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 Go-proteins by tracing their depolarization-induced in situ photoaffinity labeling with [α32P]GTP-azidoanilide (GTPAA). Labeled GTPAA was introduced into transiently permeabilized rat brain-stem synaptoneurosomes. The resealed synaptoneurosomes, while being UV-irradiated, were depolarized. Relative to synaptoneurosomes at resting potential, the covalent binding of [α32P]GTPAA to Goα1 and Goα3-proteins, but not to Goα2-isoforms, was enhanced by 5- to 7-fold in depolarized synaptoneurosomes, thereby implying an accelerated exchange of GDP for [α32P]GTP. Their depolarization-induced photoaffinity labelling was independent of stimulation of Go-protein-coupled receptors and could be reversed by membrane repolarization, thus excluding induction by transmitters release. 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 Goα-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 Goα-proteins in isolated synaptoneurosomal membranes. These findings support a possible involvement of VGSC in depolarization-induced activation of Goα-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). Goα-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 Goα1 subtype appears to be involved in the coupling of muscarinic receptors to Ca2+ channels, and the Goα2 subtype mediates inhibition of Ca2+ current activated by somatostatin receptors (19). The function of the Goα3 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 [α32P]GTPAA1 (22, 23) indicated a depolarization-induced accelerated exchange of GDP for [α32P]GTPAA in Goα1- and Goα3-proteins, implying a depolarization-induced activation of these Gα-proteins. [α32P]GTPAA was introduced into transiently permeabilized synaptoneurosomes as described before (10). Unlike the endogenously bound guanine nucleotides, [α32P]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 Gα-proteins from muscarinic receptors (12, 24, 25), we examined the possibility that VGSC can be involved in depolarization-induced activation of Goα-proteins. Our results indicated that depolarization-induced activation of Goα-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 Goα-proteins. Our results indicated that depolarization-induced activation of Goα-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 Goα-proteins. These findings suggest repeated
interactions between VGSC-α subunit and Gα-proteins, as long as membrane depolarization lasts.

MATERIALS AND METHODS

Reagents—ATP (grade 1), GPP[S], GTP[S], tetrodotoxin, dithiotheritol, carbamylcholine, atropine, serotonin, piperone, propranolol, naloxone, and yohimbine were all purchased from Sigma. 4-Azidoaniline hydrochloride was supplied by Fluka (Switzerland). α-2-amino-5-phospho- phovarlic acid was purchased from Cambridge Research Biochemicals. DPL-205-429 (DPL R enantomer; see Footnote 1) was kindly supplied by Dr. E. Rissi and Dr. D. Romer of Sandoz Ltd., Pharmacological Division, Preclinical Research (Basel, Switzerland). N,N‘-1,4-Phenylenediaminimide (PDM) was purchased from Aldrich. Pertussis toxin (PTX), cholera toxin (CTX), and the A-protomer of CTX were purchased from List Biological Laboratories, Campbell, CA. Guanosine-5′-triphosphate tetra(triethy lammonium) salt, [α-32P]GTP ([32P]GTP) (500 Ci/mmol), and [3H]-labeled antibodies against peptide derived from the amino-terminal domain of Gα-proteins (CG2) were purchased from NEN Life Science Products. [3H]Acetyl-choline ([3H]AcCh) (96 Ci/mmol, 98% pure) and [phenyl 3H]tetraphenylphosphonium bromide ([3H]TPP) (33.6 Ci/mmol) were purchased from Amersham Pharmacia Biotech. Phosphorua-[32P]HPO4 (400–800 mCi/ml) was purchased from ICN Pharmaceutics. N,N′-Methyl-4-piperidylbenzilate was prepared by catalytic tritium exchange as described in Cohen-Armon et al. (26). For detection of Gαi-isosforms, three polyclonal peptide antibodies from rabbit (AS 6, AS 201, and AS 248) were generated as described elsewhere (14, 19). AS 6, which detects all known Gαi-isosforms, is directed against amino acids 22 to 35 of all Gαi isosforms. AS 248, which recognizes Gαi1 and the closely related isoform Gαi3 (14), was directed against amino acids 310 to 323 of Gαi3. AS 201, which reacts exclusively with rodent Gαi1 (14, 18), was directed against amino acids 293 to 308 of Gαi1. Antibodies directed against the carboxyl-terminal decapeptide of Gαi2-proteins were kindly supplied by Professor G. Milligan, Glasgow University, UK. Antibodies detecting four splice variants of Gαo-proteins in mammalian brain AS 348 (27) have been used. Polyclonal antibodies raised against peptide correlating to residues 1491–1508 of a subunit of VGSC (28) (SP19) were purchased by Alomone Laboratories, Jerusalem, Israel.

