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A μ-OPIOID RECEPTOR-FILTER ASSAY

RAPID ESTIMATION OF BINDING AFFINITY OF LIGANDS AND REVERSIBILITY OF LONG-LASTING LIGAND–RECEPTOR COMPLEXES

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Abstract—A filter-associated binding technique, originally described by Leysen and Gommeren [*J. Receptor Res.* **4**, 817 (1984); Drug Dev. Res. **8**, 119 (1986)], was focused on the study of μ-opioid receptor sites. The interesting binding features of ^{3}H-Sufentanil, a μ-selective radioligand, permitted such a filter procedure to be performed.

Its application was 3-fold. A 5-min binding assay allowed us to verify that specific ^{3}H-Sufentanil binding to filter-absorbed rat brain membranes was endowed with the known kinetic and equilibrium binding properties of membrane-bound μ-opioid receptor sites.

Moreover, the filtration technique allowed us to get rapid information about the dissociation rates of unlabelled compounds from the receptor sites. In practice, membranes, preincubated with high concentrations of cold drugs, were absorbed to filters. Dissociation was achieved by repeatedly applying buffer samples on the filter, and monitored by the recovery in free specific ^{3}H-Sufentanil binding sites.

Finally, such a dissociation procedure, improved by a washing buffer at high ionic strength, was found to be much more efficient and attractive than the classical dilution–centrifugation procedure, especially for slowly-dissociating compounds. Special attention was paid to the discrimination between pseudo-irreversible binding of drugs and stable covalent labelling of μ-opioid receptor sites (either by affinity or photoaffinity probes), particularly when unlabelled ligands were the only tools available.

Affinity and especially photoaffinity labelling of opioid receptors may be considered as a promising approach to the study of the molecular origin of their apparent heterogeneity. Up to now, several affinity [1–8] and photoactivatable ligands (azido-derivatives) [9–14], derived from natural or synthetic opioid compounds, have been synthesized. Most of them, under non-radioactive form, have been described as potential irreversible ligands for opioid receptor subtypes. The use of three tritiated affinity labels afforded unambiguous evidence for stable covalent complexes involving selective labelling of δ-receptor sites [15], subsequently purified to apparent homogeneity [16], or μ-opioid receptor sites [3, 4].

Our goal was to develop new photosensitive probes (aryldiazonium derivatives) whose activation leads to highly reactive species (aryl cation) and possibly to a more specific site-directed labelling of opioid receptor subtypes. Therefore, we synthesized photoactivatable derivatives of opiate antagonists (naltrexone) or agonists (etonitazene, spirodecanone and fentanyl) able to generate the corresponding aryl cations upon light irradiation. Under reversible conditions (in the absence of light), most of our probes, with the exception of the naltrexone derivative, showed a pronounced μ-binding selectivity (unpublished results) with high binding affinity constants. Surprisingly, the photosensitive derivative of carfentanil appeared to behave as a good affinity label of the μ-opioid receptor subtype [17]. Indeed, the corresponding drug–receptor complexes resisted repetitive dilution–centrifugation steps, a dissociation procedure currently used as the only means of discriminating between reversible and stable covalent binding of an unlabelled ligand. Meanwhile, our attention was also pointed to the poor efficacy of this dilution–centrifugation technique in removing pseudo-irreversible compounds.

Such was the case for the potent opiate agonist lofentanil, involved in reversible but long-lasting binding to its specific receptor sites [18, 19].

Recently, Leysen and Gommeren developed a filtration technique to measure the dissociation rate of unlabelled drugs from several membrane-bound neurotransmitter receptors [20], including from the μ-opioid receptor subtype [21]. ^{3}H-Sufentanil, a μ-selective opiate radioligand [22], was selected as the labelled ligand of choice for a binding assay performed with filter-adsorbed membranes. Indeed, in addition to its high affinity properties, this opiate agonist displayed a fast association rate as well as a favourable specific vs non-specific binding ratio [21, 22].

Since the filter procedure required some savoir-faire [20], we looked first at a number of criteria which have to be fulfilled for specific ^{3}H-Sufentanil binding on filter-trapped membranes to be relevant of μ-opioid receptor sites.

The binding properties at μ-opioid receptor sites

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† Abbreviations used: HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid.

of several photosensitive aryldiazonium derivatives were examined in terms of apparent binding affinity constants and dissociation rate. We also compared the efficiency of the filter technique, associated or not with a washing buffer at high ionic strength, and of the classical dilution–centrifugation procedure in dissociating pseudo-irreversible drugs from the receptor sites.

