Evidence for Involvement of the Voltage-dependent Na\textsuperscript{+} Channel Gating in Depolarization-induced Activation of G-proteins

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Evidence for activation of pertussis-toxin-sensitive G-proteins by membrane depolarization in rat brain-stem synaptoneurosomes was recently reported (Cohen-Armon, M., and Sokolovsky, M. (1991) J. Biol. Chem. 266, 2595–2605; (1991) Neurosci. Lett. 126, 87–90) and is further supported in this study by the observation that the depolarization-induced effect is inhibited when G-proteins are stabilized in the non-activated state with guanosine 5'-O-(2-thiodiphosphate) (GDP\textsubscript{SS}), which was introduced into synaptoneurosomes during the process of permeabilization and resealing. In the present study, agents that either keep the voltage-dependent Na\textsuperscript{+} channel in persistently activated state (while Na\textsuperscript{+} currents are blocked) or prevent it from activation were used in an attempt to determine whether the voltage-dependent Na\textsuperscript{+} channels are involved in the depolarization-induced activation of pertussis-toxin-sensitive G-proteins. The main probe employed was the cardiotoxic and antiarrhythmic agent DPl, which is a racemic mixture of two enantiomers, one of which (the R enantiomer) reportedly prevents depolarization-induced activation of the Na\textsuperscript{+} channel while the other (the S enantiomer) inhibits Na\textsuperscript{+} channel inactivation. The results suggest that while inactivation of the voltage-dependent Na\textsuperscript{+} channel does not interfere with the putative depolarization-induced activation of G-proteins, membrane depolarization affects G-proteins and the coupled muscarinic receptors only if the voltage-dependent Na\textsuperscript{+} channels are capable of being activated. Thus, inhibition of the depolarization-induced activation of Na\textsuperscript{+} channels was accompanied by inhibition of the depolarization-induced activation of pertussis-toxin-sensitive G-proteins and by modifications of both the coupling of G-proteins to muscarinic receptors and the ADP-ribosylation of G\textsubscript{a}-proteins. These effects could be counteracted by persistent activation of the voltage-dependent Na\textsuperscript{+} channels (while Na\textsuperscript{+} current was blocked). Our observations may suggest that the voltage-dependent Na\textsuperscript{+} channel gating is involved in the depolarization-induced activation of pertussis-toxin-sensitive G-proteins and may provide evidence for a possible mechanism of membrane depolarization signal transduction in excitable cells.

We have recently reported evidence for a depolarization-induced activation of PTX\textsuperscript{1}-sensitive G-proteins (hereafter termed G-proteins) in brain-stem synaptoneurosomes. These observations revealed the same features as those observed in activation of G-proteins by neurotransmitters and hormones (1–4). They included, first, a depolarization-induced enhancement of the specific binding of [\textsuperscript{3}H]GTP/[\textsuperscript{3}H]GDP to membranes of permeabilized-resealed synaptoneurosomes (5), attributed to the exchange of GDP bound to inactivated G-proteins for labeled GTP (6–9). The enhancement in GTP binding could be prevented by PTX-catalyzed ADP-ribosylation of G-proteins and reversed by repolarization of the depolarized synaptoneurosomes (5). Second, we observed a depolarization-induced conversion of the muscarinic receptor from a high affinity to a low affinity state for agonist binding (5, 10). This effect was attributed to a depolarization-induced activation of G-proteins (5, 10), since it could be imitated by persistent activation of the G-proteins coupled to the muscarinic receptor (11, 12) and prevented by distortion of the coupling between receptor and G-proteins following PTX-catalyzed ADP-ribosylation of G-proteins (5). Third, we observed a depolarization-induced inhibition of PTX-catalyzed ADP-ribosylation of the \textalpha-subunit of G\textsubscript{a}-proteins (13). Since ADP-ribosylation of PTX-sensitive G-proteins can be inhibited by dissociation of the \textalpha- from the \textbeta\textsubscript{2}-subunits (14, 15), an event which occurs on G-protein activation (1–4), the observed inhibition of ADP-ribosylation on membrane depolarization may also indicate a depolarization-induced activation of these G-proteins (13). As previously reported, all of these depolarization-induced effects on G-proteins could be reversed by membrane repolarization, were independent of the release of transmitters (5, 13), and were not dependent on the response of serotoninergic, \textalpha- and \textbeta-adrenergic, or NMDA receptors (5).

Additional evidence in support of a depolarization-induced activation of PTX-sensitive G-proteins comes from the observation in this study that GDP\textsubscript{SS}, when introduced into synaptoneurosomes (5) in order to prevent G-protein activation (3, 6–9), prevents the depolarization-induced effect on the muscarinic high affinity agonist binding and on PTX-catalyzed ADP-ribosylation of G-proteins.

In the present work we investigated the possible involvement of the voltage-dependent Na\textsuperscript{+} channel (hereafter referred to as the Na\textsuperscript{+} channel) in the observed depolarization-induced activation of G-proteins. This was done by the use of

\textsuperscript{1}The abbreviations used are: PTX, pertussis toxin; AcCh, acetylcholine; NMB, N-methyl-4-piperidyl benzilate; BTX, batrachotoxin; GDP\textsubscript{SS}, guanosine 5'-O-(2-thiodiphosphate); Gpp\textsubscript{NHp}, 5'-guanylylimidodiphosphate; TTX, tetrodotoxin; TPP\textsuperscript{2+}, tetraphenylphosphonium ion; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; DPI, 4-[3-(4-diphenylmethyl-1-piperazinyl)-2-hydroxypropoxy]-1H-indole-2-carbonitrile.

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agents known to affect the Na⁺ channel gating, including
the inhibitor of Na⁺ channel inactivation, batrachotoxin (BTX),
(16-18), the local anesthetic tetrodotoxin (TXX) and others, is
and the cardio- and antihypertensive drug 4-[3-(4-diphen-
yl methyl-1-1-piperazinyl-2-hydroxy-propoxy]-1H-indole-2-
carbonitrile (DPI-201-106) (Sandoz), a racemic mixture of
the enantiomers DPI-205-429 (R enantiomer) and DPI-205-
430 (S enantiomer) (20). Determination of the influence of
DPI on the depolarization-induced effect on G-proteins was
of great interest, since the R enantiomer prevents the depo-
larization-induced activation of the Na⁺ channel, while the S
enantiomer, by inhibiting Na⁺ channel inactivation, prolongs
its activated state (20, 21). The enantiomers compete in an
allostERIC manner with one another and with labeled BTX for
binding to the Na⁺ channel (20, 21). The effects of the two
enantiomers of DPI on the depolarization-induced activation
of G-proteins were examined by their effect on the high affinity
binding of [³H]AcCh to the muscarinic receptor and the
specific binding of [³H]GTP/[³H]GDP to membranes of permeabilized-reseated synaptoneurosomes, on the basis of
our previous results (5, 13).
Our results indicate that inactivation of Na⁺ channels, induced by prolonged membrane depolarization (22), does not
interfere with the putative depolarization-induced activation of
G-proteins. On the other hand, prevention of the depolariza-
tion-induced activation of the Na⁺ channels (i.e. the channel
remains in the closed state (22)) prevents the depolariza-
tion-induced activation of PTX-sensitive G-proteins, modi-
fies the coupling of G-proteins to the muscarinic receptors,
and inhibits PTX-catalyzed ADP-ribosylation of the α-sub-
unit of G-proteins. These events can be counteracted by
agents that induce persistent activation of the Na⁺ channel
(with Na⁺ current blocked by TTXX) but do not themselves induce G-protein activation.
It should be noted that according to current findings, the
inactivated state of the Na⁺ channel is considered not to be
identical with the "closed state" of the Na⁺ channel although
in both states Na⁺ current is zero (17, 22 and Refs. therein).

The findings of the present study support involvement of
the voltage-dependent Na⁺ channel gating in the depolariza-
tion-induced activation of PTX-sensitive G-proteins. The
results of this study are also in line with previous indications
(5, 10) that unmodified ADP-ribosylation sites must be pres-
ent on the α-subunit of PTX-sensitive G-proteins in order
for the effect of membrane depolarization on G-proteins to
be induced. The apparent involvement of both Na⁺ channel
gating and ADP-ribosylation sites on PTX-sensitive G-pro-
teins in the putative depolarization-induced activation of
G-proteins may provide evidence for a possible mechanism of
membrane depolarization signal transduction in excitable
cells.

