediated by voltage-induced modifications in voltage-gated ion channels. In view of the reciprocal effects of VGSC activation and the activation of G-coupled muscarinic receptors (12, 24, 25), we examined the possibility that depolarization-induced activation of VGSC is involved in depolarization-induced activation of \( \Gamma \)o-proteins. Unlike in other excitable tissues, the \( \alpha \) subunit of the VGSC in the brain is covalently bound to a glycoprotein with a single transmembrane spanning segment, the \( \beta \)2 subunit of VGSC (69, 70). Evidence for a possible role of \( \beta \)2 in the anchorage of the \( \alpha \) subunit of the VGSC to phospholipids may suggest its involvement in the interaction of VGSC with other proteins anchored to the phospholipid matrix (70). In view of this, the possibility that voltage-induced modifications in the \( \alpha \) subunit of VGSC may influence other membrane proteins, including \( \Gamma \)o-proteins, should be considered and further examined.

DPI-enantiomers that interact with a common site in the \( \alpha \) subunit of VGSC (45) modulate differently its voltage-dependent activation (45). Only the DPI R enantiomer, which prevents the activation of VGSC (45), prevented both the depolarization-
Depolarization-induced Activation of G_o-proteins

**FIG. 8.** Co-immunoprecipitation of the α subunit of VGSC cross-linked with G_o-proteins in the membranes of brain synaptoneurosomes. Brain-stem and cortical synaptoneurosomes were treated with the permeable cross-linker PDM (25 μM). The cross-linked product was immunoprecipitated by anti-G_o antibodies (AS 6) and immunolabeled by antibodies directed against the α subunit of VGSC (SP19) and antibody directed against G_oα1 and G_oα2-proteins. Each lane contained 100 μg of protein. A, immunolabeling of G_o-proteins in 39-kDa protein band (10% polyacrylamide SDS-PAGE) following cross-linking during 30-s and 1-, 2-, and 3-min incubation with PDM in synaptoneurosomes, unstimulated (a), depolarized (60 mM [K⁺]) (b), pretreated with 1 μM batrachotoxin (c), or with batrachotoxin (1 μM) and tetrodotoxin (1 μM) (d). In e and f, cross-linking was performed in isolated synaptoneurosomal membranes treated with GDPβS (100 μM) and GTPγS (100 μM), respectively (n = 10). B, upper lanes, immunolabeling of G_oα-proteins by the common anti-G_oα antibody (Ab.) AS 6 following a 2-min cross-linking of membrane proteins by PDM in depolarized (60 mM [K⁺] (lane 1) and unstimulated synaptoneurosomes (lane 2). G_oα-proteins were co-immunoprecipitated with Gβγ subunit by antibodies directed against a common epitope in Gβγ-proteins. Lower lanes, immunolabeled G_oα-proteins, co-immunoprecipitated with Gβγ-subunit by antibody directed against a common epitope in Gβγ-proteins, from membranes of depolarized and unstimulated synaptoneurosomes (lanes 1 and 2, respectively). Co-immunoprecipitation was not preceded by cross-linking. Immunoprecipitated proteins were subjected to SDS-PAGE (10% polyacrylamide) and electroblotted (Western blot) (n = 3). C, immunolabeling of the α subunit of VGSC (lanes 1 and 2) co-immunoprecipitated with G_oβ-proteins by AS 6 antibody (lanes 3–5 and 7–9) after a 2- min cross-linking by PDM (25 μM) under the following treatments. Lanes 3, 5, and 6, cross-linking conducted during depolarization. Lane 4, cross-linking conducted at resting potential. Lane 6, immunoprecipitation of the cross-linked product by a common antibody directed against Gβγ-proteins. Lanes 7 and 8, cross-linking in isolated membranes treated by GDPβS (100 μM) and by GTPγS (100 μM), respectively.

**FIG. 9.** In situ [32P] phosphorylation of G_o-proteins in synaptoneurosomes at resting potential and during membrane depolarization. Brain-stem synaptoneurosomes preincubated for 30 min with [32P]phosphorus (10 μCi/sample, 400–800 μCi/ml) in Krebs-Henseleit buffer were incubated for 10 min in Krebs-Henseleit buffer containing 4.7 or 50 mM [K⁺]. Membrane proteins were separated by SDS-PAGE (10% acrylamide), electroblotted (Western blot), and immunolabeled with antibodies directed against the amino-terminal domain of G_o-proteins (GC/2). Lanes 1 and 2, autoradiogram of [32P]-phosphorylated proteins in the membranes of synaptoneurosomes at resting potential (lane 1) and during depolarization (lane 2). Lanes 3 and 4, immunolabeled G_o-proteins at resting potential and during depolarization, respectively. Each lane contained 200 μg of protein (n = 3).
Depolarization-induced Activation of G-proteins

membrane potential and the activation of muscarinic (M1) receptors (72). Thus, in addition to activation of ion channels by stimulation of G-protein-coupled receptors (1, 73–75), receptor stimulation may be modulated by activation of voltage-gated ion channels. This in turn may result in feedback mechanisms, producing long term changes in the membrane potential.

Because receptor stimulation induces activation of several receptor-coupled G-proteins (4), depolarization-induced activation of even a fraction of G-proteins may affect the activity of other G-proteins, as well (10, 24), thereby producing additional versatility in synaptic transmissions in the central nervous system.

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Activation of $G_\alpha$-proteins by Membrane Depolarization Traced by in Situ Photoaffinity Labeling of $G_\alpha$-proteins with [$\alpha^{32}$P]GTP-azidoanilide
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