MATERIALS AND METHODS

Membrane preparation

Male Wistar rats (100–150 g) were killed by decapitation. Whole brains minus cerebella were dissected out and homogenized in 10 vol. (ml/g wet weight of tissue) ice-cold 0.25 M sucrose using a Duall homogenizer. Following centrifugation at 500 *g* (10 min), the pellet was rehomogenized in 5 vol. 0.25 M sucrose and recentrifuged at 1100 *g* (10 min). The combined supernatants were adjusted to a final dilution of 45 vol. in Tris–HCl buffer 50 mM, EDTA 1 mM (pH 7.4). The mixture was then centrifuged at 35,000 *g* for 30 min at 4° and the supernatant was discarded. The pellet (mitochondrial and microsomal membranes) was homogenized in 5 vol. ice-cold 0.32 M sucrose and kept at −70° until use. The tissue was kept at 0–4° throughout the preparation procedure.

^{3}H-Sufentanil binding assays on filters

Technical conditions were essentially the same as those described by Leysen and Gommeren [20, 21].

For routine experiments, the membrane preparation was diluted up to 350 vol. with Tris–HCl buffer 50 mM, EDTA 1 mM (pH 7.4) and preincubated at 37° for 20 min.

Two millilitre aliquots of membrane suspension (5.8 mg original wet weight of tissue or 0.35 mg protein) were then layered on GF/B glass fibre filters (2.5 cm diameter, Whatman) disposed on a 30-well vacuum filtration apparatus (each well having a 7 ml maximal capacity).

Membranes were adsorbed on filters, under vacuum for 5–10 sec, until partially dried filters were obtained. Immediately thereafter, the vacuum was released.

A 0.4 ml sample of 0.5 nM ^{3}H-Sufentanil buffered solution (warmed at 37°), containing or not unlabelled drugs, was applied on the filter. After 5 min, incubation was terminated under vacuum by twice rinsing the filters with 4 ml ice-cold Tris–EDTA buffer.

The filters were transferred to counting vials and extracted by vigorous shaking in 6 ml of scintillation cocktail (Biofluor TM, N.E.N.). The vials were allowed to stand for at least 8 hr and counted for radioactivity with an efficiency of 45%.

Specific ^{3}H-Sufentanil binding was defined as the difference between total binding and non-specific binding obtained in the presence of 10^{-6} M naloxone.

^{3}H-DAGO binding assays

Rat brain membranes (0.27 mg protein/ml) were incubated at 25° for 90 min with 1 nM ^{3}H-DAGO in the absence or the presence of cold drugs at various concentrations. Incubation was stopped by rapidly filtering, under vacuum, the 1 ml assay mixture over GF/B filters, then immediately rinsing twice with 3 ml ice-cold Tris–EDTA buffer. Specific ^{3}H-DAGO binding was defined as the difference between total binding and non-specific binding obtained in the presence of 10^{-6} M naloxone. Under these conditions, a K_D-value of 1.3 nM for specific ^{3}H-DAGO binding was determined.

Analysis of binding data

Apparent binding affinity constants (K_i-values) of drugs were calculated from IC_{50}-values, determined in competition experiments, according to the Cheng and Prusoff [23] equation: $K_i = IC_{50}/(1 + L/K_d)$ where L and K_d are respectively the radioligand concentration and its equilibrium dissociation constant.

Dissociation procedures of drug–receptor complexes

Rat brain membranes (0.7 mg protein/ml) were incubated for 20 min at 37° with or without various concentrations of unlabelled opiate compounds.

Filter-adsorbed-tissue procedure. The whole procedure followed the methodological scheme previously described [20].

Drug dissociation was initiated in the batch incubation mixture by a 4-fold dilution with Tris–EDTA buffer and by further incubation at 37° for 10 min.

Two-millilitre aliquots of diluted membrane preparation (0.35 mg protein) were layered on GF/B filters and concentrated under partial vacuum for 5 sec. Then, 3 ml aliquots of Tris–EDTA buffer (pH 7.4), prewarmed at 37°, were poured on the filters and allowed to drip for 5 min through the filters. The residual liquid was filtered under partial vacuum for 5 sec and the 5 min washing procedure was repeated again until ^{3}H-Sufentanil binding assays were performed as previously described.

When Tris–EDTA buffer containing 1 M NaCl was used for the dissociation experiment, filters were washed under partial vacuum with 7 ml of Tris–EDTA buffer (at 37°), deprived of salt, prior to the ^{3}H-Sufentanil binding assay.

Dilution–centrifugation procedure. The dissociation step started with a 9-fold dilution of the assay medium with Tris–EDTA buffer and with further incubation at 37° for 10 min. Then, membranes were sedimented by centrifugation at 100,000 *g* for 12 min at 20°. The pellet was either reconstituted in 200 vol. of ice-cold Tris–HCl buffer (0.3 mg protein/ml) and assayed for ^{3}H-DAGO binding or rehomogenized in 750 vol. of Tris–EDTA buffer, incubated at 37° for 10 min, and submitted to a second centrifugation before free specific ^{3}H-DAGO binding sites were measured.