MATERIALS AND METHODS

Reagents—DPI-201-106 and its two enantiomers, DPI-205-429
(R) and DPI-205-430 (S), were kindly supplied by Dr. E. Russi and
Dr. D. Romer of Sandoz Ltd., Pharmaceutical Division, Preclinical
Research (Basel, Switzerland). Purity was >98.5% for the R enan-
tomer and >98.0% for the S enantiomer. Prior to each experiment,
solutions of the racemic mixture and the two enantiomers were
prepared in ethanol/water (1:1 v/v) solution. The final concentration
of ethanol in the incubation buffers was <0.1%. [³H]AcCh (96 Ci/
mol, 98% pure) was purchased from Amersham (Buckinghamshire,
United Kingdom). Small aliquots of the radioactive in ethanol/water
(1:1 v/v) were kept at -70 °C and dried by a gentle stream of
nitrogen prior to use. [³H]NMNB (70 Ci/mmol, 97% pure) was
prepared by catalytic tritium exchange as described elsewhere
(23). BTX, kindly supplied by Dr. J. W. Daly (Laboratory of Bioorganic
Chemistry, National Institutes of Health, Bethesda, MD) was
solved in ethanol. The storage period did not exceed 3 weeks at
-20 °C. Tetrodotoxin (TXX), dithiotreitol (DTT), dissorsipropho-
rophosphate, atropine, 5'-guanylyl imido-diphosphate (GGppNHP),
guanosine 5'-O-thiodiphosphate (GDP[S]), ATP (grade I), NAD
(grade I), sperine, propanolol, yohimbine, and tetracaine were all
purchased from Sigma. PTX and the A-protomer of PTX (ADP-
ribosyltransferase) were synthesized from Biological Laboratories
(Cambridge, MA). [Adenylate-³H]N6-phenylisopropyladenosine, di(diethyl-ammonium) salt ([³H]NAD) (1000 Ci/mmol), [phenyl-³H]
tetraphenyl phosphonium bromide ([³H]TPP) (35 Ci/mmol), and
[8,5-³H]guanosine 5'-triphosphate, tetrasodium salt ([³H]GTP) (33.6
Ci/mmol) were purchased from Du Pont. New England Nuclear.
-γ-ADP-ribophosphonic acid was from Cambridge Research Bio-
chemicals (U.K.). Sucicid acid and EDTA were from Merck
(Darmstadt, Germany). Dowex 2 X 10, 50-100 mesh (Cl-form) was from
Fluka AG (Chemische Fabrik, Buchs, Germany). Antibodies against
the N terminus of G-proteins (GC/B) were purchased from Du Pont.

Brain Tissue Preparation—Adult male rats of the CD strain were
obtained from Levenstein's Farm, Yokneam, Israel, and maintained
as described previously (23). From pooled brain-stem regions obtained
from 3- to 4-month-old rats (10), synaptoneurosomes were prepared
according to Hollingsworth et al. (24) in Krebs-Henseleit buffer containing
118.5 mM NaCl, 4.7 mM KCl, 1.18 mM MgCl₂, 2.5 mM
CaCl₂, 1.25 mM NaH₂PO₄, 10 mM glucose, and 1.18 mM KCl in an
atmosphere of 95% O₂, 5% CO₂ at 25 °C. The synaptoneurosomes were
then resuspended in the same buffer in the absence of CaCl₂.
Under the experimental conditions, synaptoneurosomes retained
their membrane potential for up to 4 h (5).

Depolarization Experiments—Synaptoneurosomes were depolar-
ized by exposure to high concentrations of the selectively permeable
cation, K⁺, in the Ca²⁺-free Krebs-Henseleit buffer solution through
the presence of Na⁺ channel blocker of the specific binding of [³H]AcCh in the
muscarinic receptor in the synaptoneurosomal membrane preparation
was measured according to the method of Gurwitz et al. (25), with a number of
modifications (5). Ca²⁺-free Krebs-Henseleit buffer in which CaCl₂
was replaced by NaCl ([Ca²⁺] <20 μM) was used (5, 26). Following
incubation for 20 min with the cholinesterase inhibitor diisopropyl-
fluorophosphate (300 μM), synaptoneurosomes (20 μl aliquots) were
added to tubes containing 20 μl of buffer and the indicated concen-
tration of [³H]AcCh. After 1 h of incubation with gentle shaking at
25 °C, the reaction was terminated by the addition of 5-6 ml of ice-
cold buffer and filtration under high pressure through GF/F filters
(Whatman, 25-mm diameter). The filters were immediately washed
with 6 ml of buffer and then counted for tritium using a scintillation
mixture (Hydroluma) and a scintillation spectrometer (LKB 1218)
at 48% efficiency. Nonspecific binding was determined in the presence
of 1 μM atropine. All determinations were carried out in triplicate and
were found to vary by no more than 15%.

The measured binding of [³H]AcCh to the muscarinic receptor in
this preparation probably does not include [³H]AcCh uptake or inter-
nalization, since it was found to be similar in synaptoneurosomal
preparations and in membranes prepared from synaptoneurosomes
either by homogenization or by lysis in hypotonic buffered solution
containing 5-50 mM Tris, 70 mg MgCl₂, 1.5 mM MgATP, and 2 mM
EGTA. Under the experimental conditions employed, binding of [³H]AcCh
to the nicotinic receptors did not occur, as indicated by measurement
of the specific binding of [³H]AcCh in the presence and absence of
the specific nicotinic blocker di-tybocurarine (27). Since di-tybocu-
arinie did not exert any effect on either the specific or the nonspecific
binding of [³H]AcCh in the presence of 1 μM atropine (25, 28), it was concluded that [³H]AcCh binding measured under these
experimental conditions represented the specific binding of [³H]AcCh
to the muscarinic receptor only.

[³H]NMNB Binding—Binding of the muscarinic antagonist [³H]
NMNB to the muscarinic receptor in synaptoneurosomes was mea-
sured as previously described (28). Nonspecific binding was determined
in the presence of 1 μM atropine (25).

Depolarization Experiments—Synaptoneurosomes were depolar-
ized by exposure to high concentrations of the selectively permeable
cation, K⁺, in the Ca²⁺-free Krebs-Henseleit buffer solution through
an exchange of Na⁺ for K⁺. In most experiments membrane depolarization
was achieved by an increase in [K⁺] from 4.7 to 50 mM. Washings and filtrations were carried out in cold high [K⁺] buffers. The
method is described in detail elsewhere (5, 10). Under the experimental
conditions employed ([Ca²⁺] <20 μM, [Mg²⁺] =1.8 mM), membrane depolarization did not induce measurable presynaptic
release of ACh into the incubation buffer (26), as also reported
previously (5, 10).

In our previous reports (5, 10, 13), we described experiments
designed to test the possible effect of batrachotoxin (BTX), which
blocks Na⁺ channels (16-18), on the 
[³H]AcCh binding, [³H]GTP binding, and PTX-catalyzed ADP-ribo-
sylation. These experiments included parallel measurements of
the
above three parameters, in synaptoneurosomes and in membrane preparations, in the presence of various concentrations of K' and Na'. The effects of high K' observed in the synaptoneurosomes were not observed in membrane preparations, suggesting that the effects observed in synaptoneurosomes are attributable to high K'-induced membrane depolarization rather than to a direct effect of high K' on the binding of [3H]AcCh or [3H]GTP/PH/GDP or on ADP-riboylation of G-proteins (5, 13).

A possible effect of inward Na' current induced by the high K'-induced depolarization was ruled out, since voltage-dependent Na' channels are inactivated during the prolonged membrane depolarization (22) induced by exposure to high K', and since the potential Na' current blocker TTX (22) did not interfere with the depolarization-induced effects on G-proteins and muscarinic receptors (5, 10).

Estimation of Membrane Potential by [3H]TPP+ Accumulation—

[3H]TPP permeates freely across cell membranes, and thus its distribution at equilibrium in various systems is dependent on the membrane potential (29, 30). Changes in membrane potential were confirmed by monitoring of the intracellular accumulation of [3H]TPP+ according to the method of Cheng et al. (31). Synaptoneurosomes (approximately 4 mg of protein/ml) were incubated with approximately 7 x 106 M [3H]TPP+ (40 µl) at 25°C for 20 min. The method is described elsewhere (5).