Preparation of Synaptoneurosomes—Adult male rats of the CD strain were obtained from Levenstein’s Farm, Yokneam, Israel, and maintained as described before (26). Synaptoneurosomes were prepared from pooled brain stems obtained from 2- to 3-month-old rats according to Hollingsworth and Hollingsworth (32). Before being lysed (10), synaptoneurosomes were washed by centrifugation according to Pfeuffer (22). The 4-azidoaniline hydrochloride was added to synaptoneurosomes preincubated for 30 min at room temperature in the dark, the mixture was incubated of 4 at 37 °C as described before (10). [α-32P]GTPAA (0.2 μM) was added to this solution. After precisely 40 min, the permeabilized synaptoneurosomes were ressealed by two suc- cessive centrifugations (10 min) with Krebs-Henseleit containing 2.5 mM Ca Cl2 and 1.18 mM Mg Cl2 followed by centrifugation (1000 g, 5 min) (10). Membrane permeabilization and res sealing were confirmed by measuring the accumulation of [3H]TPP in each step (10, 33). The ressealed synaptoneurosomes were then resuspended in Ca2+-free (10 μM) modified Krebs-Henseleit buffer containing either 4.7 or 50 mM [K+] (1), inducing either resting potential or membrane depolarization, respectively. Samples of the ressealed synaptoneurosomes, containing approximately 100 μg of protein, were UV-irradiated (300 nm, 350 W) for 5 min on ice. GTP-binding proteins were photolabeled by [α-32P]GTPAA. For estimation of the nonspecific binding of [α-32P]GTPAA, synaptoneurosomes were permeabilized in the presence of [α-32P]GTPAA and GTPyS (200 μM). The nonspecific photoaffinity labeling was determined by photoaffinity labeling of synaptoneurosomes, samples of nonpermeabilized synaptoneurosomes were subjected to photoaffinity labeling under the above conditions. Photoaffinity labeling of membrane proteins with [α-32P]GTPAA in nonpermeabilized synaptoneurosomes was negligible. Membrane proteins were separated by SDS-PAGE and autoradiographed. [2P]GTPAA-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 (33), it follows that at equilibrium the transmembrane concentration gradient of [3H]TPP is proportional to membrane potential, according to the Nernst 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 μM [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 (Hydrofluor;see description in 10). The binding of [3H]AcCh to muscarinic receptors in ressealed synaptoneurosomes was determined by the method of Chang et al. (35). [3H]AcCh binding to muscarinic receptors was measured in Ca2+-free Krebs-Henseleit buffer, pH 7.4, were incubated with 200 ng/ml PTX for 2 h at 37 °C and 95% O2, 5% CO2 as described before (11).

Binding of [3H]AcCh to Muscarinic Receptors in Ressealed Synaptoneurosomes—To determine the effect of membrane depolarization on the binding of [3H]AcCh to high affinity muscarinic receptors, synaptoneurosomes were incubated for 30 min in 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) and various concentrations of [3H]AcCh. Na+ 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 was 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 Ca2+-free Krebs-Henseleit buffer (described above), with a Cs+/[Mg2+] 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 was 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 $G_o$-proteins (residues 22–35 (AS 6) (13, 14), and residues 2–16 (GC/2) (39)) and to antibodies detecting carboxyl-terminal domains of $G_{i1}$-proteins, $G_{i2}$-proteins (AS/7) (40), and $G_s$-proteins (As 348) (27). In addition, polyclonal antibodies against carboxyl-terminal domains of $G_o1$ and $G_o2$ 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).

**Immunoprecipitation of the $\alpha$-Subunit of VGSC by anti-$G_o$ (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 diithioretil, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 units/ml aprotinin, 10 mM Tris-Cl (pH 7.4). Solubilized membranes were immunoreacted with antibodies directed against the amino-terminal domain in $G_o$-proteins (GC/2). Immunolabeled $G_o$-proteins in membranes isolated from depolarized synaptoneurosomes (B) and from synaptoneurosomes at resting potential (D) were detected. The $^{32}$P-labeling of autoradiographed $G_o$-proteins was quantified by densitometry (E). The upper and lower traces present changes in the relative optical density produced by autoradiographed labeled $G_o$-proteins in A and C, respectively. Data are from a typical experiment, one of five performed. Each sample contained 200 μg of protein.

