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Role of Fluidity of Membranes on the Guanyl Nucleotide-Dependent Binding of Cholecystokinin-8S to Rat Brain Cortical Membranes

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ABSTRACT. The binding of [^{3}H]cholecystokinin octapeptide (sulphated) ([^{3}H]CCK-8S), an agonist of the cholecystokinin receptors, to rat cortical membranes was fast, specific and saturable, with pH optimum at 6.5–7.0. The divalent cations Mg^{2+} and Ca^{2+} clearly enhanced [^{3}H]CCK-8S binding, whereas the monovalent cations Na^+ and K^+ were inhibitors. Inactivation of the ligand binding ability of these membranes was dependent on the incubation temperature and corresponding $\tau_{1/2}$ values were 11 days at 4°, 12 hr at 21°, 154 min at 30° and 51 min at 37°, which revealed the apparent activation energy of this process to be 130 ± 4 kJ/mol. Scatchard analysis of the saturation curves of [^{3}H]CCK-8S binding was best described by a one site binding model with a K_d = 0.63 ± 0.18 nM and a maximum binding of 32 ± 2 fmol/mg protein. The stable GTP analogue guanosin-5′-O-(3-thiotriphosphate) (GTPγS) decreased the affinity of [^{3}H]CCK-8S binding only up to 2-fold without significant influence on maximal binding. Modulation of membrane properties by different detergents revealed that only in the case of digitonin (0.03–0.04%) did the GTP-dependence of [^{3}H]CCK-8S binding considerably increase without significant influence on the ligand binding properties in the absence of GTPγS. Other detergents studied (sodium cholate, sodium deoxycholate, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate (CHAPS), sucrose monolaurate, series Triton X and Tween) either had little influence on GTPγS-dependence of [^{3}H]CCK-8S binding or inactivated the receptor. Parallel studies of fluorescent polarization of diphenylhexatriene (DPH) in rat cortical membranes indicated that digitonin was the only detergent which at low concentrations caused a rapid increase in membrane fluidity and thereafter stabilized it at a certain level. Other detergents studied had only moderate influence on membrane fluidity (CHAPS, cholate, deoxycholate) or caused fast and continuous increase of membrane fluidity (Triton X-100, Tween 80). These data together point to the essential influence of the fluidity of membranes on the regulation of the interactions between G proteins and CCK receptors in rat cortical membranes. Under standard experimental conditions (temperature lower than 30°), the CCK receptor-G protein complex is active for quantitative characterization of the receptors, but the membranes are too rigid for natural communication and regulation. BIOCHEM PHARMACOL 55;4:423–431, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cholecystokinin receptor; G proteins; rat brain; cholecystokinin octapeptide; CCK-8S; membrane fluidity; CCK receptor-G protein complex

Cholecystokinin (CCK)‖, a 33 amino acid peptide first purified from pig small intestine [1], also occurs in several other molecular forms and is widely distributed throughout the central and peripheral nervous systems [2]. Receptors for CCK were first found in pancreatic acinar cells (CCK_A) [3], followed by the finding of receptors with different pharmacological properties (CCK_B) in the brain [4]. Pharmacological data have also suggested the presence of gastrin and CCK-4 receptors [5], but molecular cloning studies have revealed only two different receptor types, CCK_A and CCK_B. These discrepancies in pharmacology have been explained by the different coupling with G proteins, which causes multiple affinity states for ligand binding [6]. The amino acid sequences of the rat CCK_A and CCK_B receptors with 429 and 452 amino acids, respectively, are highly homologous with 48% amino acid identity [7]. Hydropathy analysis of the structure of CCK receptors predicts seven transmembrane-spanning domains and suggests that these receptors belong to the G protein-coupled superfamily of receptors [8]. CCK_B receptors have been found throughout the brain with especially high densities in the striatum, cortex, and limbic system [9], but CCK_A receptors are also

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‖*Abbreviations:* B_{max}, maximal ligand binding capacity; CCK, cholecystokinin; CCK-8S, cholecystokinin octapeptide, sulphated; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; DPH, 1,6-diphenyl-1,3,5-hexatriene; GTPγS, guanosine 5′-O-(3-thiotriphosphate); [^{3}H]CCK-8S, [propionyl-^{3}H]-cholecystokinin octapeptide (sulphated); K_d, dissociation constant of ligand binding; MES, 4-morpholine ethanesulfonic acid; PIPES, 1,4-piperazine diethanesulfonic acid.

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found in certain brain areas [10]. While the signal transduction mechanism of the CCK_A receptors via pertussis toxin-insensitive G proteins is well documented, less is known about the CCK_B pathway. It is proposed that G proteins from the G_q family play a crucial role in CCK_B transmission, causing activation of phospholipase C, formation of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, and release of intracellular Ca^{2+} [7].

Guanyl nucleotides induce a clear shift in agonist binding affinity of several G protein-coupled receptors. For example, for muscarinic and dopaminergic receptors the shift can be considerably higher than 100-fold [11, 12]. However, the guanyl nucleotides have only modest effect on the agonist binding to the CCK_B receptors and even these data seem to be quite inconsistent [13–16]. In the present investigation we have studied the optimal binding parameters of [^{3}H]CCK-8S to rat cortical membranes and found that membrane fluidity seems to be crucial in the GTP-dependent regulation of agonist binding to CCK_B receptors.

MATERIALS AND METHODS

Materials

[Propionyl-^{3}H]-cholecystokinin octapeptide (sulphated) ([^{3}H]CCK-8S, 60–73 Ci/mmol) was from Amersham Inc., 4-morpholine ethanesulfonic acid (MES), 1,4-piperazine diethanesulfonic acid (PIPES) and Tris from United States Biochemical Corporation, HEPES and guanosine 5′-O-(3-thiotriphosphate) (GTPγS) from Boehringer Mannheim, BSA, cholic acid, digitonin, 1,6-diphenyl-1,3,5-hexatriene (DPH), polyethylenimine, Triton X-100 from Sigma Chemical Co., Tween 80 and sodium deoxycholate from Merck, sucrose monolaurate from Mitsubishi-Kasei Food Co., caerulein from Bachem AG, and 4-{[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[[1.7.7.-trimethylbicyclo [2.2.1]hept-2-yl-oxy]carbonyl]amino]propyl]amino]1-phenylmethyl] amino-4-oxo-[1S-1a.2β[S*(S*)]4a]}-butanoate *N*-methyl-D-glucamine (CAM 1028) was donated by Parke-Davis Neuroscience Researche Centre.