Treatment of Synaptoneurosomes with BTX and TTX—Synaptoneurosomes were preincubated in Ca2+-free Krebs-Henseleit buffer with BTX (1 µM) for 40 min at 37°C. In order to exclude Na' currents, TTX (1 µM) was added together with BTX (10, 32, 33). The samples were centrifuged by centrifugation at 3,000 x g, 10 min) and two washes with the same Ca2+-free buffer. Following this pretreatment, no changes in membrane potential (as measured by [3H]TPP+ accumulation) or uptake of 22Na into the pretreated synaptoneurosomes were observed (5, 10), i.e., inward Na' current was blocked and hence membrane depolarization was not induced under these experimental conditions (22).

Binding of [3H]GDP or [3H]GDP/S to Permeabilized-resealed Synaptoneurosomes—Synaptoneurosomes were permeabilized in the presence of ATP as described in detail previously (5). The procedure included incubation of the synaptoneurosomes with ATP (6 mM) in isotonic Krebs-Henseleit buffer containing 100 mM NaCl, 4.7 mM KCl, 1.18 mM KH2PO4, 24.9 mM NaHCO3, 0.5 mM succinate, pH adjusted to 8.3-8.4 (permeabilization buffer). Permeabilization was carried out in the presence of 0.2 µM [3H]GTP (5). After precisely 40 min at 25°C, the synaptoneurosomes were ressealed by two successive cycles of incubation (10 min) with Krebs-Henseleit buffer, and centrifugation (1000 x g, 5 min) (5). They were then exposed for 1-2 min to Krebs-Henseleit buffer containing either 4.7 or 50 mM [K']. In order to minimize binding of the labeled guanine nucleotide to the external surface of the synaptoneurosomes, samples (100-µl aliquots, approximately 400 µg of protein) were loaded onto an anion exchanger (5). After 5 min (200 µl), the synaptoneurosomes were centrifuged (3,000 x g, 10 min), and the sample was washed with the same Ca2+-free buffer. Following this pretreatment, no changes in membrane potential (as measured by [3H]TPP+ accumulation) or uptake of 22Na into the pretreated synaptoneurosomes were observed (5, 10), i.e., inward Na' current was blocked and hence membrane depolarization was not induced under these experimental conditions (22).

Binding of [3H]GTP to Membrane Preparations—Membranes were prepared from synaptoneurosomes by homogenization in hypotonic buffer (50 mM Tris-HCl, pH 7.4), with the use of a motor-driven Teflon pestle (950 revolutions/minute) in a glass homogenizer. The membrane preparations were incubated with [3H]GTP, at concentrations ranging from 0.1 to 2.2 µM, in Krebs-Henseleit buffer containing <20 µM Ca2+, at 25°C for 20 min (equilibrium was reached after 10 min). Each sample (100 µl) contained approximately 400 µg of protein. Incubations were terminated by the addition of cold buffer, the samples were filtered through GF/C filters and counted for tritium in a scintillation spectrometer. Nonspecific binding was measured in the presence of 300 µM Gpp(NH)p. The method was described in detail previously (5).

PTX Treatment of Synaptoneurosomes—Synaptoneurosomes in Ca2+-free Krebs-Henseleit buffer were treated with 200 ng/ml PTX (2 h, 37°C, 95% O2, 5% CO2). The PTX-treated synaptoneurosomes were then washed in the same buffer and subjected to various experiments, as described below. Similar treatment with PTX was applied to permeabilized-resealed synaptoneurosomes that were either loaded with GDP/PS (200 µM) in the permeabilization buffer or unloaded. [3H]ADP-ribosylation of Membrane Preparations—Synaptoneurosomes, either treated or untreated with PTX in Ca2+-free Krebs-Henseleit buffer, were washed and homogenized in buffer containing 10 mM Tris-HCl, 2 mM MgCl2, 0.1 mM EDTA, and 2 mM DTT, pH 7.4. The preparation was centrifuged (1,000 x g, 10 min), and the membranes were then pelleted from the supernatant (30,000 x g, 20 min) and suspended in 50 µl of the same buffer. Samples containing 200 µg of protein (determined by the Lowry method (34)) were incubated (1 h, 37°C) with 10 µg of activated PTX (5, 10) or 2 µg of the A-protomer of PTX (35), in buffer containing 2 mM MgCl2, 3 mM ATP, 75 mM Tris-HCl, 20 mM DTT, and 1 µCi of [3H]NAD. The final volume was 110 µl. Membranes were pelleted, resuspended in sample buffer (36), boiled for 2 min, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5% or 10% polyacrylamide). The dried gels were autoradiographed by exposure to Kodak X-Omat x-ray film with an enhancing screen (usually for 24 h, -70°C). Densitometry was performed with a laser densitometer (LKSB Bromma Ultrascan II).

Immunoblotting—Gels were electroblotted onto nitrocellulose paper overnight at 10°C and a constant current of 150 mA, as described by Towbin et al. (37). Dried nitrocellulose strips were immersed first in 3% gelatin and then in anti-a, GC/2 (38) (1:500 dilution) in 5% bovine serum albumin, as described elsewhere (39). The bands formed were visualized by a peroxidase-conjugated secondary antibody (37).

Results

Effects of Membrane Depolarization on the High Affinity Binding of [3H]AcCh to Permeabilized-resealed Synaptoneurosomes Loaded with GDP/PS—In order to assess the effect of membrane depolarization on G-protein activation (5, 13), we examined the depolarization-induced effect on the conversion of agonist-binding sites in the muscarinic receptor from high to low affinity (5) in the presence of GDP/PS, which keeps G-proteins in an unactivated state (3, 6-9). Because the cell membrane is not permeable to GDP/PS (40), the latter was introduced into synaptoneurosomes which were permeabilized and ressealed for the purpose, as described in "Materials and Methods." Effective permeabilization and ressealing was confirmed by [3H]TPP+ accumulation measurements, as described previously (5). Specific binding of [3H]AcCh to the loaded membranes, either unloaded or loaded with GDP/PS, was measured in buffers containing 4.7 or 50 mM [K'], i.e., at resting or depolarization membrane potential, respectively. Changes in membrane potential were confirmed by measurement of [3H]TPP+ accumulation (see "Materials and Methods"). The effect of membrane depolarization on the high affinity binding of [3H]AcCh to muscarinic receptors in unloaded permeabilized-resealed synaptoneurosomes is presented in Fig. 1A. Fig. 1B shows the corresponding effect in synaptoneurosomes loaded with GDP/PS. As demonstrated, membrane depolarization reduced the high affinity binding of [3H]AcCh in the unloaded synaptoneurosomes (Fig. 1A). This effect could be reversed by re-exposure of the depolarized synaptoneurosomes to 4.7 mM [K'] buffer (not shown). Similar results were previously observed in intact synaptoneurosomes (5, 10) and were attributed to a depolarization-induced conversion of high affinity muscarinic agonist-binding sites to a low affinity state, presumably reflecting activation of G-proteins coupled to the muscarinic receptors (5, 7, 12). In synaptoneurosomes loaded with GDP/PS, the high to low affinity conversion did not occur (Fig. 1B), thus further supporting the involvement of G-protein activation in the observed depolarization-induced effect. As reported previously, under these experimental conditions no measurable release of ACh was detected (5, 10).
Depolarization, Voltage-dependent Na⁺ Channels, G-proteins

FIG. 1. Effect of GDPβS, loaded into permeabilized-released synaptoneurosomes, on depolarization-induced changes in the high affinity binding of [³H]AcCh to the muscarinic receptor. Permeabilization was performed in the absence (A) and in the presence (B) of GDPβS (200 μM) (details under "Materials and Methods"). Specific binding of [³H]AcCh to the permeabilized-resealed synaptoneurosomes was measured in buffers containing 4.7 mM [K⁺] (resting potential) (●) and 50 mM [K⁺] (depolarization) (○). Nonspecific binding of [³H]AcCh was measured in the presence of 1 μM atropine. The data are from a typical experiment, one of three performed.

possibility that the effect of GDPβS might reflect interference with the depolarization-induced release of transmitters related to the NMDA, serotoninergic, or α- and β-adrenergic receptors was excluded by measurement of [³H]AcCh binding in both 4.7 and 50 mM [K⁺] buffers in the presence of specific antagonists to these receptors (i.e. D-2-amino-5-phosphovaleric acid (10 μM), spiperone (1 μM), yohimbine (1 μM), and propranolol (1 μM), respectively).

