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Fourier transform infrared (FTIR) spectroscopic investigation of the nicotinic acetylcholine receptor (nAChR)

Investigation of agonist binding and receptor conformational changes by flash-induced release of 'caged' carbamoylcholine

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The binding and interaction of carbamoylcholine with the nicotinic acetylcholine receptor was investigated using photolytically released carbamoylcholine ('caged' carbamoylcholine). Upon UV flash activation of this photolabile substrate analog, characteristic changes in the IR absorbance spectrum were detected. Apart from difference bands arising from the changes of molecular structure upon photolytical release, spectral features can be attributed to the agonist upon binding to the receptor as well as to conformational changes of the receptor itself. The two photo-labile agonist analogs *N*-[1-(2-nitrophenyl)ethyl] carbamoylcholine iodide (cage I) and *N*-(α-carboxy-2-nitrobenzyl) carbamoylcholine trifluoroacetate (cage II), with different structures for comparison of the 1680–1540 cm^{-1} region sensitive for protein conformation, yielded consistent results. A preliminary interpretation in terms of substrate binding and local conformational changes of the receptor upon carbamoylcholine binding is provided, in analogy to the binding of acetylcholine, activation, and subsequent deactivation taking place during signal transduction.

Nicotinic acetylcholine receptor (nAChR); Conformational change; Agonist binding; FTIR

1. INTRODUCTION

The nicotinic acetylcholine receptor (nAChR) is a ligand-gated ion channel (type-I receptor) transducing extracellular signals through plasma membranes [1–3]. Despite a wealth of structural information relatively little is known about its mechanism of action. In particular, no information exists on how the binding of the neurotransmitter to the extracellular signal-receiving part of the receptor protein causes opening of the ion channel. The channel and its gating device is presumed to be located more than 40 Å away from the agonist binding site [4].

The nAChR is an allosteric protein [2,5]. The binding sites for effector molecules and the channel are thought to interact in a way similar to regulatory and active sites in allosteric enzymes. According to this model, signal transduction would take place through conformational changes induced by agonist binding. Conformational changes have been observed by means of fluorescence measurements using intrinsic [6] and extrinsic [7] fluorescent probes. Principally, protein conformational changes of the receptor should also be detectable by infrared absorption and derivative spectroscopy of the amide I band [8,9].

In this manuscript, we propose a different approach using the high sensitivity of Fourier transform infrared (FTIR) spectroscopy, which allows to detect contributions of individual bonds to the IR spectrum of the protein. FTIR difference spectroscopy, where a specific reaction is used to obtain the difference spectra between two states of one sample, has been successfully applied to photoreactive proteins [10,11] and redox proteins [12,13]. These difference spectra are selective for the functionally important parts of the protein rather than being selective for a specific cofactor or a substrate. Recently, the concept of FTIR difference spectroscopy has been extended to more general enzyme reactions by the use of inactive photolabile substrate derivatives which can be released with a UV flash to start the reaction. As an example, conformational changes of the Ca^{2+} ATPase from the sarcoplasmic reticulum caused

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Abbreviations: FTIR, Fourier-transform infrared; AChR, nicotinic acetylcholine receptor; ACh, acetylcholine; cage I, *N*-[1-(2-nitrophenyl)ethyl]carbamoylcholine iodide; cage II, *N*-(α-carboxy-2-nitrobenzyl)carbamoylcholine trifluoroacetate.

by the release of ATP from a photolabile precursor ('caged ATP') were demonstrated by FTIR difference spectroscopy [14,15]. Here we demonstrate conformational changes in the AChR, recorded as changes in the IR spectrum, upon binding of the agonist carbamoylcholine released from a 'cage' by a UV flash.

2. MATERIALS AND METHODS

Microsacs from *Torpedo californica* were prepared as described [16], recovered from sucrose gradients by dilution in H_2O and sedimentation (35,000 × *g*/90 min). Membrane fragments were then resuspended in sodium Ringer solution and were stored at −20°C. Protein was determined according to Lowry et al. [17]. The final protein concentration was 1.5 mg/ml.

