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Evidence That Barbiturates Inhibit Antigen-Induced Responses through Interactions with a GTP-binding Protein in Rat Basophilic Leukemia (RBL-2H3) Cells

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Little is known about the mechanism of action of anesthetics at the biochemical level. The present work, however, gives evidence that barbiturates inhibit inositol phospholipid hydrolysis in both intact and permeabilized rat basophilic leukemia (RBL-2H3) cells by an effect on GTP-binding proteins (G-proteins). Inhibition of hydrolysis was observed when intact cells were stimulated with antigen (DNP_{24} BSA) or with oligomers of IgE. The inhibition was dependent on the concentration and type of barbiturate used with an order of inhibitory action of secobarbital < S(−) pentobarbital < pentobarbital < R(+) pentobarbital < phenobarbital. The relatively inactive analogue, (1'RS, 3'SR) 3-hydroxypentobarbital caused little (<30% at 1 mM) or no inhibition (at 0.1–0.5 mM). In permeabilized cells, the hydrolysis induced by DNP_{24} BSA and the nonhydrolyzable analogue of GTP, GTPγS (2–100 μM), was also inhibited by pentobarbital. The inhibition of hydrolysis was decreased as *p*H increased, and was no longer apparent at *p*H 7.8, a possible indication that the inhibitory effect was due to the unionized form of the drug. In permeabilized cells, the inhibition by pentobarbital occurred in the presence or absence of Ca^{2+} and was uncompetitive in nature (K_m = 7.1 μM for GTP in controls *vs.* 1.6 μM in the presence of 0.5 mM pentobarbital). Taken together, the data suggest that barbiturates alter the activity of G-proteins independently of Ca^{2+}, and the inhibition may depend on both the hydrophobic properties and the stereospecific and structural features of the molecule. (Key words: Anesthetics, hypnosis: barbiturates. Cell: basophil. Phospholipids: inositol; phosphatidylinositol.)

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THE MECHANISM of action of anesthetics at the biochemical level is still virtually unknown. Previously, we[1] and others[2] have demonstrated an effect of anesthetics on a biochemical mechanism, cell signal transduction, and have proposed that the effect is at the level of the GTP-binding protein (G-protein). One signal transduction mechanism, the inositol phospholipid pathway[3,4] (fig. 1), located in the plasma membrane, involves the coupling of receptors to an effector enzyme (phospholipase C) through the intervention of an unidentified G-protein(s) (Gp-type G-protein),[5–7] and the subsequent hydrolysis of inositol phospholipids. The hydrolysis generates two synergistic signals, release of intracellular Ca^{2+} from the endoplasmic reticulum by inositol 1,4,5 triphosphate, and the activation of protein kinase C by 1,2-diacylglycerol. The interaction of these signals initiates a cell response.[3,4] In previous studies,[1] we have shown that barbiturates inhibit phospholipid hydrolysis in rat aortic endothelial cells by a possible effect on a Gp-type G-protein. If barbiturates do indeed inhibit coupling of G-proteins to effector molecules, as has been suggested, the effect should be ubiquitous to any cell possessing the putative Gp. To test these hypotheses we have extended our studies to the rat basophilic leukemia (RBL-2H3) cell.[8] The RBL-2H3 cell was selected as a model for our studies because: 1) it is thought to possess the Gp-type G-protein that is insensitive to pertussis and cholera toxins[9]; 2) activation of the inositol phospholipid pathway is accomplished by a different mechanism than that in endothelial cells[1] (*i.e.*, by aggregation of receptors)[10–13]; 3) activation of the pathway leads to an easily measured physiologic response (*i.e.*, secretion) that has been shown to be inhibited by pentobarbital[14]; and 4) the inositol phospholipid pathway in these cells has been thoroughly investigated,[15] which would allow the manipulation of the various components of hydrolysis.

In RBL-2H3 cells, inositol phospholipid hydrolysis can be initiated in two ways. Hydrolysis can be initiated when receptors for immunoglobulin IgE are aggregated either with chemically linked oligomers of IgE or primed with IgE and then aggregated with antigen.[10–13] Such stimulation results in the indirect activation of the G-protein,