Effect of Membrane Depolarization on PTX-catalyzed ADP-ribosylation of Permeabilized-resealed Synaptoneurosomes Loaded with GDPβS—The effect of membrane depolarization on PTX-catalyzed ADP-ribosylation in brain-stem synaptoneurosomes was previously reported (13). In that study, membranes prepared from synaptoneurosomes that were ADP-ribosylated in high [K⁺] buffer underwent intense [³²P]ADP-ribosylation upon subsequent exposure to PTX-catalyzed ADP-ribosylation in the presence of [³²P]NAD, suggesting that membrane depolarization had inhibited the initial ADP-ribosylation possibly as a result of a depolarization-induced activation of PTX-sensitive G-proteins (14, 15). This possibility was further supported by examination of the depolarization-induced effect on ADP-ribosylation in the presence of GDPβS (3, 6–9). Brain-stem synaptoneurosomes were permeabilized (5), loaded with GDPβS (200 μM in the permeabilization buffer), and resealed, as described under "Materials and Methods." GDPβS-loaded and unloaded permeabilized-released synaptoneurosomes were ADP-ribosylated by PTX (200 ng/ml, 37 °C, 2 h, 95% O₂, 5% CO₂). Membranes prepared from loaded or unloaded synaptoneurosomes were then subjected to an additional ADP-ribosylation, performed in the presence of PTX A-protomer and [³²P]NAD (see "Materials and Methods").

Autoradiograms of the ADP-ribosylated membranes, analyzed by SDS-PAGE (10% acrylamide), are presented in Fig. 2. Lanes 1 and 2 show [³²P]ADP-ribosylation of membranes prepared from GDPβS-loaded synaptoneurosomes that were treated with PTX in 4.7 mM [K⁺] and 100 mM [K⁺] (depolarization) buffers. As shown, [³²P]ADP-ribosylation of 39-kDa G-protein (5, 13) in membranes prepared from synaptoneurosomes exposed to either of the buffers was less efficient than that in membranes prepared from control (PTX-unloaded) synaptoneurosomes in 4.7 mM [K⁺] buffer (lane 3) or in 100 mM [K⁺] buffer (lane 4), indicating that 39-kDa G-proteins in loaded synaptoneurosomes had undergone ADP-ribosylation both at resting potential and during depolarization. Lanes 5–8 present the corresponding bands for membranes prepared from unloaded permeabilized-resealed synaptoneurosomes. Lanes 5 and 6 show the 39-kDa G-proteins labeled by [³²P]ADP-ribosylation of membranes prepared from ADP-ribosylated synaptoneurosomes at rest and depolarization. As shown, [³²P]ADP-ribosylation of these proteins in membranes prepared from synaptoneurosomes that were initially treated with PTX in 4.7 mM [K⁺] buffer (lane 5) was less efficient than that in membranes prepared from control synaptoneurosomes incubated in the same buffer in the absence of PTX (lane 7), indicating that the 39-kDa proteins in synaptoneurosomes treated with PTX at resting potential had undergone ADP-ribosylation. However, in membranes prepared from synaptoneurosomes treated with PTX in 100 mM [K⁺] buffer (lane 6), [³²P]ADP-ribosylation of 39-kDa G-proteins was as efficient as in control membranes (lane 8), indicating that the ADP-ribosylation of 39-kDa G-proteins in the PTX-treated synaptoneurosomes had been inhibited by
The effect of the DPI S enantiomer (5 μM), in the presence of TTX (1 μM), and the effect of TTX (1 μM) on the depolarization-induced decrease in binding of [3H]AcCh to brain-stem synaptoneurosomes. As shown, the S enantiomer, which inhibits inactivation of the Na+ channel or prolongs its activated state (20, 21), did not affect the depolarization-induced conversion of binding sites in the muscarinic receptor from high to low affinity (Fig. 3A). Since Na+ current was blocked by TTX (32, 33), induction of membrane depolarization by inward Na+ currents was prevented (22). TTX (1 μM) did not affect the depolarization-induced high to low affinity conversion, as also observed previously (10). However, in the presence of the R enantiomer (5 μM), which prevents activation of the Na+ channel (20, 21), the depolarization-induced high to low affinity conversion was completely prevented (Fig. 3B), and treatment with the racemic mixture of the drug (5 μM) partially inhibited the depolarization-induced affinity conversion (Fig. 3C).

Since the DPI agents did not affect the total binding to the muscarinic receptors, measured by the binding of the antagonist [3H]NMPB (not shown), these results appear to provide evidence for the possible involvement of Na+ channel activation in the depolarization-induced conversion of muscarinic high affinity sites into the low affinity state.

Effects of DPI on Binding of [3H]AcCh to the Muscarinic Receptor in Membrane Preparations—In order to distinguish the effects of DPI enantiomers on the binding of [3H]AcCh to the muscarinic receptor from their effects on the depolarization-induced changes in [3H]AcCh binding, binding of [3H]AcCh in the presence of the enantiomers was measured in membrane preparations. Treatment with the R and with the S enantiomer of DPI had similar effects on the density of high affinity binding sites of [3H]AcCh in membranes prepared from rat brain-stem (Fig. 4). Binding of [3H]AcCh to brain-stem membranes treated with the S and R enantiomers of DPI is presented in Fig. 4, A and C, respectively. The corresponding Scatchard plots are presented in the insets. The calculated changes in [3H]AcCh-binding site density (Bmax) and in the apparent dissociation constant (Kd) induced by the S and R enantiomer are presented in Fig. 4, B and D, respectively (n = 10). As shown, in the presence of either the S or the R enantiomer up to a concentration of 10 μM, the decrease in density of high affinity binding sites (B*max/Bmax) was negligible. Binding affinity, however, increased in the presence of the S enantiomer (Fig. 4A, inset, and B). An effect similar to that was observed after treatment with BTX (28) (which also inhibits inactivation of the Na+ channel (16–18)). No significant change in [3H]AcCh binding affinity was observed in the presence of the R enantiomer (Fig. 4B, inset, and D).

In our subsequent experiments aimed at determining the effects of DPI on the depolarization-induced changes in [3H]AcCh binding, we used concentrations at which the binding density of [3H]AcCh to the muscarinic receptor in membrane preparations is not affected. These concentrations are similar to those used to induce prolonged activation (S enantiomer) or to prevent activation of the Na+ channel (R enantiomer) in electrophysiological studies (20, 21).