Membrane Preparation

Rat cerebral cortices were homogenized in 20 vol (wet weight/volume) of ice-cold 50 mM Tris-HCl buffer (pH 7.4) using a glass-teflon homogenizer, and centrifuged at 30,000 × g for 20 min at 4°. The membrane pellet was resuspended in 20 vol (ww/v) of fresh buffer and centrifuged under the same conditions. The final pellet was homogenized in a buffer containing 20 mM Na-HEPES, 20 mM NaCl, 5 mM $MgCl_2$, 0.5 mg/mL BSA (pH 7.0 at 21°) if not stated otherwise, and was used for binding experiments at a concentration of 15–20 mg wet weight per mL, which corresponded to approximately 0.7–1.0 mg protein per mL as determined by the modified Lowry method [17], using bovine serum albumin as standard. In the studies at different pH values, the buffering components were 20 mM Na-MES (pH 5.5), Na-PIPES (pH 6.0; 6.5; 7.0), Na-HEPES (pH 7.0; 7.5; 8.0) or Tris-HCl (pH 7.5; 8.0; 8.5). A mixture of G_i and G_o was prepared from porcine brain as previously described [18] with slight modifications [19] and inserted into cortical membranes [20].

Ligand Binding Assay

[^{3}H]CCK-8S was found to attach to plastic and glass surfaces and all experiments were therefore performed in tubes and with pipette tips which were siliconized with Sigmacote.® (Sigma Chemical Co.) For the equilibrium binding studies, the crude membrane homogenate was incubated with [^{3}H]CCK-8S (0.05–5 nM) for 90 min at 21° and free ligands were removed by fast filtration through glass-fiber filter (GF/B, Whatman International Ltd.). The filters were washed three times with 3 mL cold incubation buffer, and bound [^{3}H]CCK-8S, trapped on the filter, was counted with a liquid scintillation counter. The specific binding was defined as the difference between total and nonspecific binding, which was measured in the absence and presence of 0.1 μM caerulein or 3 μM CAM 1028, respectively. To avoid possible artefacts in determination of the relatively high levels of nonspecific binding, two different ligands were used, but with virtually similar results. The pretreatment of the filters with 0.3% (w/v) polyethylenimine had no significant influence on the nonspecific binding of [^{3}H]CCK-8S. All binding data were analyzed by nonlinear least-squares regression analysis by using a commercial program GraphPad PRISM™ (GraphPad Software) and the results are presented as mean ± SE.

Determination of Membrane Fluidity

Fluidity of rat cortical membranes was measured by the determination of fluorescence polarization of DPH in the membrane suspension, which allows one to characterize the apparent microviscosity of these membranes [21, 22]. For the insertion of the marker, the cortical membranes in the incubation buffer were incubated with 2 μM DPH for 30 min at 37°. Membranes were diluted with the incubation buffer in the presence of different concentrations of the detergents, and the fluorescent polarization of DPH was measured at 21° with a spectrofluorometer (SLM Instruments Inc) using excitation and emission wavelengths of 358 and 430 nm, respectively. The degree of fluorescence polarization P was calculated as:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}},$$

where $I_{\parallel}$ and $I_{\perp}$ are the emission intensities detected by the analyzer oriented parallel or perpendicular to the direction of polarization of the excitation light, respectively. The value of fluorescence polarization of DPH is proportional to the order of molecular packing and inversely proportional to membrane fluidity [21].

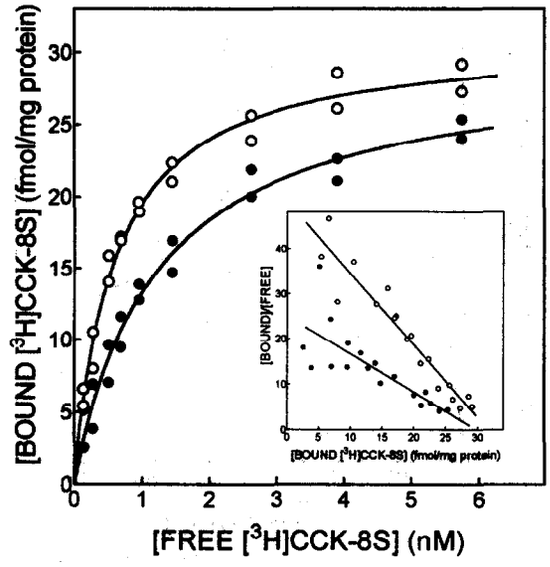

FIG. 1. Equilibrium of the specific binding of [^{3}H]CCK-8S to rat cortical membranes in the presence of 30 μM GTPγS (●) or in the absence of GTPγS (○). Rat cortical membranes in the buffer containing 20 mM Na-HEPES, 20 mM NaCl, 5 mM $MgCl_2$, 0.5 mg/mL BSA (pH = 7.0) were incubated with different concentrations of [^{3}H]CCK-8S in the absence (total binding) and presence (nonspecific binding) of 0.1 μM caerulein for 90 min at 21°. Specific binding was defined as the difference between total and nonspecific binding. Inset: Data in the Scatchard plot.