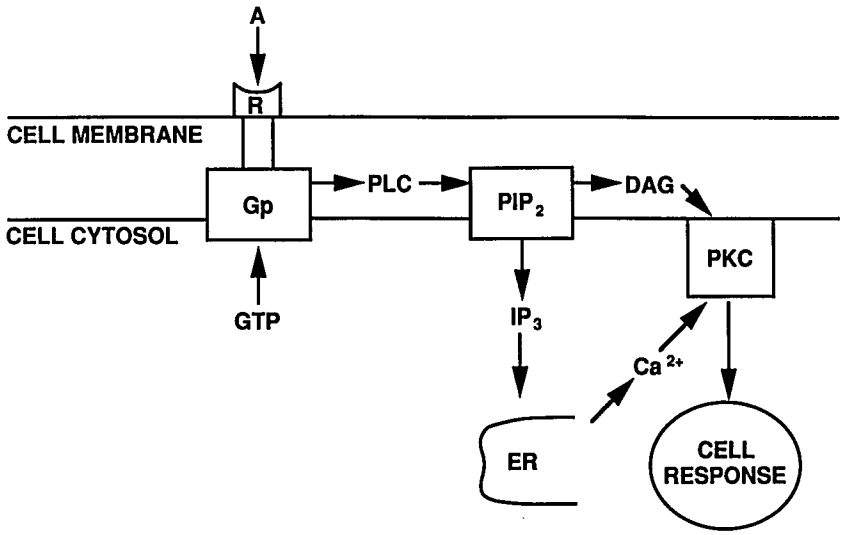

FIG. 1. Schematic illustration of G-protein-regulated inositol phospholipid hydrolysis and signal transduction. The activation of the receptor (R) by the agonist (A) stimulates phospholipase C (PLC) to cause the hydrolysis of phosphatydylinositol biphosphate (PIP_2), through the intervention of the putative GTP binding protein (Gp). The resultant hydrolysis generates two synergistic signals, release of Ca^{2+} from the endoplasmic reticulum (ER) by inositol triphosphate (IP_3), and the activation of protein kinase C (PKC) by Ca^{2+} and 1,2 diacylglycerol (DAG). The activation of PKC leads to a cell response.[5]

which leads to a physiologic response (*i.e.*, secretion of histamine and serotonin).[10,11,16] When RBL-2H3 cells[17] are made permeable with streptolysin O[18,19] to large molecules such as the nonhydrolyzable analogue of GTP, GTPγS, these cells can respond to both antigen and GTPγS. GTPγS can, however, directly activate the G-protein by substituting for GTP in the activation of the α subunit of GP.[5-7] The ability to directly activate the G-protein has allowed us to investigate the possibility that barbiturates may inhibit inositol phospholipid hydrolysis in the RBL-2H3 cell by an interaction with a G-protein(s).

Materials and Methods

The solution used (one that was optimal)[12] for the maintenance of RBL-2H3 cells in culture was Eagles Minimum Essential medium (MEM) with Earle's balanced salt solution supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin (Grand Island Biological Co., Grand Island, NY, [GIBCO]) and 15% heat-inactivated fetal calf serum (Hyclone, Logan, UT). Trypsin-EDTA (0.05%) was obtained from GIBCO.

The following chemicals and solutions used in the individual experiments were: Myo-[2 − ^{3}H] inositol, 16.9 Ci/mmole (Dupont New England Nuclear, Boston, MA); unlabelled myo-inositol (Sigma Chemical Co., St. Louis, MO); rat monoclonal antidinitrophenol specific IgE (DNP-specific IgE); dinitrophenol-conjugated bovine serum albumin ($DNP_{24}BSA$); and a chemically cross-linked higher oligomer of IgE (gifts of Dr. Henry Metzger, National Institutes of Arthritis, Musculoskeletal and Skin diseases, National Institutes of Health, Bethesda, MD). The oligomer fraction used consisted mainly of tetrameres (higher oligomers) of IgE.[11] The preparation of these materials have been described previously.[20]

Other chemicals included guanosine 5′ − (3 − 0 − thio) triphosphate (GTPγS) (Boehringer Mannheim Biochemicals, Indianapolis, IN), 40 IU, *e.g.*, reduced streptolysin O (Wellcome Diagnostics, Dartford, England); sodium pentobarbital, phenobarbital, secobarbital, and lithium chloride (Sigma). Optical isomers of pentobarbital, R(+) and S(−), and a structural analogue of pentobarbital (1′RS, 3′SR)-3′hydroxypentobarbital were gifts of Dr. F. Ivy Carroll (National Institutes of Health; Research Triangle Park, NC). Dowex-1 formate resin was obtained from Bio-Rad Laboratories (Richmond, CA); and 0.1% Pentex bovine serum albumin, fraction V, was obtained from Miles Laboratories, Inc. (Elkhart, IN).

Buffers used were: Buffer A, used for the inositol phospholipid hydrolysis experiments in intact cells, contained 119 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.01 mM $MgSO_4$, 25 mM piperazine −N,N′-bis (2-ethane-sulfonic acid) (PIPES), 5.6 mM glucose, 40 mM NaOH, and 0.1% w/v bovine serum albumin, with and without 10 mM LiCl, *p*H 7.2. Buffer B, used for experiments with permeabilized cells, contained 138.7 mM potassium glutamate, 1 μM $CaCl_2$, 110 mM LiCl, 2 mM potassium PIPES, 1 mM MgEGTA 5, mM ATP, and 5 mM glucose at *p*H 6.8. In experiments in which the *p*H was varied, the *p*H values were adjusted by the addition of 1 N HCl or 1 N NaOH. Solutions of barbiturates and stimulants were prepared as described previously.[1]

Preparation of Rat Basophilic Leukemia (RBL-2H3) Cells

RBL-2H3 cells[10] were maintained as monolayer cultures in supplemented MEM in 5% CO_2, 37° C, as previously described.[10,13,16] Cells were routinely subcultured every 48–72 h by treatment with trypsin-EDTA (0.05%,

5 min) and counted in a hemocytometer. Aliquots of subcultured cells were transferred to 24-well (Costar) cluster plates (2×10^5 cells/well) for inositol phospholipid hydrolysis experiments. Cultures were incubated for 24 h to allow for recovery from trypsinization and maximal response to antigen.