Effect of the R enantiomer of DPI on the Depolarization-induced Affinity Changes in the Muscarinic Receptor after Pretreatment with BTX—the enantiomers of DPI reportedly compete with BTX for binding to the Na+ channel (20, 21). We therefore examined the effect of the R enantiomer on the depolarization-induced changes in the muscarinic receptor when the Na+ channels were in a persistently open state, achieved in the presence of BTX (16–18).
Fig. 3. Effects of DPI enantiomers and racemic mixture on binding of [³H]AcCh to high affinity sites in the muscarinic receptor in rat brain-stem synaptoneurosomes at resting potential (in 4.7 mM [K⁺] buffer, ○, △) and upon depolarization (in 50 mM [K⁺] buffer, ●, ▴). Binding curves are shown for control preparations (circles) and for preparations treated with the S enantiomer in the presence of 1 μM TTX (A), the R enantiomer (B), and the racemic mixture (C), all at 5 μM (triangles). Also shown is the effect of membrane depolarization, induced by high [K⁺] in the presence of the Na⁺ channel blocker TTX (1 μM), on [³H]AcCh binding (D). Nonspecific binding of [³H]AcCh was measured in the presence of 1 μM atropine and was not affected by changes in [K⁺] in the incubation buffer or by the presence of the DPI racemic mixture or its enantiomers (not shown). The data shown are of a typical experiment, one of three performed with each enantiomer (A and B) and one of five performed with the racemic mixture (C).
FIG. 4. Effects of DPI enantiomers on binding of [3H]AcCh to high affinity sites in the muscarinic receptor in membranes prepared from rat brain-stem synaptoneurosomes. Specific binding curves are shown for control (○); DPI S enantiomer (μM): 10 (●); 20 (▲); 40 (▼) (A); and DPI R enantiomer (μM): 10 (○); 20 (▲), 40 (▼) (B); nonspecific binding was measured in the presence of atropine (1 μM) (not shown). Scatchard plots are presented in the insets of A and C. The effects of S and R enantiomers on the calculated density of high affinity sites and on the calculated apparent dissociation constants of [3H]AcCh binding to the high affinity sites of the muscarinic receptor.
The fact that both the DPI S enantiomer (Fig. 3C) and BTX pretreatment counteracted the inhibition imposed by the DPI R enantiomer on depolarization-induced high to low affinity conversion (Figs. 3B and 5) may suggest that the depolarization-induced conversion of muscarinic agonist-binding sites from high to low affinity could be affected by changes induced in the gating state of the voltage-dependent Na⁺ channels, i.e. in the inactivated state the Na⁺ channels do not appear to interfere with the depolarization-induced affinity conversion. Neither inactivation induced by the exposure to high [K⁺] (prolonged depolarization (22)), nor inhibition of Na⁺ channel inactivation by BTX (16-18) and DPI S enantiomer (20, 21) interfered with the depolarization-induced affinity conversion (Fig. 3A and Fig. 5, inset). However, when the depolarization-induced activation of the Na⁺ channels was prevented by the DPI R enantiomer (20, 21), the depolarization-induced high to low affinity conversion was blocked (Fig. 5). The fact that this inhibition could be counteracted by persistent activation of the Na⁺ channels (Fig. 5) may suggest that Na⁺ channel activation is involved in the depolarization-induced muscarinic high to low affinity conversion.

Effect of DPI on the Depolarization-induced Binding of Labeled GTP/GDP to Permeabilized-resealed Synaptoneurosomes—The depolarization-induced increase previously observed

(Kd) are presented in B and D, respectively, B_max* and B_max are the maximal binding values of [3H]AcCh binding to the high affinity sites of the muscarinic receptor in the presence and in the absence of DPI enantiomer, respectively; K_d* and K_d are the respective apparent dissociation constants of binding in the presence and in the absence of DPI enantiomer. The figure shows the ratios B_max*/B_max (●) and K_d*/K_d (○) calculated from 10 experiments in the presence of the S enantiomer (B) and the R enantiomer (D). Bars represent the calculated S.D. values.

Synaptoneurosomes were pretreated with BTX (1 μM, 37 °C, 40 min), with the addition of TTX (1 μM) to abolish Na⁺ current (see "Materials and Methods"). Binding of [3H]AcCh to pretreated and control synaptoneurosomes was then measured in 4.7 and 50 mM [K⁺] buffers in the presence and absence of the DPI R enantiomer (5 μM). Fig. 5 shows the effects of the R enantiomer on the depolarization-induced decrease in high affinity binding of [3H]AcCh to the muscarinic receptor in control and in BTX-pretreated preparations. As shown, BTX prevented the inhibition exerted by the R enantiomer on the depolarization-induced decrease of [3H]AcCh high affinity binding (see also Fig. 3B).

It should be noted that BTX in the presence of TTX had no effect on the depolarization-induced conversion of muscarinic high affinity sites to the low affinity state (Fig. 5, inset). Since inward Na⁺ current (and hence additional membrane depolarization) was excluded under these experimental conditions, as confirmed by measurements of [3H]TPP accumulation and 22Na⁺ uptake by the pretreated synaptoneurosomes (not shown) (see Ref. 10), exposure of BTX- and TTX-pretreated synaptoneurosomes to high [K⁺] buffers induced membrane depolarization, as it did in control preparations. As in our previous study (10), we could therefore observe the depolarization-induced effect on [3H]AcCh high affinity binding when the Na⁺ channels were in a persistently activated state (Fig. 5, inset).

See Ref. 37.
served in the specific binding of $[^3H]GTP/[^3H]GDP$ to permeabilized-resealed synaptoneurosomes was attributed to a depolarization-induced activation of PTX-sensitive G-proteins (5). We examined the depolarization-induced effect on $[^3H]GTP/[^3H]GDP$ binding in the presence of DPI and its enantiomers (20, 21). Synaptoneurosomes prepared from rat brainstem were permeabilized in the presence of ATP loaded with $[^3H]GTP$ (0.2 $\mu$M $[^3H]GTP$ in the permeabilization buffer) and resealed (see "Materials and Methods"). Paper chromatography of the guanine nucleotides extracted from the loaded synaptoneurosomes revealed only $[^3H]GDP$, although $[^3H]GTP$ was still detectable in the incubation medium (for details see Ref. 5).

Accumulation of $[^3H]GTP/[^3H]GDP$ (Fig. 6, upper panel) and intrasynaptoneurosomal binding (Fig. 6, lower panel) were measured after exposure (1-2 min) of the resealed synaptoneurosomes to buffers containing either 4.7 or 50 mM $[K^+]$ in the absence (control) and presence of the racemic mixture of DPI and each of its enantiomers (see "Materials and Methods"). Changes in membrane potential were confirmed by measurements of $[^3H]TPP^+$ accumulation (5). As shown, similar amounts of $[^3H]GTP/[^3H]GDP$ were loaded into the various preparations (Fig. 6, upper panel). In control preparations, the amount of $[^3H]GTP/[^3H]GDP$ binding to depolarized synaptoneurosomes (in 50 mM $[K^+]$ buffer) was approximately 4-fold higher than the binding to synaptoneurosomes at resting potential (in 4.7 $\mu$M $[K^+]$ buffer) (Fig. 6, lower panel). A similar depolarization-induced enhancement in $[^3H]GTP/[^3H]GDP$ binding was previously reported (5).

The effects of the racemic mixture and enantiomers of DPI on the depolarization-induced increase in intrasynaptoneurosomal binding of labeled GTP/GDP are presented in Fig. 6 (lower panel). In the presence of the $R$ enantiomer ($5 \mu$M) (i.e. where activation of Na$^+$ channels was prevented (20, 21)), no depolarization-induced increase in $[^3H]GTP/[^3H]GDP$ binding was detected. On the other hand, the $S$ enantiomer ($5 \mu$M), which was added in the presence of TTX (1 $\mu$M to exclude inward Na$^+$ current (10, 32, 33), did not affect the depolarization-induced enhancement in $[^3H]GTP/[^3H]GDP$ binding which was observed in control preparations (Fig. 6, lower panel). Under these experimental conditions, TTX by itself did not interfere with $[^3H]GTP/[^3H]GDP$ binding (not shown). In the presence of the racemic mixture ($5 \mu$M) the depolarization-induced enhancement in $[^3H]GTP/[^3H]GDP$ binding was partially inhibited (Fig. 6, lower panel).

Corresponding measurements were carried out in synaptoneurosomes pretreated with PTX (200 ng/ml, 37 °C, 2 h, 95% O$_2$, 5% CO$_2$) (see "Materials and Methods"). As reported previously (5), the depolarization-induced enhancement in labeled GTP/GDP binding was not detected in synaptoneurosomes subjected to PTX-catalyzed ADP-riboylation (Fig. 6, lower panel).

These results may suggest the involvement both of ADP-ribosylation sites on PTX-sensitive G-proteins and of the voltage-dependent Na$^+$ channel gating in the depolarization-induced enhancement of $[^3H]GTP/[^3H]GDP$ binding attributed to G-protein activation (5).

These measurements were all carried out in the absence of

![Fig. 6. Effects of DPI enantiomers and racemic mixture on binding of $[^3H]GTP/[^3H]GDP$ to permeabilized-resealed synaptoneurosomes prepared from rat brainstem.](image-url)
Ca\(^{2+}\) in order to avoid possible interference by transmitter release (5). This possibility was further excluded by measurement of the specific binding of \(^{3}H\)GTP/\(^{3}H\)GDP to permeabilized synaptoneurosomes exposed (after rescaling) to antagonists of serotoninergic (siperoxine, 1 \(\mu\)M), \(\alpha\)- and \(\beta\)-adrenergic (yohimbine, 1 \(\mu\)M and propanolol, 1 \(\mu\)M, respectively), muscarinic (atropine, 1 \(\mu\)M), and NMDA (d-2-amino-5-phosphovaleric acid, 10 \(\mu\)M) receptors. No significant changes in the effects of DPI on the depolarization-induced increase in \(^{3}H\)GDP binding were observed in the presence of these antagonists. This supports our earlier suggestion (5) that a possible depolarization-induced activation of these receptors does not interfere with the depolarization-induced increase in \(^{3}H\)GTP/\(^{3}H\)GDP binding (5) nor does it interfere with the effects of DPI on the depolarization-induced increase in \(^{3}H\)GTP/\(^{3}H\)GDP binding.