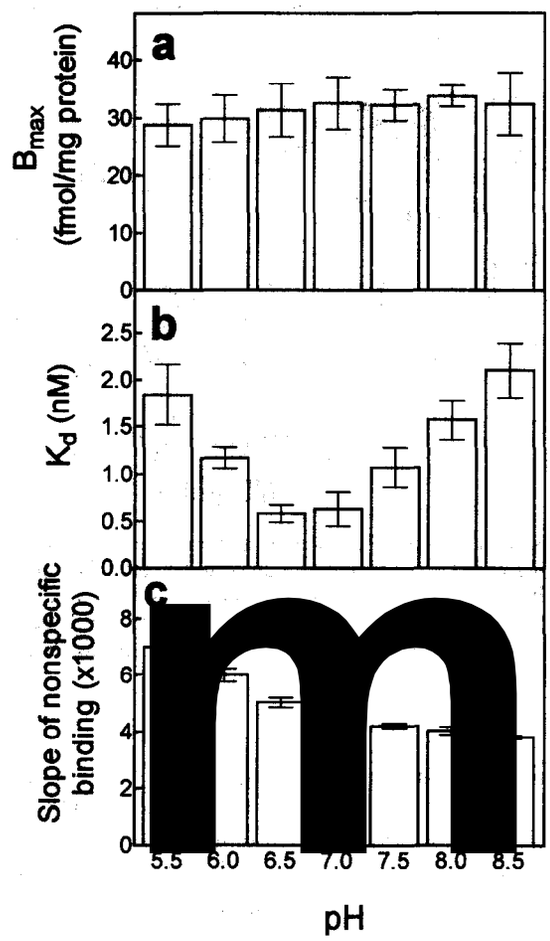

FIG. 2. Influence of pH on the binding parameters B_{max} (a), K_d (b), and the slope of the nonspecific binding (c) of [^{3}H]CCK-8S binding to rat cortical membranes. Rat cortical membranes in 20 mM Na-MES (pH = 5.5), Na-PIPES (pH = 6.0; 6.5; 7.0), Na-HEPES (pH = 7.0; 7.5; 8.0) or Tris-HCl (pH = 7.5; 8.0; 8.5) with 20 mM NaCl, 5 mM $MgCl_2$, 0.5 mg/mL BSA were incubated with different concentrations of [^{3}H]CCK-8S in the absence (total binding) and presence (nonspecific binding) of 0.1 μM caerulein for 90 min at 21°. Specific binding was defined as the difference between total and nonspecific binding. The slope of nonspecific binding was calculated from the linear relationship of [^{3}H]CCK-8S binding in the presence of 0.1 μM caerulein vs. [^{3}H]CCK-8S concentration. Data are presented as mean ± SEM.

RESULTS

The curve of [^{3}H]CCK-8S binding to rat cortical membranes was saturable (Fig. 1), and the straight line obtained by Scatchard analysis (Fig. 1, inset) indicates the presence of a single class of [^{3}H]CCK-8S binding sites. The dissociation constant (K_d) and maximal binding capacity (B_{max}) were estimated to be 0.63 ± 0.18 nM and 32 ± 2 fmol/mg protein, respectively. The studies of [^{3}H]CCK-8S binding to rat cortical membranes were carried out at room temperature in the reaction medium, which contained 20 mM Na-HEPES, 20 mM NaCl, 5 mM $MgCl_2$, 0.5 mg/mL BSA (pH = 7.0). These conditions were found to be an optimal compromise between different factors influencing [^{3}H]CCK-8S binding.

The pH value 7.0 was selected as optimum of three different parameters: highest ligand binding affinity, highest number of specific binding sites, and lowest level of nonspecific binding (Fig. 2). The pH had no significant influence on the number of binding sites, but had a clear optimum for the affinity of [^{3}H]CCK-8S binding at 6.5–7.0. The nonspecific binding of [^{3}H]CCK-8S decreased with an increase in pH. None of the parameters studied depended on the type of buffer if several buffers (PIPES, HEPES, Tris) were used at the same pH values.

Regarding the ions studied, the presence of Mg^{2+} or Ca^{2+} was essential for high affinity [^{3}H]CCK-8S binding. The $MgCl_2$ at concentrations above 3 mM caused a 6-fold increase in the specific binding of 2 nM [^{3}H]CCK-8S, whereas in the case of $CaCl_2$, the increase in specific binding was also accompanied by a significant increase in nonspecific binding. The effects of Mg^{2+} and Ca^{2+} were not cumulative and usage of both cations did not initiate additional binding of [^{3}H]CCK-8S. Sodium as well as potassium ions were not essential for the specific binding of [^{3}H]CCK-8S and had a moderate inhibitory effect at concentrations above 50 mM. One of these monovalent cations was required at a concentration of 10–40 mM to reduce nonspecific binding (data not shown). BSA was also necessary to decrease nonspecific binding and had no inhibitory effect on [^{3}H]CCK-8S binding, as was described in the case of [^{125}I]-Bolton-Hunter labelled CCK-8S [23].

The stability of CCK receptors in rat cortical membranes

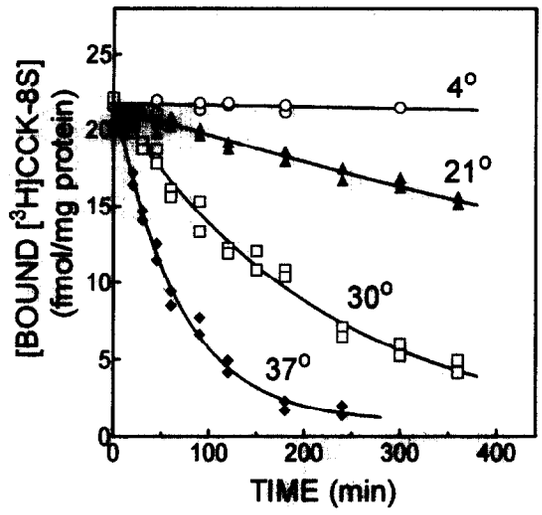

FIG. 3. Inactivation of [^{3}H]CCK-8S binding sites in rat cortical membranes. Rat cortical membranes in 20 mM Na-HEPES, 20 mM NaCl, 5 mM $MgCl_2$, 0.5 mg/mL BSA (pH = 7.0) were incubated at the different temperatures. At the indicated time, aliquots of incubated samples were taken and specific binding of [^{3}H]CCK-8S was determined.