Protein determination

Protein concentrations were determined according to Spector [24] using bovine gamma-globulin as a standard.

Chemicals

^{3}H-Sufentanil (12 Ci/mmol), fentanyl and lofentanil were from Janssen Pharmaceutica (Beerse, Belgium). (Tyrosyl-3,5-^{3}H)-D-Ala-Gly-NMe-Phe-Gly-ol (^{3}H-DAGO, 43 Ci/mmol) was from the

C.E.A. (Saclay, France). Levorphanol and dextrorphan, etonitazene, and bremazocine were respectively gifts from Hoffmann LaRoche, Ciba–Geigy and Sandoz (Basel, Switzerland). Naloxone was supplied by Du Pont de Nemours (Glenolden).

Aryldiazonium salts of opiate derivatives. Fentanyl- and carfentanil-aryldiazonium derivatives have been synthesised as previously reported [17]. Synthesis of spirodecanone-, etonitazene- and naltrexone- aryldiazonium derivatives will be described elsewhere.

All aryldiazonium salts were purified by HPLC on a TM-C_{18} column (Waters μBondapack, 3.9 × 300 mm) and eluted at a flow rate of 2 ml per min under gradient conditions (100% H_2O, 0.05% TFA to 100% CH_3CN in 40 min). Eluted products were monitored for absorbance at 229 nm.

Photolysis of the aryldiazonium derivatives. Irradiation experiments of the aryldiazonium salts were carried out in water, in a quartz cell, at the maximal absorption wavelength of the chromophore (380 nm for all derivatives except for the fentanyl one which was irradiated at 340 nm). Photolysis was monitored by UV spectroscopy until complete disappearance of the diazonium absorption peak.

We verified that, in the absence of light, all diazonium salts were stable for hours in Tris–EDTA buffer (pH 7.4), with or without rat brain membranes.

RESULTS

^{3}H-Sufentanil binding characteristics using filter-absorbed membrane material

Several experiments were carried out in order to define all parameters required for a safe determination of the specific ^{3}H-Sufentanil binding component to be obtained.

(a) When increasing amounts of membranes were incubated on filters with 0.5 nM ^{3}H-Sufentanil (Fig. 1), specifically-bound ^{3}H-Sufentanil was found to vary linearly with tissue amounts up to 6.7 mg per filter (0.4 mg protein). All further experiments were carried out using 5.8 mg of tissue per filter which allowed accurate determinations to be obtained.

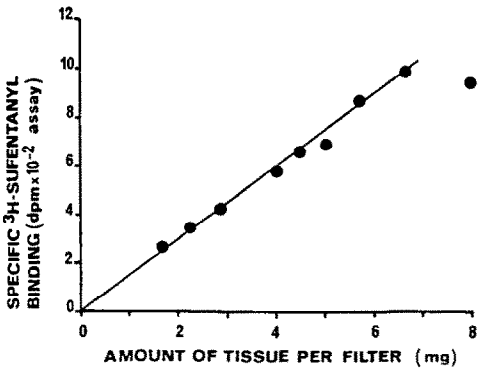


Fig. 1. Specific ^{3}H-Sufentanil binding as a function of tissue amount adsorbed to filters. Two-millilitre aliquots containing increasing amounts of rat brain membranes were layered on GF/B filters and incubation with 0.5 nM ^{3}H-Sufentanil was performed as described under Methods. Presented values are the mean values of two independent experiments performed in duplicate.

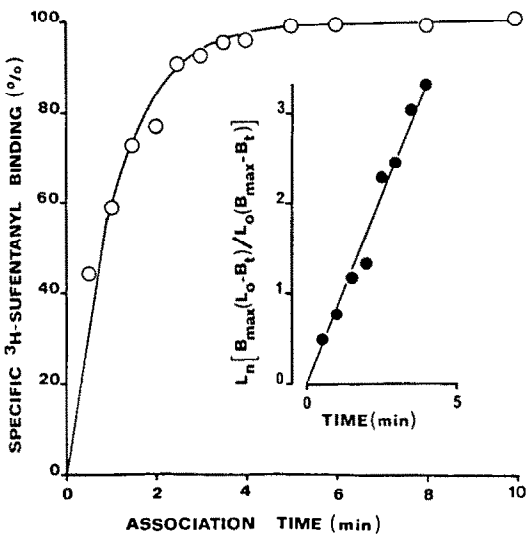


Fig. 2. Time-dependence, at 37°, of specific ^{3}H-Sufentanil binding to filter-adsorbed membrane material. Samples were incubated separately following the standard conditions as described under Methods. The association rate constant was derived from the slope of the plot (inset) according to the equation [25]:

$$\text{Ln}\,(B_{max}(L_0\text{-}B_t)/L_0(B_{max} - B_t)) = k_1 t(1/(L_0 - B_{max})$$

yielding $k_1 = 0.36\,\text{nM}^{-1}\cdot\text{min}^{-1}$, with L_0, the ^{3}H-Sufentanil concentration (0.54 nM), B_{max}, the specific binding at equilibrium (102 pM) and B_t, the specific binding at time t.