**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 Ca$^{2+}$-free Krebs-Henseleit containing 4.7 or 50 mM [K$^+$]. Cross-linking was stopped by the addition of 8 mM 2-mercaptoethanol (4).

**Fig. 1. Effect of membrane depolarization on the specific in situ photoaffinity labeling of $G_o$-proteins with $[^{32}\text{P}]$GTPAA in membranes prepared from transiently permeabilized synaptoneurosomes.** $[^{32}\text{P}]$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 $[^{32}\text{P}]$GTPAA-labeled proteins were immunolabeled by antibodies directed against the amino-terminal domain in $G_o$-proteins (GC/2). Immunolabeled $G_o$-proteins in membranes isolated from depolarized synaptoneurosomes (B) and from synaptoneurosomes at resting potential (D) were detected. The $^{32}$P-labeling of autoradiographed $G_o$-proteins was quantified by densitometry (E). The upper and lower traces present changes in the relative optical density produced by autoradiographed labeled $G_o$-proteins in A and C, respectively. Data are from a typical experiment, one of five performed. Each sample contained 200 μg of protein.
aptoneurosomes were loaded with \[^{32}P\]GTPAA and UV-irradiated from depolarized synaptoneurosomes (B). After incubation for 5–10 min at room temperature, \[^{32}P\]GTPAA was depolarized and photoaffinity labeled in the presence of \[^{32}P\]GTPAA and GDP/S (100 μM), i.e. under conditions preventing exchange of GDP for GTP in Go-proteins (1–3). The isoelectric pH of the photosylated Go-proteins in depolarized synaptoneurosomes was shifted by approximately 0.5 pH unit toward a more acidic pH relative to that in synaptoneurosomes at resting potential (Fig. 1). These results are consistent with an accelerated exchange of GDP for \[^{32}P\]GTPAA in Go-proteins in depolarized synaptoneurosomes.

A further identification of the photoaffinity-labeled Go-proteins has been enabled by immunolabeling with antibodies directed against the carboxyl-terminal domain of two Go-protein subtypes: Goα1- and its isoform, Goα3, the most abundant Go-proteins in the brain, and Goα2-proteins (see “Materials and Methods”). Immunolabeling indicated a depolarization-induced shift in the isoelectric pH of Goα1- and Goα2-proteins toward a more acidic pH (Fig. 2), which could result from a depolarization-induced exchange of GDP for \[^{32}P\]GTPAA. The isoelectric pH of these Goα-proteins was not altered (Fig. 2).

Photoaffinity-labeled Go-proteins were also immunolabeled with antibodies directed against a common carboxyl-terminal domain of Goα1- and Goα2-proteins (see “Materials and Methods”). Under the experimental conditions employed, the isoelectric pH of these Goα-proteins was not significantly altered by membrane depolarization (Fig. 3).

Depolarization-induced Activation of Goα-Proteins Was Not Mediated by Stimulation of G-protein-coupled Receptors—The enhanced photoaffinity labeling of Goα-proteins with \[^{32}P\]GTPAA in depolarized synaptoneurosomes may suggest that, apart from being activated by stimulation of G-protein-coupled receptors, Go-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 \[^{32}P\]GTPAA in Goα-proteins, all experiments were conducted under conditions preventing transmitter release (see “Materials and Methods”). Moreover, depolarization-induced activation of Goα-proteins was examined in synaptoneurosomes treated with antagonists of G-proteins-coupled and abundantly spread receptors in the central nervous system. Resealed synaptoneurosomes preloaded with \[^{32}P\]GTPAA were depolarized and photolabeled in the presence of antagonists of muscarinic, NMDA-glutamate, dopaminergic, serotoninergic, or adrenergic receptors in concentrations that inhibit 90% of agonist binding to these receptors. However, the increase in the photoaffinity labeling of Goα-proteins with \[^{32}P\]GTPAA in the depolarized synaptoneurosomes was preserved despite their treatment with antagonists (Fig. 4).