[^{3}H]Acetylcholine (ACh) binding was measured at 20°C by an equilibrium centrifugation assay. The membrane suspension (protein concentration 0.1 mg/ml) was incubated for 30 min at room temperature with 10^{-4} M eserine to block acetylcholinesterase. After 15 min incubation with varying ACh concentrations (0.4×10^{-7} to 4.5×10^{-3} M), 165 μl aliquots were centrifuged for 30 min in a Beckman airfuge. Before and after centrifugation 50 μl aliquots were taken and counted in 5 ml of Supertron (Kontron, München) in a liquid scintillation counter to obtain the total and free concentrations of [^{3}H]ACh, respectively. The specific activity of the ACh stock solution (New England Nuclear) was 50 mCi/mmol.

The following photolabile agonist precursors ('caged' carbamoylcholine) described in [18,19] were obtained from Molecular Probes (Eugene, Oregon): *N*-[1-(2-nitrophenyl)ethyl] carbamoylcholine iodide or *N*-(α-carboxy-2-nitrobenzyl)carbamoylcholine trifluoroacetate (termed 'cage I' or 'cage II', resp.). The structures of the cages and their presumed photolysis products according to [18,19] are shown in Fig. 1. Photolysis was triggered by a Xenon flash at a flash energy of approx. 150 mJ in the spectral range from 305 nm to 424 nm at the area of the sample, giving a photolysis yield of up to 27%. Photolysis of caged carbamoyl choline I or II without protein was investigated at a concentration of 50 mM and with 15 mM sodium bisulfite.

IR samples of the receptor were obtained by centrifuging an aliquot of the receptor suspension containing 100–200 μg protein in a Beckman airfuge (120,000 × *g*; 10 min) and spreading the pellet onto a CaF_2 window. Excess water was removed from the surface of the pellet by a gentle stream of dry N_2. A typical receptor IR sample contained about 100 μg protein, 20 mM cage I or II, and 6 mM sodium bisulfite in a sample volume of approximately 1 μl. Sodium bisulfite was added in order to prevent inactivation of the receptor by 2-nitrosoacetophenone, a primary cage photolysis product [18]. An IR microcell was formed with a second CaF_2 window separated by a 6 μm spacer.

FTIR spectra were obtained on a Bruker IFS 25 instrument equipped with a HgCdTe detector of selected sensitivity as follows: 7 subsequent single beam spectra (I_n, n=1,...,7) of the sample were recorded. Between I_3 and I_4 the photolysis flash was applied. For all spectra 100 scans in 75 s were collected except for spectra I_2 and I_4 which were computed from 5 scans. All spectra were recorded with a resolution of 4 cm^{-1} and a triangular apodization. A Ge bandpass filter transmitting from 2000 cm^{-1} to 600 cm^{-1} was used in the IR beam to cut off unwanted IR radiation. The absorbance difference (D_n) between spectrum I_3, immediately before the flash, and spectrum I_n was obtained by calculating $D_n = -\log(I_n/I_3)$. The first difference spectrum, D_1, calculated from spectra recorded before the flash, served as baseline control for difference spectra recorded after the flash. The difference spectra $D_4 \ldots D_7$ showed absorbance changes after application of the flash [15].