Effects of DPI on the Binding of Labeled GTP/GDP to Membrane Preparations—In order to examine the effects of DPI on the binding of \(^{3}H\)GTP (attributed to G-protein activation (5)) in synaptoneurosomal membranes when changes in membrane potential are excluded, we examined the binding of \(^{3}H\)GTP/\(^{3}H\)GDP in membranes prepared from rat brain-stem synaptoneurosomes in the presence of the enantiomers of DPI.

Binding of \(^{3}H\)GDP to the membranes, which determines the steady-state amount of \(^{3}H\)GTP that displaces GDP (3, 6–9) in the presence and absence of DPI, is presented in Fig. 7. The calculated apparent dissociation constant (Fig. 7, inset) for \(^{3}H\)GDP binding to control membranes was 0.2 ± 0.03 \(\mu\)M, and the calculated highest amount of \(^{3}H\)GDP bound was 38 ± 2 pmol/mg protein (n = 3). Nonspecific binding was determined in the presence of 300 \(\mu\)M Gpp(NH)\(P\). Similar amounts of \(^{3}H\)GTP bound/milligram of protein were previously measured in this preparation (5). As shown, the \(R\) or \(S\) enantiomer of DPI did not significantly alter either the specific or the nonspecific binding of \(^{3}H\)GDP, i.e. neither enantiomer affected the displacement of GDP by \(^{3}H\)GTP in the membrane preparations (Fig. 7). Also pretreatment with 1 \(\mu\)M BTX, which resulted in activation of Na\(^{+}\) channels in these membrane preparation (10, 28), did not affect the binding of \(^{3}H\)GTP/\(^{3}H\)GDP to the membranes (not shown). These results may indicate that neither activation nor inactivation of the voltage-dependent Na\(^{+}\) channel (22) by itself induces activation of G-proteins as reflected by the displacement of GDP by \(^{3}H\)GTP (1–4, 6–9).

Effects of DPI on Coupling of the Muscarinic Receptor to G-proteins—Uncoupling of the muscarinic receptor from G-proteins, triggered by G-protein activation (2, 41, 42), induces conversion of the muscarinic receptor from a high affinity to a low affinity state for agonist binding (11, 12). Persistent activation of G-proteins should therefore be accompanied by a low affinity state of \(^{3}H\)AcCh binding. In order to determine whether agents that affect the gating state of the Na\(^{+}\) channel also affect the coupling between the muscarinic receptor and G-proteins, we examined whether persistent activation of G-proteins can affect the muscarinic affinity for \(^{3}H\)AcCh (11, 12, 25) in the presence of the DPI \(R\) enantiomer (i.e. under conditions where Na\(^{+}\) channel activation is prevented) or \(S\) enantiomer (i.e. when Na\(^{+}\) channels are persistently activated) (20, 21).

Fig. 8A shows the Gpp(NH)\(P\)-induced decrease in high affinity binding of \(^{3}H\)AcCh in control membrane preparations. Since no parallel change was observed in the density of muscarinic binding sites, as measured by the binding of the antagonist \(^{3}H\)NMPB (not shown), the decrease in \(^{3}H\)AcCh binding was attributed to a conversion of agonist-binding sites in the muscarinic receptor from a high to a low affinity (10, 28). Similar results were obtained in the presence of the DPI racemic mixture (5 \(\mu\)M) (Fig. 8B) or the \(S\) enantiomer (5 \(\mu\)M) (not shown). In the presence of the \(R\) enantiomer (5 \(\mu\)M), however, no such Gpp(NH)\(P\)-induced decrease in high affinity binding of \(^{3}H\)AcCh was observed (Fig. 8B). It thus appears that in the presence of the \(R\) enantiomer the coupling between muscarinic receptors and G-proteins was distorted, so that the muscarinic receptor remained in its high affinity state even when the G-proteins were persistently activated in the presence of Gpp(NH)\(P\).

Effects of Modifiers of the Voltage-dependent Na\(^{+}\) Channel on \(^{3}P\)IPTX-catalyzed ADP-ribosylation of G-proteins—The ADP-ribosylation site on \(\alpha\)- and \(\alpha\)-proteins apparently plays an essential role in the coupling between G-proteins and muscarinic receptors (41–45). Therefore, in view of the observed effect of the \(R\) enantiomer on the coupling of the muscarinic receptors to G-proteins (Fig. 8), we examined the effects of agents capable of modifying the gating state of the Na\(^{+}\) channel on IPTX-catalyzed ADP-ribosylation of G-proteins.

Persistent activation of the Na\(^{+}\) channels, without induction of Na\(^{+}\) current, was obtained by pretreatment of synaptoneurosomes with BTX or the DPI \(R\) enantiomer in the presence of TTX (32, 33). Inhibition of Na\(^{+}\) channel activation was induced by the DPI \(R\) enantiomer (20, 21) or the local anesthetic tetracaine (19, 46). Membranes prepared from synaptoneurosomes incubated with these agents (37 °C, 1 h, 95% O\(_2\), 5% CO\(_2\)) were then \(^{3}P\)IPTX-catalyzed in the presence of the \(A\)-protomer of PTX, as described under "Materials and Methods." Fig. 9 presents the autoradiograms obtained following SDS-PAGE (10% acrylamide) analysis of
the membrane proteins. Fig. 9A shows the effect of persistent activation of the Na" channel (induced by BTX or the S enantiomer), and the effect of prevention of the Na" channel activation (induced by the R enantiomer) on the ADP-ribosylation of 39-kDa G-proteins. Lanes 1–4 show [32P]ADP-ribosylation of 39-kDa G-proteins (5, 10, 13) in membranes prepared from untreated synaptoneurosomes (lane 1) and from synaptoneurosomes treated with the S enantiomer (5 μM) in the presence of TTX (1 μM) (lane 2), the racemic mixture (5 μM) (lane 3), and the R enantiomer (5 μM) (lane 4). Lane 5 shows [32P]ADP-ribosylation of G-proteins in membranes prepared from synaptoneurosomes pretreated with BTX (1 μM) and TTX (1 μM); the effect of the R enantiomer on the BTX- and TTX-pretreated synaptoneurosomes is shown in lane 6.

These results indicate that the inhibitory effect of the R enantiomer on the [32P]PTX-catalyzed ADP-ribosylation of the 39-kDa G-proteins (lane 4) could be counteracted by the S enantiomer in the DPI racemic mixture (lane 3) or totally abolished as a result of persistent activation of the voltage-dependent Na" channels by BTX (lane 6) while Na" current was blocked. Weak 32P-labeling was detected in a 30 kDa protein band. The effects of the above modifiers of the Na" channel gating on the labeling of this protein band were similar to those observed for the 39-kDa G-proteins (Fig. 9A).

Fig. 9B, lanes 1–4, illustrate the inhibitory effect of the potent local anesthetic drug tetracaine (19, 46) on [32P]ADP-ribosylation of the 39-kDa G-proteins in membranes prepared from synaptoneurosomes incubated with increasing concentrations of tetracaine (0.5, 1, 2, and 5 μM). The effective concentrations of tetracaine were similar to those obtained for blocking of Na" channels and for inhibition of the binding of labeled BTX to the Na" channel in brain preparations (46, 47). The effects induced by tetracaine on the 32P-labeling of a 30-kDa protein band were similar to those observed for the 39-kDa G-proteins (Fig 9B). [32P]ADP-ribosylation of membranes prepared from control and PTX-pretreated (100 ng/ml, 37 °C, 2 h, 96% O2, 5% CO2) synaptoneurosomes is presented in lanes 5 and 6, respectively. As expected for PTX-sensitive G-proteins, labeling of the 39-kDa protein band was weak in membranes prepared from ADP-ribosylated synaptoneurosomes, thus indicating ADP-ribosylation of these proteins in PTX-treated synaptoneurosomes. 32P-Labeling of the 30-kDa protein band was, however, not affected.