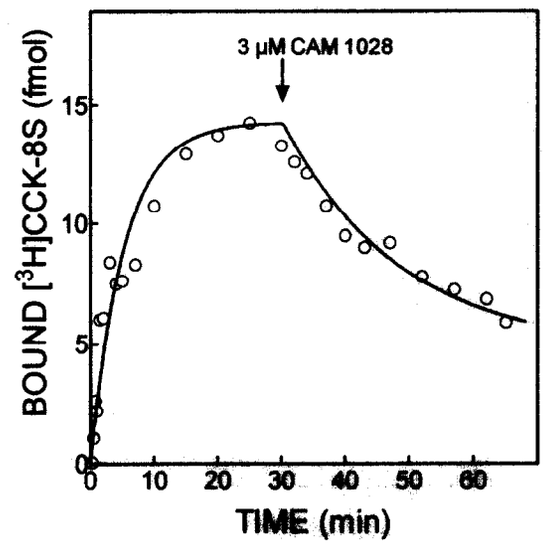

FIG. 4. Association and dissociation of [^{3}H]CCK-8S. Rat cortical membranes in 20 mM Na-HEPES, 20 mM NaCl, 5 mM $MgCl_2$, 0.5 mg/mL BSA (pH = 7.0) were incubated with 1.9 nM [^{3}H]CCK-8S at 21°, and at the indicated time aliquots of the incubation medium were taken and specifically bound [^{3}H]CCK-8S was determined. The dissociation was initiated by 3 μM CAM 1028 after 30 min preincubation with [^{3}H]CCK-8S.

was measured at different temperatures (4°, 21°, 30° and 37°). The decrease in the specific binding of [^{3}H]CCK-8S was dependent on the incubation temperature (Fig. 3), and the half-lives were estimated to be 51 ± 6 min at 37°, 154 ± 10 min at 30°, 12 ± 1 hr at 21° and 11 ± 3 days at 4°. The stability of the receptors was not affected by the addition of protease inhibitors such as pepstatin (1 μg/mL), benzamidine (0.5 mM), or PMSF (0.1 mM), or by dithiotreitol (1 mM), indicating that proteolytic degradation and oxidation of sulfhydryl groups are not the major factors in determining the rate of inactivation. The short half-lives of the cortical CCK receptors at 30° and 37° complicated the use of these temperatures for the characterization of ligand binding properties and the incubation temperature for this study was thus selected to be 21°.

The Arrhenius plot of the inactivation rate constants of [^{3}H]CCK-8S binding sites in rat cortical membranes revealed a straight line (r^2 = 0.97), indicating a single mechanism of the process at all temperatures studied. The activation energy was estimated to be 130 ± 4 kJ/mol, which is within the range obtained in other studies (120–250 kJ/mol) for the inactivation of many solubilized proteins [24], but considerably higher than the value for the muscarinic receptors in rat cortical membranes (57 ± 8 kJ/mol) [25], in which case the determining role of surrounding lipids was proposed.

Specific binding of [^{3}H]CCK-8S to rat cortical membranes was reversible and time-dependent. The association of 1.9 nM [^{3}H]CCK-8S had a half-life of $\tau_{1/2}$ = 3.7 ± 0.6 min, reaching a saturation level within 30 min (Fig. 4). Dissociation of [^{3}H]CCK-8S from these membranes, which was initiated with 3 μM CAM 1028, was monophasic with $k_{-1} = (9.0 \pm 1.6)\cdot10^{-4}$ s^{-1}, giving a half-life for the complex $\tau_{1/2}$ = 13 ± 2 min. The dissociation of the [^{3}H]CCK-8S from the membranes was clearly monophasic without the signs of heterogeneity which had been described earlier [23]. These kinetic data revealed that the apparent second-order rate constant of the [^{3}H]CCK-8S binding, which corresponds to the simple bimolecular association reaction scheme, is $k_1 = (1.17 \pm 0.27)\cdot10^6$ s^{-1} M^{-1}, and the dissociation constant K_d calculated from the ratio of rate constants k_{-1}/k_1 = 0.77 ± 0.18 nM is in good agreement with the constant K_d = 0.63 ± 0.18 nM determined from the equilibrium binding isotherm (Fig. 1).

The addition of the nonhydrolyzable GTP analogue GTPγS to the reaction medium caused a 2-fold increase in K_d (from 0.63 ± 0.18 nM to 1.27 ± 0.24 nM), without a significant effect on the number of binding sites (30 ± 3 fmol/mg protein, Fig. 1). The effect of the GTPγS reached its maximal value at 3 μM and did not change as a result of a further increase in the concentration of the nucleotide (data not shown). Similar results were obtained using the artificial G protein activator AlF_4^- at concentration 30 μM. The insertion of purified G proteins (G_o, G_i) into cortical membranes did not cause significant changes in [^{3}H]CCK-8S binding parameters in the absence or presence of guanine nucleotides (data not shown), suggesting that a low concentration of G proteins is not the reason for the moderate effect of GTPγS.

The role of membrane properties on the GTP-dependent binding of [^{3}H]CCK-8S in rat cerebral cortex was studied by modulation of these membranes by different detergents. Seven detergents, listed in Table 1, were examined for their

TABLE 1. Influence of detergents on the GTP dependence of the specific binding of [^{3}H]CCK-8S to the rat brain cortical membranes

Detergent	Studied concentrations of the detergent % (w/v)	Maximal effect of GTPγS: Optimal concentration of the detergent % (w/v)	Specific binding of [^{3}H]CCK-8S: No GTPγS (% of control)*	Specific binding of [^{3}H]CCK-8S: Decrease by 30 μM GTPγS (%)†
Control	—	—	100 ± 2	23 ± 3
Triton X-100	0.02–0.2	—	0	0
Tween 80	0.02–0.2	0.02	88 ± 5	40 ± 2
CHAPS	0.02–0.2	0.02	74 ± 3	36 ± 6
Na-cholate	0.01–0.2	0.04	58 ± 2	71 ± 3
Na-deoxycholate	0.01–0.1	0.01	56 ± 1	52 ± 7
Digitonin	0.01–0.2	0.04	78 ± 4	82 ± 1
Sucrose monolaurate	0.005–0.1	0.03	84 ± 9	62 ± 6

The membranes were incubated with 2.6 nM [^{3}H]CCK-8S and different concentrations of the detergents in the absence and presence of 30 μM GTPγS. Data are presented as percentage of the specific binding of [^{3}H]CCK-8S without detergents (*) and as percentage of decrease in [^{3}H]CCK-8S by GTPγS at the optimal detergent concentration in comparison with [^{3}H]CCK-8S binding in the absence of GTPγS (†).