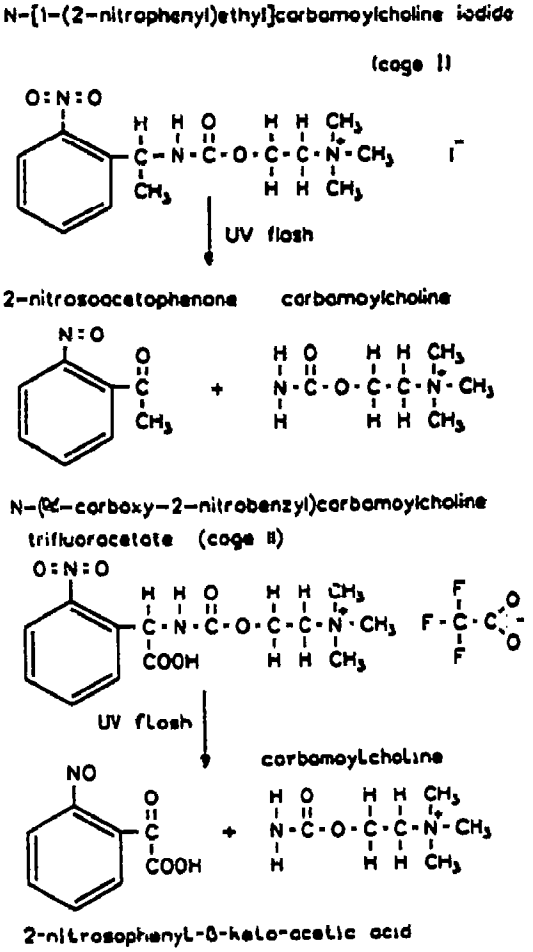

Fig. 1. Structures of *N*-[1-(2-nitrophenyl)ethyl] carbamoylcholine iodide (cage I) and *N*-(α-carboxy-2-nitrobenzyl)carbamoylcholine trifluoroacetate (cage II) and their proposed primary reaction products according to [18,19].

3. RESULTS

In order to obtain samples of the nACh receptor suitable for IR spectroscopy, which are transparent even in the region of high water absorbance around 1650 cm^{-1}, excess water had to be removed. The [^{3}H]acetylcholine binding assay described above was used to address the question if this partial dehydration might affect receptor activity. An untreated nAChR sample and a sample which had been prepared as an IR sample on a CaF_2 window as described above, but later resuspended in Ringer solution, were compared. Within the limits of the assay, no significant difference in the activity can be detected (data not shown). On the basis of this assay, we conclude that our nAChR samples used for FTIR spectroscopy are fully active and undergo the same agonist binding and signal transduction as in the native state.

Fig. 2 shows a FTIR difference spectrum of a nAChR sample obtained with cage I as a trigger (full line). For