Fig. 9C compares [32P]ADP-ribosylation performed in synaptoneurosomal membranes in the presence and absence of the reducing agent DTT (20 mM). It should be noted that [32P]ADP-ribosylation of the membranes was performed in the presence of the PTX A-protomer, so DTT was not required for activation of the ADP-ribosyltransferase subunit of PTX (35, 45). Fig. 9C presents the autoradiograms of [32P]ADP-ribosylated membranes following SDS-PAGE analysis when ADP-ribosylation was conducted in the presence of 20 mM DTT (lane 1) and in its absence (lanes 2–4). Like the 39-
kDa protein band, the 30-kDa protein band was also labeled only in the presence of 20 mM DTT (lane 1). Other protein bands labeled in the absence of DTT (lane 2) were labeled also in the absence of the A-protomer of PTX (lane 3), as well as in membranes prepared from synaptoneurosomes pretreated with PTX (200 ng/ml, 37 °C, 2 h, 95% O₂, 5% CO₂) synaptoneurosomes, respectively. Also shown are the corresponding effects of these Na⁺ channel modifiers on the 30-kDa protein band observed after longer autoradiographic exposure (3 days instead of 24 h). Each lane contained 200 μg of protein, as measured by the Lowry method (34).

**Immunolabeling of [³²P]ADP-ribosylated G-proteins in Membranes Prepared from Synaptoneurosomes Pretreated with Modifiers of the Voltage-dependent Na⁺ Channel**—Immunolabeling was performed in order to identify the 39-kDa G-proteins modified by agents affecting the Na⁺ channel gating. Synaptoneurosomes were pretreated with the Na⁺ channel activators BTX (16-18) and the local anesthetic drug tetracaine, on PTX-catalyzed [³²P]ADP-ribosylation of 39-kDa G-proteins in membranes prepared from brain-stem synaptoneurosomes treated with the drug. Lanes 1–4 show an enhancement of the inhibitory effect of tetracaine on PTX-catalyzed [³²P]ADP-ribosylation of 39-kDa G-proteins with increasing drug concentrations (0.5, 1, 2, and 5 μM). Lanes 5 and 6 show [³²P]ADP-ribosylation of membranes prepared from control and PTX-pretreated synaptoneurosomes, respectively. Also shown are the corresponding effects of tetracaine and PTX pretreatment on a 30-kDa protein band observed after longer autoradiographic exposure (3 days instead of 24 h). Each lane contains 200 μg of protein, as measured by the Lowry method (34).

Fig. 10A presents autoradiograms of the [³²P]ADP-ribosylated 39-kDa G-protein in control membranes (prepared from untreated synaptoneurosomes) (lane 1) and in membranes prepared from synaptoneurosomes treated with TTX (1 μM) and either the S enantiomer of DPI (5 μM) (lane 2) or BTX (1 μM) (lane 3), from synaptoneurosomes treated with tetracaine (1 μM) following pretreatment with BTX (1 μM) and TTX (1 μM) (lane 4), and from synaptoneurosomes pretreated with the R enantiomer (5 μM) (lane 6). In lane 5, membranes prepared from untreated synaptoneurosomes were subjected to [³²P]ADP-ribosylation in the absence of DTT. As shown, no [³²P]ADP-ribosylation was achieved in this non-reducing environment.

Fig. 10B presents immunoblots of the same preparations following the reaction with antibodies to the N terminus of the α-subunit of Gₛ-proteins. These antibodies were specifically chosen in order to avoid possible interference by the ADP-ribosylation of αₛ-proteins assumed to be on the C terminus (39) with an immunological reaction at the C terminus of the αₛ-subunit. Labeling of this protein band was previously observed following reaction with antibodies to the C terminus of the αₛ-subunit of Gₛ-proteins (5, 10, 13).
**Depolarization, Voltage-dependent Na⁺ Channels, G-proteins**

![Image](image_url)

**Fig. 10.** Immunolabeling of [³²P]ADP-riboeylated G-proteins in membranes prepared from synaptoneurosomes exposed to modifiers of the Na⁺ channel gating. [³²P]ADP-riboeylated membranes prepared from control or pretreated synaptoneurosomes were analyzed by SDS-PAGE (10% polyacrylamide). Except for membranes analyzed in lane 5, ADP-riboeylation was performed in a reducing environment (20 mM DTT) (see "Materials and Methods"). A, autoradiograms presented are of the blots (Western blots) obtained with membranes prepared from: lane 1, control synaptoneurosomes (no pretreatment); lane 2, synaptoneurosomes pretreated with the S enantiomer of DPI (5 μM) in the presence of TTX (1 μM); lane 3, synaptoneurosomes pretreated with BTX (1 μM) and TTX (1 μM); lane 4, synaptoneurosomes pretreated with BTX (1 μM), TTX (1 μM), and tetracaine (1 μM); lane 5, membranes prepared from untreated synaptoneurosomes were subjected to [³²P]ADP-riboeylation in a non-reducing environment (in the absence of DTT); lane 6, membranes prepared from synaptoneurosomes pretreated with the R enantiomer of DPI (5 μM). B, immunoblots of these preparations immunolabeled with antibodies to the N-terminal of the α-subunit of G₆-proteins (38). Each lane contains 200 μg of protein, as measured by the Lowry method (34).

modifications of the Na⁺ channel gating apparently did not affect the amounts of α₆-subunit in the membrane, while specifically affecting their ability to be [³²P]ADP-riboeylated in the presence of PTX A-protemer.

As shown, G₆-proteins in membranes prepared from synaptoneurosomes subjected to a persistent opening of Na⁺ channels with Na⁺ current prevented (lane 2) were more efficiently [³²P]ADP-riboeylated than the rest. This effect was partially inhibited following incubation of the synaptoneurosomes with tracaine (1 μM), which inhibits BTX binding to the Na⁺ channel (46, 47) (lane 4). Thus, in membranes prepared from synaptoneurosomes pretreated with inhibitors of the Na⁺ channel activators (lanes 4 and 6) the [³²P]ADP-riboeylation of α₆-proteins was inhibited.

It should be noted that although the 39-kDa G-protein was equally immunolabeled in the presence (lanes 1–4 and 6) and absence of DTT (lane 5), no [³²P]ADP-riboeylation was achieved in the non-reducing environment, suggesting that cleavage of S-S bonds in the α₆-protein is essential for PTX-catalyzed ADP-riboeylation to occur.

**DISCUSSION**

The results presented in this study provide evidence for involvement of the voltage-dependent Na⁺ channel gating in depolarization-induced activation of PTX-sensitive G-proteins. The effects of membrane depolarization on G-proteins and on muscarinic receptors were previously reported in brain-stem synaptoneurosomes and myocytes (5, 13). Inhibition of the depolarization-induced effect on G-proteins by GDP/βS (Figs. 1 and 2), which causes persistent inactivation of G-proteins (1–4), supports the contention that membrane depolarization induces G-protein activation. The present results demonstrate that this putative depolarization-induced activation of G-proteins can be inhibited by agents that prevent the activation of the voltage-dependent Na⁺ channel (Figs. 3, 5, and 6). It should be noted that only the channel gating was modified here, as induction of Na⁺ current and its consequent changes in membrane potential were prevented by addition of the potent specific Na⁺ channel blocker TTX (1 μM) (32, 33). Prevention of the voltage-dependent Na⁺ channel activation was accompanied by distortion of the coupling of G-proteins to the muscarinic receptor (Fig. 8) and inhibition of the PTX-catalyzed ADP-riboeylation of G₆-proteins (Figs. 9 and 10). Accordingly, agents that inhibit the Na⁺ channel inactivation (22), such as BTX (16–18) and the S enantiomer of DPI (20, 21) (with Na⁺ current abolished), could counteract the inhibition induced by inhibitors of the Na⁺ channel activation such as the DPI R enantiomer (20, 21) and the local anesthetic tetracaine (46) (Figs. 3C, 5, 6, 8B, 9, A and B, and 10).