influence on the binding of 2.6 nM of [^{3}H]CCK-8S to rat cortical membranes in the presence and absence of 30 μM GTPγS. Triton X-100 totally abolished specific binding of [^{3}H]CCK-8S at the concentration of 0.02%, whereas in the presence of all other detergents at least some specific [^{3}H]CCK-8S binding could be determined and an increase in GTP dependence was found (Table 1). Tween 80 and sodium deoxycholate were also harmful to the receptors, leading to the disappearance of specific binding of [^{3}H]CCK-8S at a concentration of 0.04%. CHAPS, sodium cholate and sucrose monolaurate caused the loss of the specific binding of 2.6 nM [^{3}H]CCK-8S at concentrations of 0.2%, 0.15% and 0.1%, respectively, whereas 0.2% of digitonin decreased the binding by only 56%. Binding isotherms of [^{3}H]CCK-8S binding were studied in the presence of different concentrations of sodium cholate and digitonin, the detergents which caused the greatest effects of GTPγS in preliminary experiments (Table 1). The apparent effect of sodium cholate seems to be caused mainly by the decrease in binding affinity of [^{3}H]CCK-8S to receptors in presence of this detergent. The dissociation constants of the [^{3}H]CCK-8S binding in the presence of 0.02%, 0.04%, and 0.08% of sodium cholate could be estimated to be 1.28 ± 0.21, 2.27 ± 0.43 and 3.65 ± 0.58 nM, respectively, and in the presence of 30 μM GTPγS 3.2 ± 0.7, 6.4 ± 1.1, 8.0 ± 1.2 nM, respectively. Thus, the decrease in binding affinity by GTPγS was on average 2.4-fold and did not depend on the concentration of the cholate. Digitonin caused only a moderate decrease in [^{3}H]CCK-8S binding affinity without changes in the number of binding sites, but dramatically enhanced the effect of GTPγS on [^{3}H]CCK-8S binding (Table 2). The low affinity of [^{3}H]CCK-8S binding in the presence of GTPγS at a digitonin concentration above 0.03% did not allow direct calculations of K_d and B_{max} (Fig. 5), and data presented in Table 2 were calculated on the presumption that the number of binding sites did not change as a consequence of the addition of GTPγS.

Detergents listed in Table 1 can be divided into three groups according to their influence on the fluidity of rat cortical membranes. The first group contains CHAPS, sodium cholate and deoxycholate, which caused only a moderate increase in membrane fluidity at concentrations up to 0.4% (Fig. 6). Digitonin and sucrose monolaurate are members of the second group, which caused a rapid increase in membrane fluidity at concentrations up to 0.02% and a polarization parameter 0.255. The subsequent increase in detergent concentrations had little influence on the fluidity of these membranes (Fig. 6). Detergents Triton X-100 and Tween 80 represent the third group, which caused a fast and continuous increase in membrane fluidity up to a concentration of 0.1% and a polarization parameter of 0.17 without the stable phase found for the second group.

An increase in temperature also increased fluidity of membranes. Thus, the degree of fluorescent polarization in rat cortical membranes was 0.297 ± 0.003, 0.273 ± 0.002 and 0.256 ± 0.006 at 21°, 30° and 37°, respectively.

TABLE 2. Influence of digitonin on [^{3}H]CCK-8S binding affinity to rat brain cortical membranes in the absence and presence of 30 μM GTPγS

Concentration of digitonin (%)	No GTPγS K_d (nM)	30 μM GTPγS K_d (nM)	Change by GTPγS (fold)
0	0.63 ± 0.18	1.27 ± 0.24	2.02
0.02	0.84 ± 0.16	2.84 ± 0.31	3.38
0.03	1.01 ± 0.21	13.2 ± 2.8*	13.1
0.04	1.29 ± 0.15	45 ± 6*	35
0.10	1.82 ± 0.12	n.d.**	n.d.**

* Estimated value with the presumption that the number of binding sites was not changed (Fig. 5).

** No specific [^{3}H]CCK-8S binding could be detected.

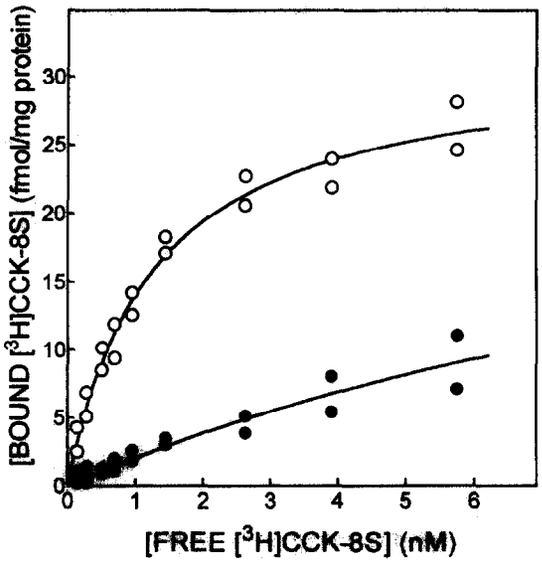

FIG. 5. Equilibrium of the specific binding of [^{3}H]CCK-8S to rat cortical membranes in the presence of 0.03% digitonin. Rat cortical membranes in the buffer containing 20 mM Na-HEPES, 20 mM NaCl, 5 mM $MgCl_2$, 0.5 mg/mL BSA, 0.03% digitonin (pH = 7.0) in the presence of 30 μM GTPγS (●) or in the absence of GTPγS (○) were incubated with different concentrations of [^{3}H]CCK-8S in the absence (total binding) and presence (nonspecific binding) of 0.1 μM caerulein for 90 min at 21°. Specific binding was defined as difference between total and nonspecific binding.

Despite inactivation, the tentative binding parameters of [^{3}H]CCK-8S binding to rat cortical membranes at 30° were K_d = 1.14 ± 0.38 nM and B_{max} = 24 ± 7 fmol/mg protein, and no specific binding could be determined in the presence of 30 μM GTPγS.