Furthermore, the possible effect of transmitters on photoaffinity labeling of Goα-proteins with \[^{32}P\]GTPAA was examined in isolated synaptoneurosomal membranes rather than in synaptoneurosomes, thereby eliminating possible effects induced by changes in membrane potential. Photoaffinity-labeled proteins in these preparations were specifically immunolabeled by anti-Goα-antibodies (AS 6) (Fig. 5, A and B). In isolated...
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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 synaptoneurosesomes 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 [α-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 synaptoneurosesomes 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 [Ca2+] (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 synaptoneurosesomes. [32P]ADP-ribosylated Go-proteins were immunoprecipitated by antibody AS 6 (13, 14).

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

Fig. 4. A, photoaffinity labeling of Go-proteins with [α-32P]GTPAA in transiently permeabilized synaptoneurosesomes at resting potential (lane 1) and during membrane depolarization (lane 2), in the presence of antagonists of muscarinic and adrenergic receptors (atropine (1 μM), propranolol (1 μM), and yohimbine (1 μM)), at resting potential (lane 3) and during membrane depolarization (lane 4), and in the presence of antagonists of the dopaminergic, serotoninergic, and NMDA-glutamate receptors (naloxone (10 μM), spiperone (1 μM) and 5-2-amino-5-phosphovaleric acid (100 μM)) at resting potential (lane 5) and during depolarization (lane 6). Membrane proteins were separated by SDS-PAGE (10% acrylamide), blotted (Western blot), and autoradiographed. B, blotted proteins photoaffinity-labeled with [α-32P]GTPAA were immunolabeled by antibodies directed against the amino-terminal domain of Goγ-proteins (GC/2). Each lane contained 200 μg of protein. Data are from a typical experiment, one of five performed.

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

Blocking the activation of VGSC Prevented the Depolarization-induced Photoaffinity Labeling of Go-proteins with [α-32P]GTPAA—Depolarization-induced photoaffinity labeling of Go-proteins with [α-32P]GTPAA was prevented by the R enantiomer of the cardiotonic and antiarrhythmic drug DPI 201–106 (10 μM), which prevents activation of VGSC (45) (Fig. 7). [α-32P]GTPAA was introduced into transiently permeabilized control synaptoneurosesomes and synaptoneurosesomes treated with the R enantiomer of DPI. After sealing, synaptoneurosesomes were UV-irradiated at either resting potential or depolarization in Ca2+ (free buffered Krebs-Henseleit. Photolabeled membrane proteins were separated by two-dimensional SDS-PAGE and electroblotted. [α-32P]GTPAA-photolabeled membrane proteins were immunolabeled with common anti Goγ-proteins antibodies (GC/2) (Figs. 7, A and B). The en-
hanced photoaffinity labeling of $G_a$-proteins with $[\alpha^{32}\text{P}]$GTPAA in depolarized synaptoneurosomes, as well as the shift in their isoelectric pH (see Figs. 1 and 2), were eliminated following pretreatment of the synaptoneurosomes 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 osubunit of VGSC (45), did not interfere with the depolarization-induced photoaffinity labeling of $G_a$-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 $G_a$-proteins. Blocking of the voltage-dependent Na$^+$ current by tetrodotoxin (48) did not affect the depolarization-induced photoaffinity labeling of $G_a$-proteins, ruling out a possible involvement of Na$^+$-current in the activation of $G_a$-proteins. This is in consistence with previous findings (10, 12).

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

Co-immunoprecipitation of the $\alpha$-Subunit of VGSC Cross-linked with $G_a$-proteins—We examined the possibility that VGSC and $G_a$-proteins interact with each other. Membrane proteins of unstimulated and depolarized brain-stem and cortical synaptoneurosomes were cross-linked by the permeable cross-linker, PDM (4), which reacts with SH sulphydryl groups, producing uncleanable products (4) (Fig. 8A). As a result, a fraction of $G_a$-proteins co-immunoprecipitated with the osubunit of VGSC (see Figs. 8, B and C). The cross-linked product (approximately 300 kDa) was immunoprecipitated by a common anti-$G_a$-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 $G_{\alpha_1}$ and $G_{\alpha_3}$-proteins (AS 248) (Fig. 8C).