Since prolonged membrane depolarization induced by exposure of the synaptoneurosomes to high [K⁺] buffer results in inactivation of the voltage-dependent Na⁺ channel (22), the depolarization-induced effect on G-proteins cannot be attributed to inward Na⁺ currents. Moreover, it appears that inactivation of the Na⁺ channels does not interfere with the depolarization-induced activation of G-proteins (Figs. 1–3 and 5–7 and Refs. 5, 10, 13). However, prevention of activation of the Na⁺ channel (22) did abolish the depolarization-induced effects on G-proteins and on muscarinic receptors (Figs. 3B, 5, 6, 8B, 9, A and B, and 10).

The fact that agents that prevent activation of the Na⁺ channel appear to modify both the coupling of G-proteins to muscarinic receptors (Fig. 8) and the ADP-riboeylation of G₆-proteins (Figs. 9, A and B, and 10), and the evidence that PTX-catalyzed ADP-riboeylation also prevents depolarization-induced activation of G-proteins, may suggest that the ADP-riboeylation sites on PTX-sensitive G-proteins are involved in the depolarization-induced activation of G-proteins.

Binding of [³H]GTP to membrane preparations (Fig. 7), thought to indicate the displacement of GDP by [³H]GTP (3, 6–9), was not affected by pretreatment with agents that either prevent Na⁺ channel inactivation in this preparation, such as BTX (10, 28) and the DPI S enantiomer, or inhibit Na⁺ channel activation, such as the DPI R enantiomer (Fig. 7). Thus, since the displacement of GDP by [³H]GTP reportedly reflects G-protein activation (1–14), these results may suggest that activation or inactivation of the Na⁺ channel does not itself cause activation of G-proteins, although inhibition of the Na⁺ channel activation interferes with the depolarization-induced activation of G-protein (Figs. 3B, 5, and 6).

The fact that BTX and DPI-201 affect Na⁺ channels in a reversible manner (16–21) does not necessarily exclude the possibility that the effects on ADP-riboeylation of G₆-protein, which were detected in membranes prepared from pretreated synaptoneurosomes, were mediated by persistently activated or persistently blocked Na⁺ channels. One cannot exclude a possible secondary effect induced by reversible changes in the Na⁺ channel and causing irreversible changes in the ADP-riboeylation sites; interference with an endogenous ADP-riboeylation of G-proteins would be reflected in the subsequent [³²P]ADP-riboeylation performed in membranes prepared from synaptoneurosomes exposed to modifiers of the Na⁺ channel gating.
pared from the pretreated synaptoneurosomes. However, because of the hydrophobicity of these effectors, it is possible that they are not removed by washes and homogenization, and this could explain their sustained effect on the [3H]ADP-ribosylation of membranes prepared from the treated synaptoneurosomes. In a previous study, our attempts to remove [3H]BTX from brain homogenates met with difficulties (10). Similar inhibition of ADP-ribosylation of G-proteins in the presence of tricyclic antidepressants was recently reported (48).

Previous reports of inhibition by local anesthetics of the formation of second messengers, such as adrenergically induced cAMP formation (49) and PI turnover (50, 51), are consistent with the present results and may suggest that the effect of these agents on the formation of second messengers is mediated by their modification of the coupling of G-proteins to receptors (and possibly also to other effectors in the membrane), which may be achieved in turn by modification of the ADP-ribosylation site on G-proteins.

The compatibility between the effects induced by membrane depolarization on the muscarinic receptors and on the coupled G-proteins (Figs. 1 and 2, 3 and 6, 5 and 9, and 10) may support previous evidence (5) for an effect of membrane depolarization on G-proteins in the postsynaptic membrane, which houses the muscarinic receptors (11, 12). The possibility that other effectors coupled to G-proteins are also changed in response to membrane depolarization should be investigated. Such a phenomenon might be of major significance with regard to signal transduction in the central nervous system.

The suggested modifications of the ADP-ribosylation site(s) on the α-subunit of G-proteins, induced by changes in the state of the Na+ channel gating, are consistent with previously reported evidence suggesting a mutually positive effect of the activation of the Na+ channel and agonist high affinity binding to the muscarinic receptor mediated by G-proteins (10, 28, 52, 53). Thus, net TTX-blockable and atropine-blockable 22Na+ influx was induced by carbamylcholine in brain-stem synaptoneurosomes, when the muscarinic receptor was in the high affinity state (10), i.e. coupled to G-proteins (2, 41, 42, 44). Also, the binding of labeled BTX to activated Na+ channels (16–18) in membranes prepared from rat brain-stem and atria was increased in the presence of muscarinic agonists (10, 28). Reciprocally, activation of Na+ channels by pretreatment with BTX increased the binding affinity of [3H]AcCh for the high affinity sites of the muscarinic receptor (28, 52) in these preparations. These mutual effects could all be abolished by persistent activation of G-proteins or by uncoupling of the muscarinic receptor from the G-proteins, i.e. in the low affinity state of the muscarinic receptor (10, 28). It should be noted that PTX-catalyzed ADP-ribosylation of the synaptoneurosomes (200 ng/ml PTX, 37 °C, 2 h, 95% O2, 5% CO2) did not abolish this effect and therefore apparently does not induce uncoupling of the muscarinic receptor from G-proteins (5, 10), whereas it did prevent the depolarization-induced exchange of GDP by [3H]GTP on G-proteins (Fig. 6 and Refs. 5, 10). This may suggest that PTX-sensitive G-proteins contain more than one type of ADP-ribosylation sites, which are involved in the interaction of G-proteins with the muscarinic receptor and in the response to membrane depolarization. The fact that G-proteins are ADP-ribosylated by PTX-A promoter only in a reducing environment (Figs. 9C and 10) may also suggest that this reaction involves changes in the spatial structure of the α-subunit and may therefore point to the involvement of several sites on this protein in the ADP-ribosylation. This suggestion is in line with recent observations (54).

A one-to-one stoichiometry in the interference with G-protein activation induced by inhibition of Na+ channel activation seems unlikely, since the density of Na+ channels measured in the membrane of brain preparation is lower than the density of G-proteins (1–2 pmol/mg protein (16, 55)) versus 40 pmol/mg protein in this preparation or 100–200 pmol/mg protein in various brain preparations (2, 56). However, a similar discrepancy exists also with respect to the density of receptors known to activate G-proteins versus the density of G-proteins in the membrane, i.e. the density of muscarinic receptors in brain preparation is 200 fmol/mg protein (brain-stem) and 1 pmol/mg protein (cortex) (25, 28). A possible explanation was recently proposed by Coulter and Rodbell (57), who suggested on the basis of cross-linking performed in membranes of brain synaptoneurosomes that groups of G-proteins ("multimeric structures") might interact with receptors, while a single G-protein in the multimeric complex might be activated by a single receptor at a time. Such multimeric structure of G-proteins allows for signal amplification (57).

Figs. 9 and 10 present labeling of a 30-kDa protein band which was affected similarly to that of the 39-kDa G-protein by pretreatment of the brain-stem synaptoneurosomes with agents that modify the voltage-dependent Na+ channel. Labeling of this 30-kDa protein band in the presence of [3H]NAD could be detected only in a reducing environment (i.e. in the presence of 20 mM DTT) and even in the absence of PTX (Fig. 9, B and C). Further studies are now being carried out in an attempt to identify the 30-kDa protein band and find out whether it has any function in a possible coupling between Na+ channels and G-proteins in brain tissue. The β3-subunit (30–40 kDa) of the voltage-dependent Na+ channel complex found in brains of most vertebrates (58), and which couples to the α-subunit of the Na+ channel complex (280 kDa) (55, 58, 59) by disulfide bonds (58), is one of the proteins that might be represented by the 30-kDa protein band.

In summary, according to previously reported evidence (5, 13) and the present results, membrane depolarization may induce G-protein activation similarly to activation of G-proteins induced by binding of ligands to G-protein-coupled receptors (1–3, 6–9). The results presented here provide evidence that the Na+ channel gating may be involved in membrane depolarization-induced activation of PTX-sensitive G-proteins in brain-stem synaptoneurosomes. In terms of this model, since the voltage-dependent Na+ channel is the first element known to respond to membrane depolarization under physiological conditions (22, 56), membrane depolarization signals that induce a threshold potential (22) in the postsynaptic membrane (60 and Refs. therein) may be transduced into a "message" in the postsynaptic excitable cell as a result of G-protein activation. Elucidation of the molecular mechanism causing the depolarization-induced activation of G-proteins may provide information on a depolarization signal transduction in excitable cells.

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