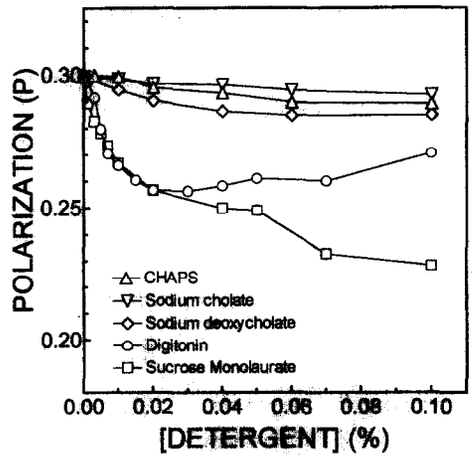

FIG. 6. Influence of detergents on the fluidity of rat cortical membranes. Rat cortical membranes in the buffer containing 20 mM Na-HEPES, 20 mM NaCl, 5 mM $MgCl_2$, (pH = 7.0) were incubated with 3 μM 1,6-diphenyl-1,3,5-hexatriene for 30 min at 37° and the fluorescence polarization (P) of the preparation was determined in the presence of different detergents at 21°.

DISCUSSION

Several earlier studies have indicated specific and saturable binding of [^{3}H]CCK-8S to rat cortical membranes, but always with a low number of binding sites and high nonspecific binding, especially in comparison with cortical membranes from guinea-pig and mouse [13, 23]. Nevertheless, psychopharmacological and behavioural studies on CCK-ergic neurotransmission make the biochemical characterization of [^{3}H]CCK-8S binding sites in rat cerebral cortex appealing [26]. In the present study, we have found that the binding of [^{3}H]CCK-8S to rat brain membranes was relatively fast, reversible and saturable, whereas optimal experimental conditions for binding isotherms appear to be an incubation for 90 min at 21° in a buffer containing 20 mM Na-HEPES, 20 mM NaCl, 5 mM $MgCl_2$, 0.5 mg/mL BSA at pH = 7.0. These conditions are a compromise between many different factors which influence binding and had to be taken into account during the experiments. The proposed pH = 7.0 is slightly higher than pH = 6.0–6.5 proposed for radioiodinated ligands [27], but was preferred due to a lower nonspecific binding and to its being closer to the optimal pH for G proteins [18]. The 40 mM Na^+ was also a compromise between the inhibitory effect of Na^+ salts at concentrations over 100 mM and their ability to decrease nonspecific binding. The inhibitory effect of Na^+ ions was not ion-specific, as K^+ had a similar inhibitory effect at concentrations over 100 mM. It is proposed that the inhibitory effect of monovalent ions at higher concentrations on [^{3}H]CCK-8S binding is caused by their desalting effect on peptic ligands or by their influence on the stability of receptor-G protein complexes, as is the case for agonist binding to several G protein-coupled receptors [20, 28, 29]. The presence of Mg^{2+} in millimolar concentrations was essential for high-affinity [^{3}H]CCK-8S binding, as could be predicted for agonist binding to G protein-coupled receptors. It is interesting to note that the same effect as that of Mg^{2+} could also be achieved with Ca^{2+}. These data are in agreement with data published earlier for CCK-8S binding to cortical membranes [27, 30], but not with the understanding of the high specificity of Mg^{2+} for the activation of G proteins [12, 31], which has been confirmed by the recently published tertiary structures of G proteins [32, 33]. Thus, it can be proposed that the requirement of Mg^{2+} for [^{3}H]CCK-8S binding is not only connected with the activation of G proteins. Alternatively, G proteins coupled to CCK receptors in rat cortical membranes may not be so highly specific for Mg^{2+} as has been described for G_0 and transducin.

A compromise between different influences was also needed for the choice of the incubation time and temperature of [^{3}H]CCK-8S binding. In comparison with several other G protein-coupled receptors, the cortical CCK_B receptors were quite labile, losing a considerable number of binding sites during incubation at 30° or 37°. Incubation for 90 min at 21° inactivated approximately 8% of the [^{3}H]CCK-8S binding sites, but taking into account the

stabilizing effect of bound ligands, inactivation can be neglected during the following analysis. Other receptors (e.g. the muscarinic acetylcholine and the dopaminergic) are much more stable in *in vitro* experiments, but the stability of these receptors has been measured by their ability to bind specific antagonists [11, 34]. In the present study, the stability of CCK receptors was characterized by their ability to bind a specific agonist, which requires the active conformation of whole receptor-G protein complexes. Earlier studies have indicated that receptor-G protein complexes [20], as well as G proteins themselves [35], are much more labile than receptors; therefore, it can be proposed that the loss of [^{3}H]CCK-8S binding activity is not mainly connected to the thermoinactivation of CCK receptors but also by the decomposition of receptor-G protein complexes.

The lability of the binding ability of receptors was the reason for the choice of an incubation time of 90 min, although the kinetic studies indicated long incubation times to reach equilibrium at all studied [^{3}H]CCK-8S concentrations. Thus, 95% of the saturation (5 $\tau_{1/2}$) of [^{3}H]CCK-8S binding during 90 min incubation can be achieved only at a radioligand concentration of 0.4 nM or higher. At lower concentrations, for example 0.1 nM, the half-life of [^{3}H]CCK-8S binding was 70 min, reaching only the level of 59% of the level predicted by equilibrium binding after 90 min incubation. In all cases presented here, the K_d values for [^{3}H]CCK-8S binding were higher than 0.6 nM, and it can thus be predicted that these values were not overestimated since equilibrium was not achieved. That the binding was not complete as well as being very close to the lower limit of detection of specific binding may be reasons for the scattering of the points in the Scatchard plot (Fig. 1) and for the proposed heterogeneity of [^{3}H]CCK-8S binding in some reports [36].