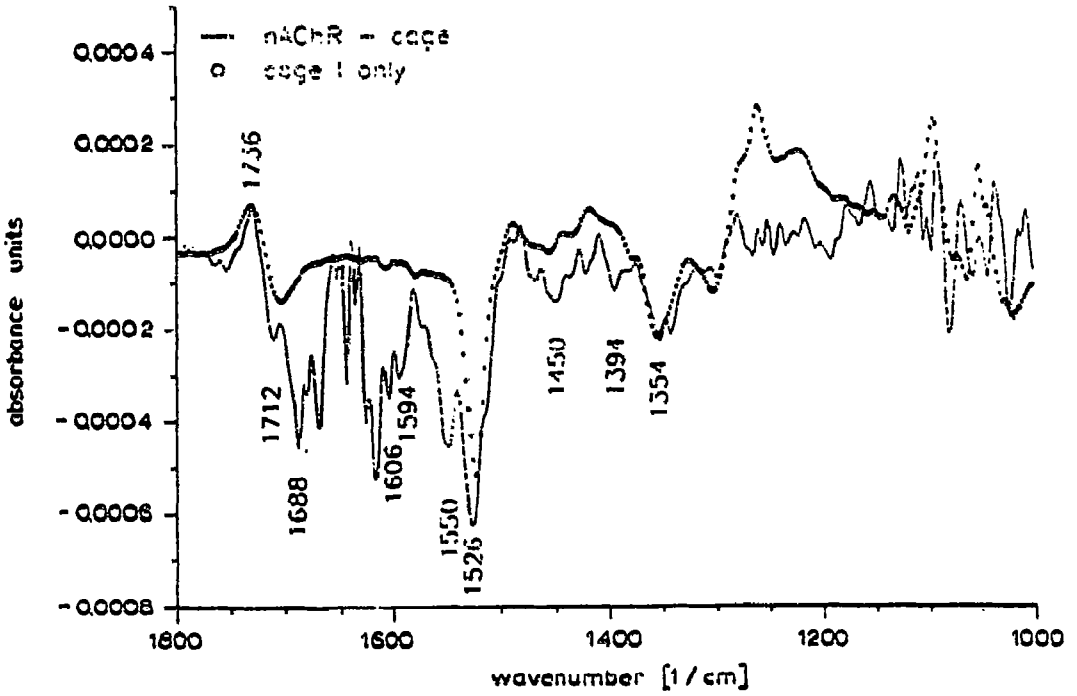

Fig. 2. FTIR difference spectra of a nAChR sample with cage I as a trigger (full line). Negative bands correspond to the state before the flash, positive bands to the state after the flash. The IR sample contained ca. 100 μg receptor protein, 20 mM cage I, and 6 mM sodium bisulfite. The difference spectrum represents an average of 4 spectra, each of them obtained from 100 interferometer scans. Temperature was 10°C. The dotted line represents the difference spectrum of cage I obtained from a 50 mM cage I solution. This spectrum was normalized to the receptor spectrum using the 1526 cm^{-1} difference band.

comparison, the difference spectrum obtained with cage I without the receptor protein is shown (dotted line). Although the spectral range from 2000 to 1000 cm^{-1} was recorded, we restrict here to the 1800 cm^{-1} to 1000 cm^{-1} range which contains most of the bands of diagnostic value. The maximum amplitude of the difference signals observed is in the order of 0.5×10^{-3} absorbance units. Since the absorbance maximum of the sample around 1650 cm^{-1} (amide I + water O–H bending mode) was kept around 1, the changes of the amide I compositions and extinction are significantly below 1/1000. In spite of the small size of the difference bands, difference spectra from different samples reproducibly show signals at the same frequencies and with comparable band amplitudes. In the 1620–1670 cm^{-1} region the noise level is significantly increased with respect to other spectral regions, only partly due to the high background absorbance. The main reason for an increased noise level in this spectral region is residual water vapour absorbance in the spectrophotometer: since cage I slowly inactivates the sample (see below), the spectra had to be recorded rapidly after preparation of the IR samples, thus resulting in incomplete dry-air purging of the instrument. Although reproducible band features are observed in this range, we shall keep in mind these possible sources of error for our discussion.

A slow inactivation (desensitization) of membrane vesicles enriched with nAChR upon prolonged incubation with cage I has been previously reported [19]. We have used this inactivation to collect further evidence that the observed spectral changes upon photolytic release of cage I (see Fig. 2, solid line) are indeed related to receptor activation. A series of FTIR spectra was started more than one hour after incubation with cage I. After this period, the receptor should be desensitized and no spectral contributions from the protein should be expected. Indeed, the difference spectrum only shows the band features obtained upon release of 'caged' carbamoylcholine (data not shown). We thus conclude that no contribution of contaminating proteins or of membrane constituents are manifest in the difference spectra. This conclusion is supported by experiments where the receptor was preincubated with carbamoylcholine before the cage was added, resulting in a difference spectrum of cage release without any protein contribution.

Fig. 3 shows a FTIR difference spectrum of a nAChR sample obtained with cage II as a trigger (full line). For comparison, the difference spectrum obtained with cage II without the receptor protein is shown (dotted line). Although approximately the same amounts of receptor and of cage were used for sample preparation, the removal of excess water, differences in sample thickness, and the slightly different photochemical yield of cage I may account for the different size of the absorbance