Labelling of RBL-2H3 Cells

For experiments with both intact or permeabilized cells, the cells were labelled with [^{3}H]inositol as described previously.[13] Cultures in 24-well cluster plates were incubated for 24 h (37° C) in supplemented MEM (as stated above) and incubated for an additional 16–17 h in supplemented MEM with 6 μCi/ml [^{3}H]inositol and primed with 5 μg/ml DNP-specific IgE. The medium was then removed and the cultures washed twice with 1 ml of buffer A or buffer B for intact cell experiments or permeabilized cell experiments, respectively. In all batches of buffer, 0.1% BSA was added to minimize spontaneous degranulation of cells.¶ For intact cell experiments, buffer A was removed before the addition of 200 μl of buffer A supplemented with 10 mM LiCl. LiCl was added to inhibit inositol-1-phosphatase and thereby allowed accumulation of [^{3}H]labelled inositol 1-monophosphate within the cell cytosol upon stimulation.[21] Cultures were incubated for 10 min (37° C) with medium that contained not only 10 mM LiCl, but also barbiturates as indicated. The medium was removed by aspiration and barbiturates and stimulants were added for a further 15-min incubation. In all experiments, appropriate controls without stimulants were performed with barbiturates.

Cell Permeabilization

Cells were permeabilized by the procedure of Howell and Gomperts[19] as modified by Ali *et al.*[17] Cells, labelled with [^{3}H]inositol and washed as in the above, were incubated with reduced streptolysin O (0.2 U/ml, 37° C) for 7 min (shaking water bath). The medium was aspirated and barbiturates and stimulants in buffer B (with 10 mM LiCl) were added as described above. For each batch of reduced streptolysin O used in these studies, the degree of cell permeabilization was assessed by assaying for cell porosity with fluorescein diacetate/ethidium bromide dye.[22] From 85–99% of the cells were found to be permeabilized. These cells still exhibit stimulatory and secretory responses to antigen.[16,17] They, however, leak cytosolic proteins and therefore eventually lose their response to stimulants.[17]

¶ Beaven MA: Personal communication.

Assay of [^{3}H]Labelled Inositol Phosphates and Inositol Phospholipids

The assay of total [^{3}H]inositol phosphates was performed as previously described.[13] The reaction was terminated by the addition of 750 μl chloroform/methanol (1:2) to each culture well. The mixture in each well was transferred to a separate polypropylene tube (Falcon #2063). Extracts were separated into two phases by the addition of chloroform and H_2O (250 μl each). The water-soluble [^{3}H]inositol phosphates in the upper aqueous phase were assayed by Dowex-1 formate resin in columns. The [^{3}H]labelled lipids were assayed by adding 3 ml chloroform-methanol (2:1) to the lower organic phase, and washing the mixture three times with 2 ml methanol that contained 1 M KCl and 10 mM inositol (1:1). The chloroform-methanol was then evaporated and the ^{3}H content of the lipid residue was determined by liquid scintillation.

The assay of [^{3}H]inositol phosphates in total (*i.e.*, inositol-1-monophosphate, inositol-1,4 biphosphate, and inositol 1,4,5 triphosphate, etc.) was used to assess the relationship between receptor activation and signal transduction. However, the time course of the appearance of the individual inositol phosphates in both intact and permeabilized cells in response to the various stimulants used here has been described in detail.[13,17,23]

Expression of Results

In studies on the hydrolysis of inositol phospholipid, with both intact and permeabilized cells, data were normalized as in previous studies:[13,17] the amount of agonist-stimulated [^{3}H]inositol phosphates released into the cell cytosol was expressed as a percent of the amount of membrane [^{3}H]inositol phospholipids originally present in unstimulated cells. The percent spontaneous release (3–5% for both intact and permeabilized cells), as determined in matched unstimulated cells, was subtracted from this value to obtain percent release of [^{3}H]inositol phosphates. When barbiturates were present, values for percent spontaneous release in the presence of the barbiturate alone were subtracted. These calculations allowed comparison of values among experiments in which different amounts of [^{3}H]label were incorporated into membrane inositol phospholipids (~45,000 dpm/2×10^5 cells). The amount of [^{3}H]inositol phospholipids remaining in the membrane after hydrolysis was expressed as a percent of [^{3}H]inositol phospholipids in unstimulated cells at zero time. The ratio of stimulated:spontaneous hydrolysis ranged from 2 to 6× for 15 min of incubation, with 1 and 100 μM GTPγS, respectively, in permeabilized cells; and 5.5 and 3× for 15 min of incubation with $DNP_{24}BSA$ and the oligomer of IgE in intact cells. Bars for SEM were omitted when these were smaller than the symbol.