The effect of GTP, which decreased the affinity of [^{3}H]CCK-8S binding to rat brain membranes ca. 2-fold, was considerably smaller than could be predicted by the model of the agonist binding to G protein-coupled receptors, where the effects of guanyl nucleotides on K_d values are usually in the order of a 10- to 1000-fold increase [8]. At the same time, the data obtained are in good agreement with earlier published data on the GTP dependence of [^{3}H]CCK-8S binding to brain membranes of different species [14, 15, 37, 38]. Higher concentrations of GTPγS (up to 300 μM) or use of the artificial G protein activator AlF_4^- did not result in additional inhibition of [^{3}H]CCK-8S binding, indicating that: 1) either the addition of these reagents is not enough to switch the CCK receptors into the low affinity state; or 2) [^{3}H]CCK-8S binding sites are heterogeneous and regulated by different effector systems; or 3) the affinities of the low- and high affinity sites of [^{3}H]CCK-8S binding are very similar to what has been found for D_3 receptors [29]. The first proposal was supported by data showing that mild modulation of membrane properties dramatically increased the GTP dependence of [^{3}H]CCK-8S binding. This most clearly appeared using digitonin, which considerably increased the GTP-dependence of [^{3}H]CCK-8S binding to rat cortical membranes at very low concentrations (Table 2). At concentrations which cause this effect (0.03–0.04%), digitonin does not yet solubilize receptors from membranes [39], but this concentration is much higher than is necessary for the permeabilization of membranes. It is proposed that the effect of digitonin on GTP-dependent binding of [^{3}H]CCK-8S is connected to its effect on membrane fluidity, as only this detergent had the ability to increase membrane fluidity and thereafter stabilize the complex (Fig. 6). All other detergents caused a rapid increase in fluidity and probably destruction of membrane structures, which led to the inactivation of receptors (Triton X-100, Tween 20, Tween 80) or had only a moderate influence on the fluidity of membranes as well on the GTP dependence of [^{3}H]CCK-8S binding (cholate, deoxycholate, CHAPS). In addition to digitonin, sucrose monolaurate also had some stabilizing effect on the membranes after a fast increase in fluidity at low concentrations of this detergent, but at higher concentrations it caused an additional increase in membrane fluidity (Fig. 6). Therefore, the solubilized receptors in sucrose monolaurate are more labile than in digitonin [40], and in the present study the detergent concentration of 0.04% caused a considerable loss of [^{3}H]CCK-8S binding sites.

A controlled increase in membrane fluidity can also be achieved with increasing incubation temperatures, but in this case the critical point is inactivation of the receptors. The tentative binding parameters of [^{3}H]CCK-8S and its GTP dependence as well as fluidity of membranes determined at 30° were very close to the analogous parameters at 21° in the presence of 0.04% digitonin. Taken together, these results support the idea of the crucial role of membrane fluidity on the guanyl nucleotide-dependent binding of [^{3}H]CCK-8S to rat cortical membranes. The temperature of 21°, which was selected to be optimal for the characterization of [^{3}H]CCK-8S binding to rat cortical membranes, seems to be below the transition temperature of membranes and corresponds to the "frozen" state, where fatty acyl chains of lipids are packed together in an ordered crystalline form [41]. In this case, the free communication and movement of proteins in membranes is greatly hindered. Thus, most CCK_B receptor-G protein complexes in the membranes appear to be in such a frozen state, and guanine nucleotides are not able to break these complexes and turn the receptors into low-affinity state. Most membrane proteins require the lipids to be in a "fluid" state for optimal activity, and this also seems to be the case for CCK_B receptors and G proteins in rat cortical membranes. The transition temperature is a characteristic of the membrane and is affected by several membrane components such as cholesterol and proteins [41]. It is proposed that the effect of digitonin in the present study is connected with the decrease in transient temperature and/or with the decrease in the structural order of the membranes. Thus, room temperature is suitable for the determination of the number

of receptors and their affinity, but cannot be used for the characterization of interactions between CCK receptors and G proteins. For the latter purpose, a higher incubation temperature has to be used or some digitonin added to increase membrane fluidity for adequate communication between proteins to occur.