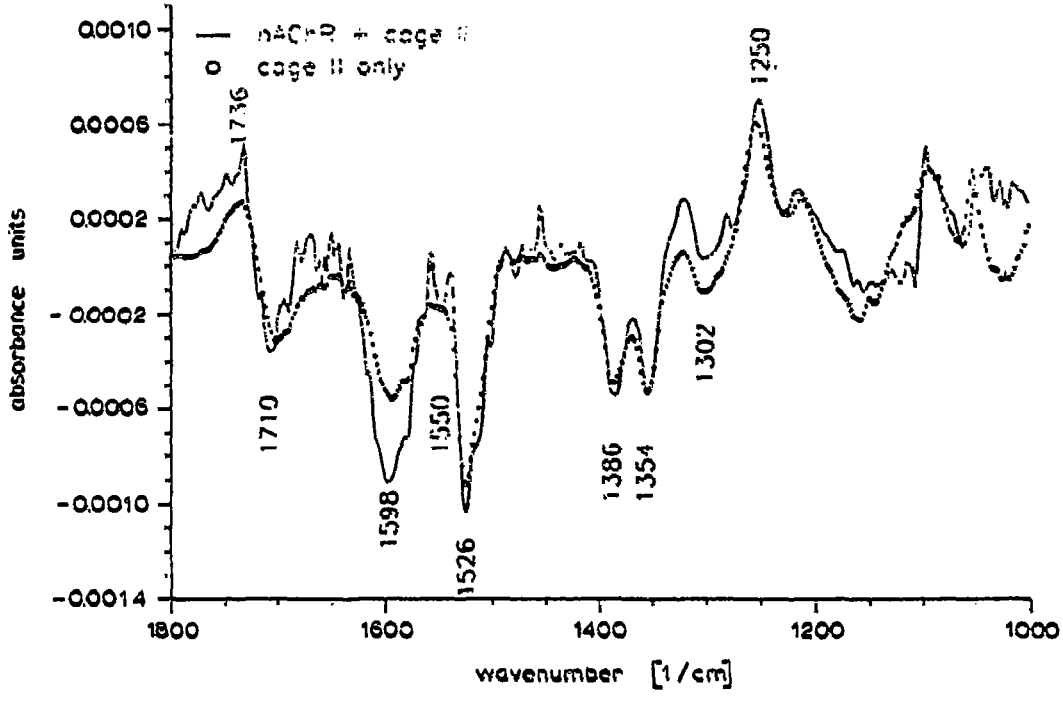

Fig. 3. FTIR difference spectra of a nAChR sample with cage II (full line). Conditions were as in Fig. 1. The dotted line represents the difference spectrum of the pure cage II normalized to the receptor spectrum as in Fig. 2.

change. The presence of closely coinciding bands in the nAChR + cage and the pure cage spectra in Figs. 2 and 3 suggest that a subtraction (nAChR+cage minus cage) of the difference spectra appears possible; nevertheless, we prefer the superposition of both, since (even nonspecific) binding of the cage to the protein might change its spectrum.

4. DISCUSSION

Upon photolysis of 'caged' carbamoylcholine, release and binding of carbamoylcholine as well as activation and subsequent deactivation (desensitization) of the receptor should occur. According to the time scale of the experiment and the rapid deactivation of the receptor (within several hundred msec under these conditions [20]), the difference spectra D_4 through D_7 should only reflect structural changes upon carbamoylcholine binding and receptor deactivation. We conclude from the small size of the difference bands that only minor conformational changes occur upon these processes. The amount of cage I or II in the nAChR-containing IR samples was chosen high enough to provide enough carbamoylcholine for the saturation of all receptors molecules even with only 27% photolysis yield. In fact, a second UV flash applied after several minutes yielded difference spectra D_4–D_7 very close to that obtained with only the cage.

The difference spectra of cage I and II photolysis (dotted lines in Figs. 2 and 3) can be used to identify the vibrational modes of the caged, unbound, and the released, receptor-bound carbamoylcholine. The highest frequency band in these cage difference spectra can be assigned to the carbamoyl C=O group, leading to a negative lobe at ca. 1706–1710 cm^{-1} for the caged form and a positive lobe at 1736 cm^{-1} for the bound form. Both receptor difference spectra show this mode at comparable frequencies in the caged and bound form, respectively. However, we note a sharpening of the positive band for the bound carbamoylcholine (Fig. 2, full line) with respect to the free carbamoylcholine, which might be a hint for a constraint structure of the bound molecule.