Cross-linking of $G_a$-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, $G_a$-proteins were, apparently, cross-linked to inactivated VGSC in depolarized synaptoneurosomes. $G_a$-proteins were also most efficiently cross-linked with the $\alpha$ subunit of VGSC in synaptoneurosomes 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 synaptoneurosomal membranes, the $\alpha$ subunit of VGSC cross-linked most efficiently with $G_a$-proteins bound to GDP/S rather than to GTPyS (Fig. 8C). In accordance, the addition of muscarinic agonists did not induce cross-linking of $G_a$-proteins with the VGSC $\alpha$ subunit (not shown). This may exclude the possibility that VGSC act as effectors of $G_a$-proteins (5–7).

Without cross-linking, $G_a$-proteins and the $\alpha$ subunit of VGSC did not co-immunoprecipitate (Fig. 8C). The VGSC $\alpha$ subunit was not cross-linked with the $\beta\gamma$ subunit of G-proteins in depolarized synaptoneurosomes (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 $G_a$-proteins and the $\alpha$ subunit of VGSC is possible once VGSC have been activated. Regarding the GTPase activity of $G_a$-proteins, these findings also suggest that $G_a$-proteins may repeatedly interact with VGSC $\alpha$ subunits as long as membrane depolarization lasts.

Effect of Membrane Depolarization on Phosphorylation of $G_a$-proteins—The isoelectric pH of $G_a$-proteins photolabeled with $[\alpha^{32}\text{P}]$GTPAA was lower by approximately 0.5 pH units in depolarized synaptoneurosomes than in synaptoneurosomes at
Depolarization-induced Activation of Go-proteins

**FIG. 6. Effect of membrane depolarization on the ADP-ribosylation of Go- and Go-proteins.** A, synaptoneurosomes were pretreated in the presence of atropine (1 μM) with PTX (200 ng/ml, 37 °C, 2 h, 95% O2/5%CO2) (lanes 1 and 2) during depolarization (lane 1) and at resting potential (lane 2). PTX-catalyzed [32P]ADP-ribosylation of Go-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). [32P]ADP-ribosylated Go-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 Go-proteins is presented in the lower frame. Go-proteins were immunodetected in the immunoprecipitates by antibody AS 248. The extent of [32P]ADP-labeling of Go-proteins was quantified by densitometry (n > 5). B, synaptoneurosomes were pretreated in the presence of propranolol (1 μM) with CTX (10 μg/ml, 37 °C, 2 h, 95% O2/5%CO2) during depolarization (lane 1) or at resting potential (lane 2). [32P]ADP-ribosylation of Go-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). [32P]ADP-ribosylation of Go-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). Go-proteins were immunolabeled by antibodies AS 348. Autoradiograms of the [32P]ADP-ribosylated proteins are presented in the upper frame. Immunolabeling of [32P]ADP-labeled Go-proteins is presented in the lower frame. The extent of [32P]ADP labeling was quantified by densitometry (O.D., absorbance) (n = 3).

The experiments described in this study provide evidence for a depolarization-induced activation of Go-protein and its isoform Go-protein in membranes of rat brain-stem synaptoneurosomes. The voltage-induced effect was clearly demonstrated by comparing their in situ photoaffinity labeling with [α32P]GTPAA in membranes of depolarized synaptoneurosomes with that in synaptoneurosomes at resting potential (Figs. 1, 2, and 4). The increased photoaffinity labeling of Go-and Go-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 Go-proteins (Fig. 6). The depolarization-induced exchange of GDP for [α32P]GTPAA in Go-proteins was reversed by repolarization and was independent of transmitters release and stimulation of muscarinic, NMDA-glutamate receptors, dopaminergic, or serotonergic 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 Go-and Go-proteins in depolarized membranes (Fig. 2) could also be associated with Go-protein phosphorylation. This seems unlikely, however, in view of the negligible effect of membrane depolarization on their labeled in situ phosphorylation under

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DISCUSSION

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 Go-proteins. This possibility was examined by in situ labeled phosphorylation of membrane proteins in depolarized synaptoneurosomes.

Synaptoneurosomes were incubated for 30 min with [32P]phosphorus (10 μCi) and then resuspended in either 4.7 or 50 mM [K+] in Ca2+-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 Go-proteins was 32P-phosphorylated (Fig. 9). Although high [K+] induced membrane depolarization increased their photoaffinity labeling with [α32P]GTPAA by approximately 5–7-fold (Figs. 1 and 2), it did not significantly alter their 32P-phosphorylation (Fig. 9), suggesting that the effect of membrane depolarization is not attributable to phosphorylation of these proteins.