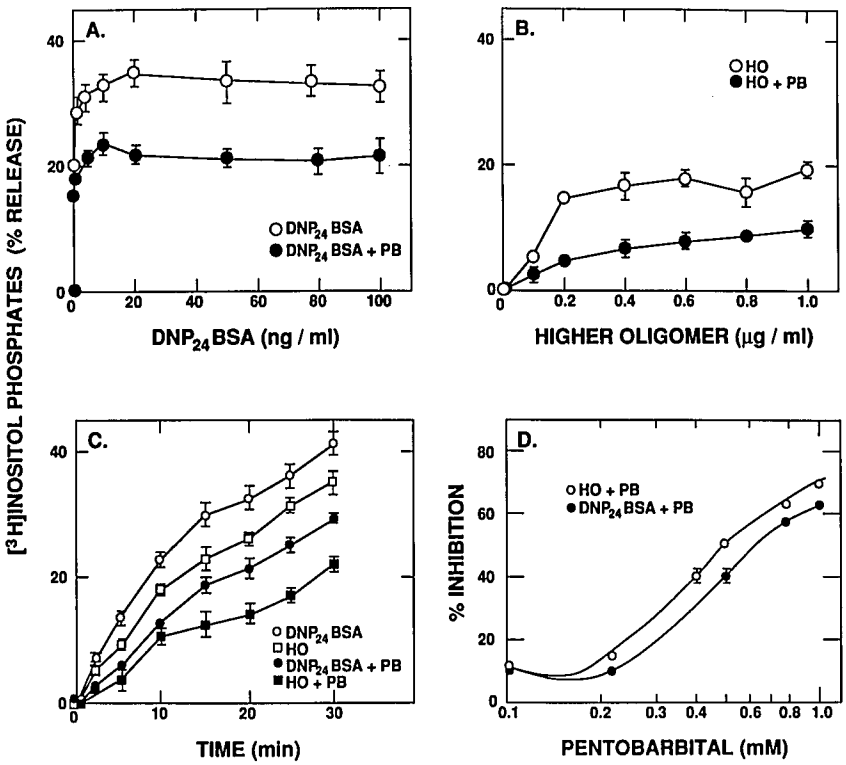

FIG. 2. Time and dose-response curves of antigen and oligomer-induced [^{3}H]inositol phospholipid hydrolysis in intact RBL-2H3 cells: effect of pentobarbital. *A* The effect of pentobarbital on hydrolysis as induced by increasing concentrations of $DNP_{24}BSA$, and *B* a higher oligomer of IgE (HO); *C* A time course of the inhibition by pentobarbital of $DNP_{24}BSA$ (10 ng/ml) and HO (1 μg/ml)-induced hydrolysis; *D* Concentration curves of the inhibition by pentobarbital of $DNP_{24}BSA$ (10 ng/ml) and HO (1 μg/ml)-induced hydrolysis. The concentration of pentobarbital in panels *A*, *B*, and *C* was 0.5 mM and the time of incubation in panels *A*, *B*, and *D* was 15 min. The release of inositol phosphates is expressed as a percent of [^{3}H]labelled inositol lipids in matched unstimulated cultures at zero time. The average amount of [^{3}H]labelled lipids at zero time was ~45,000 ± 1,000 dpm/2 × 10^5 cells. Each panel represents the mean ± SEM of four to five experiments (three wells per experimental point for each experiment). Response to stimulants in the presence of pentobarbital was significantly different from controls ($P < 0.01$ for panels *A*, *B*, and *C*; $P < 0.03$ for panel *D*). PB = pentobarbital. $DNP_{24}BSA$ = dinitrophenol-conjugated bovine serum albumin. HO = higher oligomer of IgE.

DATA ANALYSIS

Curves of percent [^{3}H]inositol phosphate release were fitted to experimental data points by cubic spline interpolation, and curves of percent inhibition were drawn by third-order polynomial regression (Sigma Plot, Jandell Scientific, Corte Madera, CA). Levels of significance were determined by two-tailed *t* test (Statworks, C. A. Cricket, Malvern, PA).

Results

INTACT RBL-2H3 CELLS: EFFECT OF BARBITURATES ON INOSITOL PHOSPHOLIPID HYDROLYSIS

The stimulation of intact RBL-2H3 cells by the antigen ($DNP_{24}BSA$) (figs. 2 *A* and *C*) and by the oligomer of IgE (HO, figs. 2 *B* and *C*), led to the release of [^{3}H]labelled inositol phosphates into the cell cytosol. Pentobarbital (0.1–1 mM) inhibited the stimulation in a concentration-dependent manner (fig. 2 *D*). Pentobarbital (0.5 mM) caused a greater ($P < 0.05$) inhibition when the oligomer was used as the stimulant (IC_{50} = 0.5 mM) than when $DNP_{24}BSA$ was used (IC_{50} = 0.6 mM). However, no difference in inhibition was seen at a low pentobarbital concentration (0.1 mM) (fig. 2 *D*). With both stimulants, inhibition was observed with as little as 0.1 mM pentobarbital (fig. 2 *D*).