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References

1. Mutt V and Jorpes JE, Structure of porcine cholecystokinin-pancreozymin. 1. Cleavage with thrombin and with trypsin. *Eur J Biochem* **6:** 156–162, 1968.
2. Morency MA and Mishra RK, Cholecystokinin (CCK) receptors. In: *Peptide Hormone Receptors*, pp. 385–436. Walter de Gruyter & Co, Berlin, 1987.
3. Sankaran H, Goldfine ID, Deveney WC, Wonga KY and Williams JA, Binding of cholecystokinin to high affinity receptors on rat pancreas. *J Biol Chem* **255:** 1849–1853, 1980.
4. Innis RB and Snyder SH, Distinct cholecystokinin receptors in brain and pancreas. *Proc Natl Acad Sci USA* **77:** 6917–6921, 1980.
5. Menozzi D, Gardner JD, Jensen RT and Maton PN, Properties of receptors for gastrin and CCK on gastric smooth muscle cells. *Am J Physiol* **257:** G73–G79, 1989.
6. Boden P, Hall MD and Hughes J, Cholecystokinin receptors. *Cell Mol Neurobiol* **15:** 545–559, 1995.
7. Wank SA, Cholecystokinin receptors. *Am J Physiol* **269:** G628–G646, 1995.
8. Dohlman HG, Thorner J, Caron MG and Lefkowitz RJ, Model systems for the study of seven-transmembrane-segment receptors. *Annu Rev Biochem* **60:** 653–688, 1991.
9. Beinfeld MC, Cholecystokinin in the central nervous system: a minireview. *Neuropeptides* **3:** 411–427, 1983.
10. Hill DR, Shaw TM, Graham W and Woodruff GN, Autoradiographical detection of cholecystokinin-A receptors in primate using ^{125}I-Bolton-Hunter CCK-8 and ^{3}H-MK-329. *J Neurosci* **10:** 1070–1081, 1990.
11. Seeman P and Grigoriadis D, Dopamine receptors in brain and periphery. *Neurochem Int* **10:** 1–25, 1987.
12. Shiozaki K and Haga T, Effects of magnesium ion on the interaction of atrial muscarinic acetylcholine receptors and GTP-binding regulatory proteins. *Biochemistry* **31:** 10634–10642, 1992.
13. Durieux C, Pham H, Charpentier B and Roques BP, Discrimination between CCK receptors of guinea-pig and rat brain by cyclic CCK8 analogues. *Biochem Biophys Res Commun* **154:** 1301–1307, 1988.
14. Wennogle L, Wysowskyj H and Petrak B, Regulation of central cholecystokinin recognition sites by guanyl nucleotides. *J Neurochem* **50:** 954–959, 1988.
15. Kaufmann R, Schöneberg T, Henklein P, Meyer R, Martin H and Ott T, Effects of guanyl nucleotides on CCK_B receptor binding in brain tissue and continuous cell lines: A comparative study. *Neuropeptides* **29:** 63–68, 1995.
16. Lallement J-C, Oiry C, Lima-Leite A-C, Lignon M-F, Fulcrand P, Galleyrand J-C and Martinez J, Cholecystokinin and gastrin are not equally sensitive to GTPγS at CCK_B receptors: importance of the sulphated tyrosine. *Eur J Pharmacol Mol Pharmacol* **290:** 61–67, 1995.
17. Peterson GL, Determination of total protein. *Meth Enzymol* **91:** 95–119, 1983.
18. Sternweis PC and Robishaw JD, Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *J Biol Chem* **259:** 13806–13813, 1984.
19. Haga K, Haga T and Ichiyama A, Reconstitution of the muscarinic acetylcholine receptor. Guanine nucleotide-sensitive high affinity binding of agonists to purified muscarinic receptors reconstituted with GTP-binding proteins (G_i and G_o). *J Biol Chem* **261:** 10133–10140, 1986.
20. Rinken A, Formation of the functional complexes of m2 muscarinic acetylcholine receptors with GTP-binding regulatory proteins in solution. *J Biochem* **120:** 193–200, 1996.
21. Shinitzky M and Barenholz Y, Fluidity parameters of lipid regions determined by fluorescence polarization. *Biochim Biophys Acta* **515:** 367–394, 1978.
22. Bashford CL, Measurement of membrane fluidity and membrane fusion with fluorescent probes. In: *Biomembrane Protocols: II. Architecture and Function* (Eds. Graham JM and Higgins JA), pp. 177–187. Humana Press Inc., Totowa, 1994.
23. Wennogle LP, Steel DJ and Petrack B, Characterization of central cholecystokinin receptors using a radioiodinated octapeptide probe. *Life Sci* **36:** 1485–1492, 1985.
24. Joly M, *A Physico-Chemical Approach to the Denaturation of Proteins*. Academic Press, London, 1965.
25. Rinken AA, Langel ÜL, Tähepõld LJ and Järv JL, Kinetics of the inactivation of the muscarine cholinoreceptor, solubilized by digitonin. *Biokhimiia (Russ)* **49:** 1533–1537, 1984.
26. Harro J, Vasar E and Bradwejn J, CCK in animal and human research on anxiety. *Trends Pharmacol Sci* **14:** 244–249, 1993.
27. Saito A, Goldfine ID and Williams JA, Characterization of receptors for cholecystokinin and related peptides in mouse cerebral cortex. *J Neurochem* **37:** 483–490, 1981.
28. Bacopoulos NG, Dopamine receptors in rat brain regions. Optimal conditions for ^{3}H-agonist binding, pH dependency and lack of inhibition by ascorbic acid. *Biochem Pharmacol* **31:** 3085–3091, 1982.
29. Malmberg A and Mohell N, Characterization of [^{3}H]quinpirole binding to human dopamine D_{2A} and D_3 receptors: Effects of ions and guanine nucleotides. *J Pharmacol Exp Ther* **274:** 790–797, 1995.
30. Gut SH, Demoliou-Mason CD, Hunter JC, Hughes J and Barnard E, Solubilization and characterization of the cholecystokinin$_B$ binding site from pig cerebral cortex. *Eur J Pharmacol Mol Pharmacol* **172:** 339–346, 1989.
31. Higashijima T, Ferguson KM, Smigel MD and Gilman AG, The effect of GTP and Mg^{2+} on the GTPase activity and the fluorescent properties of G_o. *J Biol Chem* **262:** 757–761, 1987.
32. Wall MA, Coleman DE, Lee E, Iniguez-Lluhi JA, Posner BA, Gilman AG and Sprang SR, The structure of the G protein heterotrimer $G(\alpha_{i1}\beta_{1\gamma 2})$. *Cell* **83:** 1047–1058, 1995.
33. Lambright DG, Sondek J, Bohm A, Skiba NP, Hamm HE and Sigler PB, The 2.0 Å crystal structure of a heterotrimeric G protein. *Nature* **379:** 311–319, 1996.
34. Järv J, Arukuusk P, Kõiv A and Rinken A, Muscarinic receptor—biomembrane interaction. In: *Highlights of Modern Biochemistry* (Eds. Kotyr A, Skoda J, Paces V and Kostka V), pp. 1073–1082. Zeist, Prague, 1989.
35. Haga T, Ikegaya T and Haga K, The interaction of acetylcholine receptors in porcine atrial membranes with three kinds of G proteins. *Jpn Circ J* **54:** 1176–1184, 1990.
36. Durieux C, Coppey M, Zajac J-M and Roques BP, Occurrence of two cholecystokinin binding sites in guinea-pig brain cortex. *Biochem Biophys Res Commun* **137:** 1167–1173, 1986.
37. Chang RSL, Chen TB, Bock MG, Freidinger RM, Chen R,

Rosegay A and Lotti VJ, Characterization of the binding of [^{3}H]L-365,260: A new potent and selective brain cholecystokinin (CCK-B) and gastrin receptor antagonist radioligand. *Mol Pharmacol* **35:** 803–808, 1989.

38. Lallement JC, Galleyrand JC, Lima-Leite AC, Fulcrand P and Martinez J, Gastrin(13) and the C-terminal octapeptide of cholecystokinin are differently coupled to G-proteins in guinea-pig brain membranes. *Eur J Pharmacol Mol Pharmacol* **267:** 297–305, 1994.
39. Rinken A, Kameyama K, Haga T and Engström L, Solubilization of muscarinic receptor subtypes from baculovirus infected Sf9 insect cells. *Biochem Pharmacol* **48:** 1245–1251, 1994.
40. Rinken A and Haga T, Solubilization and characterization of atrial muscarinic acetylcholine receptors in sucrose monolaurate. *Arch Biochem Biophys* **301:** 158–164, 1993.
41. Stubbs CD, Membrane fluidity: Structure and dynamics of membrane lipids. *Essays Biochem* **19:** 1–39, 1983.