**Fig. 6. Effect of membrane depolarization on the ADP-ribosylation of Go- and Go-proteins.** A, synaptoneurosomes were pretreated in the presence of atropine (1 μM) with PTX (200 ng/ml, 37 °C, 2 h, 95% O2/5%CO2) (lanes 1 and 2) during depolarization (lane 1) and at resting potential (lane 2). PTX-catalyzed [32P]ADP-ribosylation of Go-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). [32P]ADP-ribosylated Go-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 Go-proteins is presented in the lower frame. Go-proteins were immunodetected in the immunoprecipitates by antibody AS 248. The extent of [32P]ADP-labeling of Go-proteins was quantified by densitometry (n > 5). B, synaptoneurosomes were pretreated in the presence of propranolol (1 μM) with CTX (10 μg/ml, 37 °C, 2 h, 95% O2/5%CO2) during depolarization (lane 1) or at resting potential (lane 2). [32P]ADP-ribosylation of Go-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). [32P]ADP-ribosylation of Go-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). Go-proteins were immunolabeled by antibodies AS 348. Autoradiograms of the [32P]ADP-ribosylated proteins are presented in the upper frame. Immunolabeling of [32P]ADP-labeled Go-proteins is presented in the lower frame. The extent of [32P]ADP labeling was quantified by densitometry (O.D., absorbance) (n = 3).
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 $G\alpha_o$-proteins to phospholipids (53–59). Recent reports have indicated that modifications in the binding of $G\alpha_o$-proteins to myristate and palmitate in the phospholipid matrix affect their interaction with the $G\beta\gamma$ subunit, (53, 57, 60) as well as with $G\alpha_o$-protein-coupled receptors (61). Changes in palmitylation and myristoylation of $G\alpha_o$-proteins apparently affect the stability of GDP binding (55, 57, 61, 62) and thereby may affect $G\alpha_o$-protein activation (62). Depolarization-induced modifications of the anchorage of $G\alpha_o$-protein subunits in the phospholipid matrix might occur via modification of labile thioester bonds between cysteine residues in $G\alpha_o$-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 $G\alpha_o$-protein subunits of VGSC (12, 24, 25), we examined the possibility that depolarization-induced activation of VGSC is involved in depolarization-induced activation of $G\alpha_o$-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 $G\alpha_o$-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-
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Recent findings indicated a voltage-dependent modulation of the affinity of presynaptic muscarinic receptors (M2) providing an autoregulation of transmitter release (71). Also, modulation of the oscillatory properties of entorhinal cortex layer II neurons has been attributed to a reciprocal effect of post-synaptic induced exchange of GDP for [\textsuperscript{32}P]GTPAA in G\textsubscript{o}-proteins (Figs. 7, A and B) and the depolarization-induced uncoupling of G-proteins from muscarinic receptors (Fig. 7C). This supports the assumption that depolarization-induced activation of VGSC may be involved in depolarization-induced activation of G\textsubscript{o}-proteins. This assumption was further supported by the efficient cross-linking of VGSC-\alpha subunit with G\textsubscript{o}-proteins during depolarization (Figs. 8, A and C). In addition, GDP-bound G\textsubscript{o}-proteins most efficiently cross-linked with the \alpha subunit of VGSC in isolated synaptoneurosomal membranes (Fig. 8C). Regarding the GTPase activity of G\textsubscript{o}-proteins (7), these findings might suggest repeated interactions between \alpha subunit of VGSC and G\textsubscript{o}-proteins, as long as depolarization lasts (Fig. 8). Taken together, these findings support a possible depolarization-induced interaction between these proteins, which might be involved in depolarization-induced activation of G\textsubscript{o}-proteins.

Voltage-dependent activation of G\textsubscript{o}-proteins would imply a voltage-induced modulation of signal transduction cascades triggered by stimulation of G\textsubscript{o}-coupled-receptors. An example of reciprocal influence of membrane potential and receptor activity has been observed in muscarinic receptors (10, 12).

Voltage-dependent activation of G\textsubscript{o}-proteins would imply a voltage-induced modulation of signal transduction cascades triggered by stimulation of G\textsubscript{o}-coupled-receptors. An example of reciprocal influence of membrane potential and receptor activity has been observed in muscarinic receptors (10, 12).
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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 Gα-proteins may affect the activity of other G-proteins, as well (10, 24), thereby producing additional versatility in synaptic transmissions in the nervous system.

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