The order of the inhibitory effect on antigen-induced hydrolysis by the various barbiturates tested was (1'RS, 3'SR) 3' – hydroxypentobarbital < phenobarbital < R(+) isomer < racemic pentobarbital < S(−) isomer < secobarbital) (fig. 3). The apparent IC_{50} values are shown in table 1. Maximal inhibition by all barbiturates, except (1'RS, 3'SR) 3' – hydroxypentobarbital, occurred between 6–8.5 mM barbiturate.

Pentobarbital alone had no significant effect on the amount of label in the membrane inositol phospholipid pool (data not shown) and it did not significantly inhibit the basal stimulated hydrolysis of inositol phospholipids in unstimulated cells (from 3–5% in cells with buffer alone to 3.3–3.5% in cells with buffer and pentobarbital).

PERMEABILIZED RBL-2H3 CELLS: EFFECT OF BARBITURATES ON INOSITOL PHOSPHOLIPID HYDROLYSIS

The addition of the nonhydrolyzable analogue of GTP, GTPγS, to permeabilized [^{3}H]labelled RBL-2H3 cells resulted in hydrolysis of inositol phospholipids (3–30% over the range of 1–100 μM GTPγS (figs. 4 *A* and *B*). Pentobarbital (0.5 mM) inhibited this hydrolysis. Inhibition was absent with low (<2 μM) concentrations of GTPγS and pronounced at high concentrations (100 μM) of GTPγS (80% inhibition). Concentrations of GTPγS greater than 20 μM did not reverse the inhibitory effect

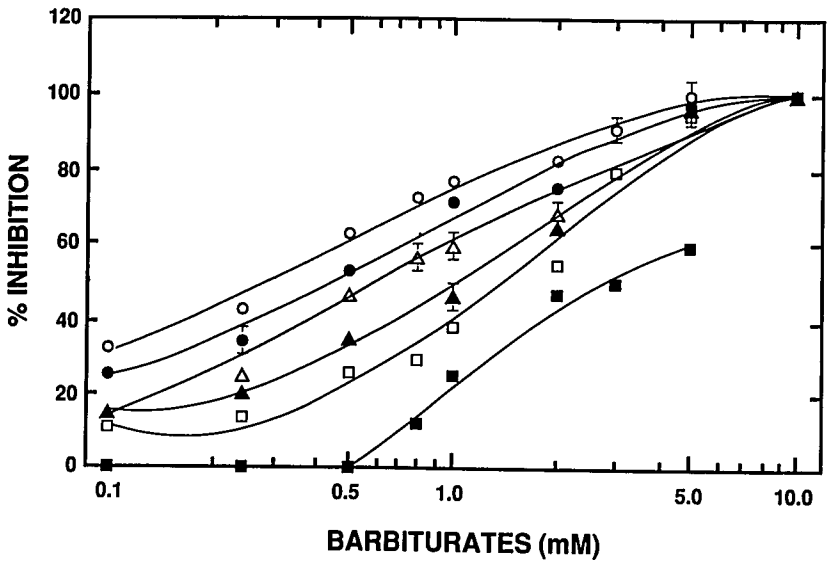

FIG. 3. Efficacy of different barbiturates in inhibiting antigen-induced [^{3}H]inositol phosphate release in intact rat RBL-2H3 cells. Cells were incubated with the barbiturates for 10 min before addition of $DNP_{24}BSA$ (10 ng/ml, 15 min), and experiments were performed as stated in "Material and Methods." Results are expressed as percent inhibition of the release of [^{3}H]inositol phosphates and are the mean ± SEM of six experiments (three wells per experimental point for each experiment). The individual curves were significantly different from each other ($P < 0.05$). ■ = (1'RS, 3'SR)-3'hydroxypentobarbital. □ = phenobarbital. ▲ = R(+) optical isomer of pentobarbital. △ = racemic pentobarbital. ● = S(−) optical isomer of pentobarbital. ○ = secobarbital.

of the barbiturates (figs. 4 *A* and *B*). This data indicated the absence of a competitive effect of pentobarbital on GTPγS-induced hydrolysis.

Characteristics of the Inhibition by Barbiturates of Inositol Phospholipid Hydrolysis in Permeabilized Cells

Previous studies had indicated that the inhibitory effects of barbiturates in intact cells were optimal at *p*H 7.2 (data not shown, but similar to fig. 5 *A*). The effect of external *p*H on intracellular *p*H was not determined. In permeabilized cells, where extracellular *p*H could be varied by buffering the external medium, the effects of barbiturates were found to be *p*H dependent. The inhibition by pentobarbital became progressively smaller with increase in *p*H with both antigen (fig. 5 *A*) and GTPγS (fig. 5 *B*), while the effect of pentobarbital on hydrolysis was opposite at high *p*H values. As in the intact cell, there was no effect of pentobarbital on the amount of radiolabel in the inositol phospholipid pool. However, unlike in the intact cell, the most potent barbiturates (secobarbital, S(−)-pentobarbital, pentobarbital, and R(+)-pentobarbital) suppressed the percent spontaneous hydrolysis of inositol phospholipids in the absence of stimulants (from 3–5% to a minimum of 1.5–2.5%), whereas the least effective barbiturates (phenobarbital and the [1'RS, 3'SR']3' hydroxypentobarbital analogue) had no effect on spontaneous hydrolysis.