Conversion of the NO_2 group to a NO group and cleaving of a C–N bond upon photolysis of the cages lead to distinct band features in the difference spectra with and without the receptor. The negative bands at 1526 cm^{-1} and at 1354 cm^{-1} can be assigned to the disappearing antisymmetric and symmetric stretching modes of the nitro group upon 'cage opening', analogous to the release of ATP from caged ATP described by Barth et al. [14,15]. The immediate photolysis products 2-nitrosoacetophenone (cage I) and 2-nitrosophenyl-β-keto-acetic acid (cage II) proposed in [18] and [19], respectively, should also lead to a relatively strong keto C=O mode (see structures in Fig. 1) contributing around 1680 cm^{-1}. The appearance of this band upon release of caged ATP or ADP could be effectively suppressed with glutathione [15], although the reaction mechanisms and reaction products are not quite clear

(D. Trentham, J.E.T. Corrie, A. Barth, and W. Mäntele, unpublished observations). In order to prevent the cage photolysis byproducts from reacting with the receptor, sodium bisulfite was added, resulting in a spectral region from ca. 1530 cm^{-1}–1700 cm^{-1} free from difference bands due to cage release for cage I (Fig. 2, dotted line). In this region, however, cage II exhibits a strong negative band around 1598 cm^{-1} which we attribute to the antisymmetric C–O mode of the carboxylate group. This corresponding symmetric C–O mode would be expected between 1450 cm^{-1} and 1350 cm^{-1} and might well account for the negative band at 1386 cm^{-1} observed only with cage II. A shift of the pK of this carboxyl group by photolysis might account for these two difference bands. Although this cage appears well-suited for the triggering of the receptor reaction and does not lead to inactivation, the additional negative band at 1598 cm^{-1} may mask conformationally sensitive bands. A possible alternative would be the use of an isotopically labelled (^{13}C, ^{18}O, or both) cage II analogue, which would have its C–O modes downshifted by at least 40–50 cm^{-1}.

Both receptor difference spectra exhibit a number of common difference bands in the 1500–1700 cm^{-1} spectral region which are not present in the spectra of the pure cages. The most significant one is the negative 1550 cm^{-1} band (present in Figs. 2 and 3). Although weaker bands at higher frequencies have also been marked in Fig. 3, we again emphasize that this spectral region exhibits an increased noise level. A likely attribution of the 1550 cm^{-1}, however, is in terms of an amide II signal arising from a local conformational change of the receptor upon carbamoylcholine binding and the resulting inactivation.

Baenziger et al. [21] have used attenuated reflection spectroscopy to detect changes in the IR spectrum of the nAChR upon desensitation. Upon binding of carbamoylcholine, small but reproducible IR difference bands were detected. The signals differ in sign and amplitude from those reported here. A possible explanation for this discrepancy may be the difference in receptor preparation. While, in the work reported here, native receptor complexes were investigated, the receptor preparation used by Baenziger et al. was subjected to an alkaline extraction. The removal of the peripheral 43K protein by this procedure was reported to modify the conformation of the cytoplasmic domains of all subunits [22].

5. CONCLUSIONS

We consider this use of photolytically released carbamoylcholine in the IR spectroscopic analysis of the nAChR a first attempt for a direct detection of conformational changes in its signal transduction. Although the relatively strong difference band features from cage release may still mask protein signals, the presently detected signals indicate small but specific conformational changes, probably involving a few amino acids only, upon binding of the agonist. These signals, however, should encourage to design better 'cages', which do not exhibit inactivation as does cage I, and which are also optimized for minimum difference signals in the 1500 cm^{-1} to 1700 cm^{-1} spectral range sensitive for the protein conformation. The use of isotopically labelled cages may represent a first step into this direction.

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