TABLE 1. IC_{50} Values for the Inhibition of DNP_{24} BSA-Induced Inositol Phospholipid Hydrolysis by Various Barbiturates in Intact RBL-2H3 Cells

Barbiturate	IC_{50} (mM)	r
(1'RS, 3'SR) 3'-hydroxypentobarbital	2.9 ± 0.30	0.986
Phenobarbital	1.6 ± 0.10	0.992
R(+)-pentobarbital	1 ± 0.24	0.996
Pentobarbital	0.6 ± 0.1	0.994
S(−)-pentobarbital	0.44 ± 0.18	0.995
Secobarbital	0.28 ± 0.07	0.994

Data are expressed as the mean ± SEM of values from six experiments giving 50% of maximal inhibition.
r = correlation coefficient of polynomial regression.

The GTPγS-induced hydrolysis of inositol phospholipid and the inhibition of hydrolysis by pentobarbital was apparent both in the presence and absence of Ca^{2+} (fig. 4 *A* and *B*). Ca^{2+} alone, at concentrations up to 1 mM, however, produced little or no stimulation of hydrolysis (maximum percent hydrolysis with 1 mM Ca^{2+} was 2.5% in 15 min). The IC_{50} values for the inhibition by pentobarbital or secobarbital of GTPγS-induced hydrolysis, taken from log-linear plots of percent inhibition (fig. 4 *C*), were 0.35 and 0.20 mM, respectively. The studies with both intact and permeabilized cells indicated that pentobarbital and secobarbital (table 1; fig. 4 *C*) were inhibitory at submillimolar concentrations. Although the IC_{50} for pentobarbital was significantly lower ($P < 0.05$) in permeabilized cells. Preliminary studies with antigen-induced permeabilized cells also indicated that the IC_{50} values for pentobarbital was less than 0.5 mM (data not shown).

Analysis of the inhibition by Lineweaver-Burke plot (data not shown), indicated uncompetitive inhibition, as determined by a decrease in the K_m (X intercept = $-1/K_m$) and a corresponding decrease in V_{max} (Y intercept $1/V_{max}$). In the presence of pentobarbital (0.5 mM), the apparent K_m of 7.1 ± 0.8 μM was reduced to a K_m of 1.6 ± 0.95 μM.

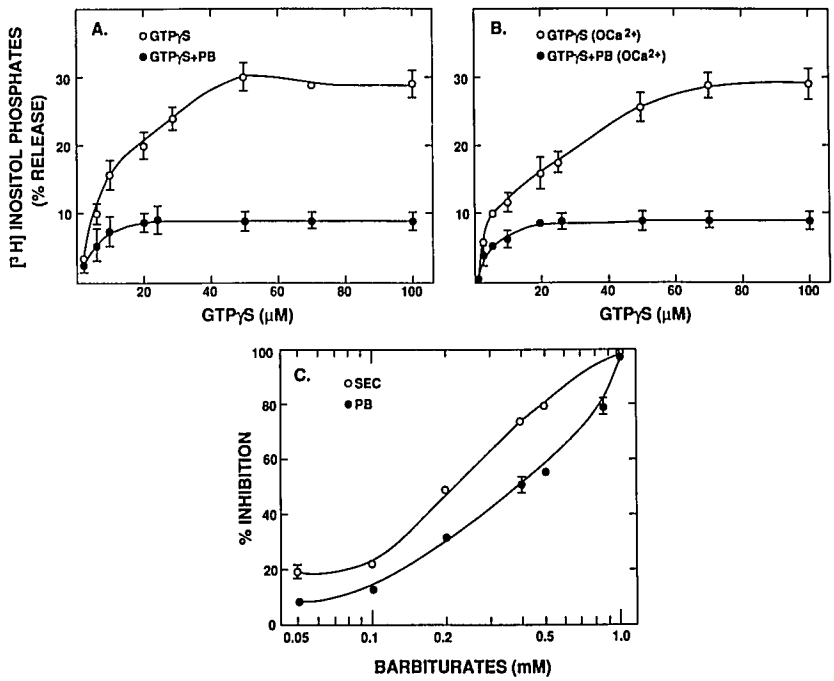

FIG. 4. Inhibition of GTPγS-induced [^{3}H]-inositol phosphate release in permeabilized RBL-2H3 cells by pentobarbital and secobarbital. Permeabilized cells were treated for 10 min with pentobarbital, secobarbital, or buffer. Cells were treated for a further 15 min as follows: *A* In the presence of 1 μM Ca^{2+} with GTPγS alone, or with GTPγS plus pentobarbital (0.5 mM); *B* in the absence of Ca^{2+} with GTPγS alone, or with GTPγS plus pentobarbital (0.5 mM). *C* Cells were treated with pentobarbital and secobarbital (0.1 to 1 mM) and GTPγS (10 μM) in the presence of 1 μM Ca^{2+}. Results are expressed as percent inositol phospholipid hydrolysis as stated in figure 1. Data represent the mean ± SEM of 3–4 experiments per panel (three wells per experimental point for each experiment; $P < 0.01$ for panels *A* and *B*; $P < 0.05$ for panel *C*). PB = pentobarbital. SEC = secobarbital.

Discussion

This study demonstrates that barbiturates inhibit stimulated inositol phospholipid hydrolysis in intact and permeabilized rat RBL-2H3 cells. In intact cells, barbiturates inhibited hydrolysis that was induced by either the antigen $DNP_{24}BSA$ or the oligomers of IgE. The inhibition was also observed in permeabilized cells stimulated with either antigen or GTPγS. The studies with GTPγS in particular suggested that the effects of pentobarbital were possibly exerted at the level of the G-protein (designated Gp) or the effector protein, phospholipase C, which is thought to couple with this putative G-protein.[5–7] Previous studies with rat endothelial cells[1] also indicated the possibility that barbiturates may suppress inositol phospholipid hydrolysis at a site other than at the ligand-receptor complex.

Intact RBL-2H3 cells respond to antigen with rapid hydrolysis of inositol phospholipid. As reported previously,[23] the rate of hydrolysis varies with the agent used to cross link the receptors of IgE (fig. 2). The aggregation of IgE receptors, whether by antigen in cells primed with antigen-specific IgE or by the oligomers of IgE, provides the trigger for the activation of the inositol phospholipid-specific phospholipase C. Because the antigen-stimulated hydrolysis of inositol phospholipids is blocked in permeabilized RBL-2H3 cells by GDPβS in a concentration-dependent manner, the activation of the enzyme is thought to involve the putative Gp-protein.[16] The actions of GTPγS reported here are also blocked by GDPβS. Although this protein has not been fully identified, use of nonhydrolyzable analogues of GTP to stimulate hydrolysis provides indirect evidence of its involvement in the activation of phospholipase C.[16,17]

Our data show that in the intact cell, barbiturates, at concentrations of 0.1 to >1 mM, inhibited hydrolysis whether hydrolysis was stimulated by antigen or by direct crosslinking of receptors with the IgE oligomer. The inhibitory activities of the barbiturates against oligomer stimulation (*e.g.*, IC_{50}) was similar to those reported for inhibition by pentobarbital in intact rat aortic endothelial cells.[1,24] However, in the intact RBL-2H3 cell the barbiturates were marginally less effective in inhibiting antigen-induced hydrolysis of inositol phospholipids than in inhibiting oligomer-induced hydrolysis. The reason for this may be intrinsic to the extent of crosslinking of receptors: $DNP_{24}BSA$ will promote extensive crosslinking of receptors whereas the crosslinking by oligomers will be restricted to aggregates of a few receptors.[11,20]

Pentobarbital also demonstrated stereoselectivity in the intact cell. Inhibition by the racemic pentobarbital was intermediate of its R(+) and S(−) optical isomers, with the S(−) isomer showing the greatest inhibition. The relatively inactive analogue of pentobarbital, (1′RS, 3′SR) 3′hydroxypentobarbital,[25] showed little inhibitory activity even at high concentrations or no activity at low concentrations (0.1–0.5 mM). These results were consistent with those of Barker *et al.*[26] for the inhibition by pentobarbital of glutamate depolarizing responses in cultured mam-

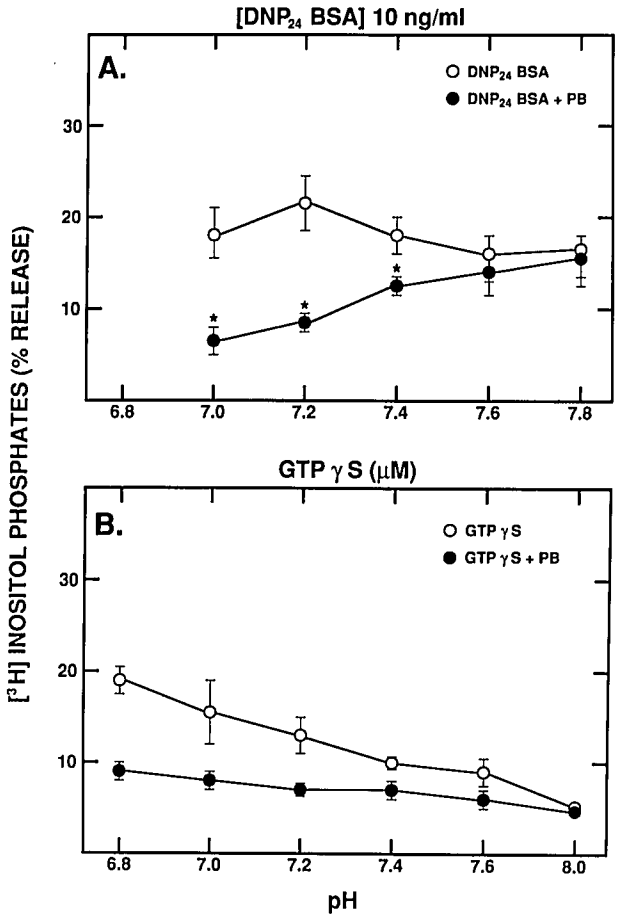

FIG. 5. Inhibition by pentobarbital of $DNP_{24}BSA$ and GTPγS-induced [^{3}H]inositol phosphate release in permeabilized RBL-2H3 cells: effect of *p*H. *A* Effect of *p*H on the inhibition by pentobarbital of $DNP_{24}BSA$-induced hydrolysis. *B* Effect of *p*H on the inhibition by pentobarbital of GTPγS-induced hydrolysis. Cells permeabilized with streptolysin O were incubated for 10 min with pentobarbital in buffer at the indicated *p*H (abscissa) followed by a 15 min incubation with $DNP_{24}BSA$ (10 ng/ml) or GTPγS (10 μM). The concentration of pentobarbital was 0.5 mM. The percent release of inositol phosphates was expressed as stated in figure 2. Results are representative of three experiments (three wells per experimental point). $*P < 0.05$ in panel *A*. $P < 0.01$ in panel *B*.

malian neurons, with those of Christensen *et al.*[25,27] for the order of anesthetic potency of the drugs in mice, and with the conclusions of Andrews and Mark[28] that there are structurally specific target sites for barbiturates. Olsen[29] has shown that barbiturates can bind to specific picrotoxin/barbiturate binding sites on the GABA-receptor ionophore complex in membranes from mammalian brain. Therefore, our studies and the studies of others have indicated that the inhibition by pentobarbital appears not to be due to its hydrophobic properties alone but also to stereospecific and structural features of the barbiturate. The present study thus raises the possibility that barbiturates act at specific sites or in a specific manner with membrane components in RBL-2H3 cells.

In addition, barbiturates can inhibit GTPγS and antigen-induced hydrolysis in permeabilized RBL-2H3 cells. As in the intact cell stimulated with antigen, the inhibition of hydrolysis by barbiturates was dependent on *p*H (fig. 5) and on the concentration of both the barbiturate and the stimulant (fig. 4). However, differences were noted between intact and permeabilized cells. In permeabilized cells, the IC_{50} value for pentobarbital was less than that observed with antigen-induced intact cells. The reason for the decrease in IC_{50} is unclear. These data, however, could reflect the differences in the intracellular *p*H in the two cell preparations and the effect of *p*H on the distribution of pentobarbital between the aqueous and lipid phases of the cell. In permeabilized cells, the effect of *p*H was notable in that the inhibition was decreased as the *p*H increased and was no longer apparent at *p*H 7.8, a possible indication that inhibition was due to the unionized form of the drug.[30] However, an explanation for the opposite effect of pentobarbital with increasing *p*H on antigen and GTPγS-induced hydrolysis (fig. 5) is not apparent but is consistent with the data of others.[16] The studies with permeabilized cells avoided the complications of the effects of *p*H on the extracellular/intracellular distribution of the drug and possible changes in intracellular *p*H that occur with antigen-stimulated intact cells.[16]

As previously reported,[16,17] the presence of extracellular Ca^{2+} was not necessary for stimulation by GTPγS in permeabilized RBL-2H3 cells. Although there is evidence for a receptor-mediated mobilization of intracellular and extracellular Ca^{2+} in RBL-2H3 cells,[31] our data suggest that the inhibition of GTPγS-induced stimulation by pentobarbital is independent of any effect that barbiturates might have on Ca^{2+} influx or mobilization. The studies with GTPγS also indicate that pentobarbital does not act in a simple competitive manner with GTPγS. The barbiturate may instead alter the functional response of the G-protein, phospholipase C, or the coupling of these two components.

The concentrations of pentobarbital used to inhibit inositol phospholipid hydrolysis (0.1 to >1 mM) in these studies are consistent with those used in other *in vitro* studies (0.05–4 mM) with isolated tissue and cell preparations.[32–35] Concentrations required to provide anesthesia in humans and animals have been reported to range from 0.05–0.2 mM,[33–36] with maximum concentrations in the extracellular fluid before distribution into the total body water of 0.5 mM.[32] Barbiturates are also used to control intracranial pressure in neuroresuscitation with serum concentrations of pentobarbital reported to range from 0.1 mM to as high as 0.5 mM.[37–39] A value of 0.5

mM is consistent with the IC_{50} values for pentobarbital in inhibiting antigen-induced hydrolysis in the intact, and GTPγS-induced hydrolysis in the permeabilized RBL-2H3 cell (0.6 and 0.35 mM, respectively). However, significant inhibition of hydrolysis was observed with concentrations as low as 0.1–0.2 mM. The concentrations of barbiturates used in these studies thus overlap those used pharmacologically and clinically.

The high affinity of barbiturates for lipids allows them to dissolve into biological membranes. Their presence in membranes has been suggested to alter the geometric configuration of lipids and/or proteins.[40] This work, therefore, does not rule out the possibility that barbiturates may inhibit the activity of other proteins involved in signal transduction and alter the membrane configuration of lipids, it gives evidence of an effect at the level of activity of G-proteins and phospholipase C. If barbiturates do affect membrane coupling events, this may in part account for the actions of anesthetics on many cell and tissue types.[40] However, a more through evaluation of the effect on different systems and in different cell types will be required.

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