(b) Kinetic analysis of the association rate of ^{3}H-Sufentanil binding to filter-adsorbed membrane material is shown in Fig. 2. Receptor–ligand association was very rapid (equilibrium was reached within 5 min) and fitted a pseudo-first order reaction plot [25], with an association rate constant $k_1 = 3.6$ $10^8\,\text{M}^{-1}.\text{min}^{-1}$.

(c) The binding of increasing concentrations of ^{3}H-Sufentanil to filter-adsorbed membranes is presented in Fig. 3. At 1.5 nM, specific ^{3}H-Sufentanil binding reached its saturation level which still represented 50% of the total radioligand binding. Scatchard analysis of 5 independent saturation curves resulted in linear plots which (mean values ± SEM) yielded a K_d of 0.25 ± 0.04 nM and a B_{max} value of 182 ± 21 fmol/mg protein (10.9 ± 1.2 fmol/mg tissue).

(d) Opiate agonists and antagonists belonging to different chemical families were tested for their ability to compete with ^{3}H-Sufentanil binding to filter-adsorbed membranes (Fig. 4). ^{3}H-Sufentanil binding displayed a strong stereoselectivity (levorphanol vs dextrorphan) while an accurate plateau of non-specific binding, defined at 10^{-6} M naloxone or 10^{-7} M etonitazene, represented only 25–30% of the total binding of 0.5 nM ^{3}H-Sufentanil.

Apparent drug inhibition constants (K_i-values) for specific ^{3}H-Sufentanil binding are listed in Table 1A.

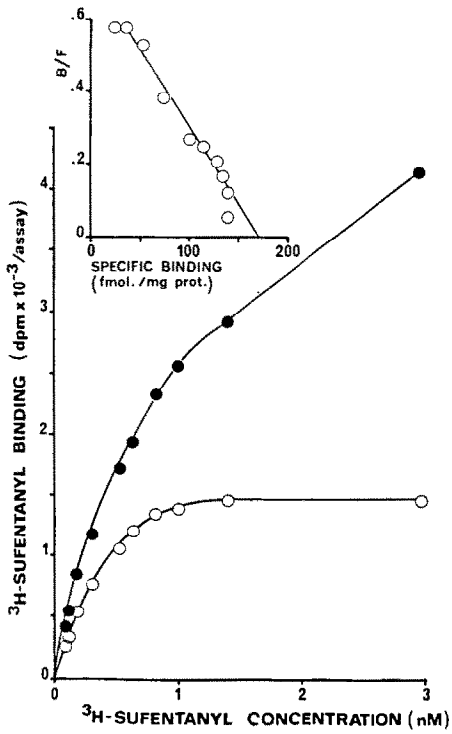


Fig. 3. Saturation curve at equilibrium of specific (○——○) ^{3}H-Sufentanil binding to filter-adsorbed membranes. Samples (5.8 mg tissue preparation) were incubated for 5 min at 37° with increasing concentrations (0.08 nM–3 nM) of ^{3}H-Sufentanil in the absence (total binding: ●——●) or presence of 10^{-6} M naloxone (non-specific binding). Inset: K_d and B_{max}-values for specific ^{3}H-Sufentanil binding were calculated from Scatchard plots. Forty-six percent (at 0.08 nM) to 13% (at 3 nM) of added ^{3}H-Sufentanil contributed to its total binding, thus free ^{3}H-Sufentanil concentrations were calculated by subtracting the totally bound from the totally added radioligand.

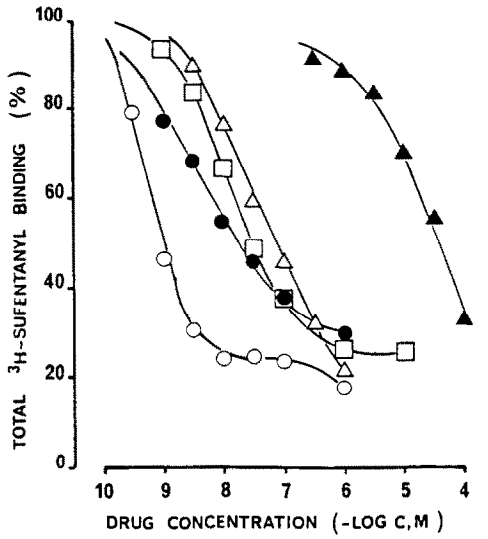


Fig. 4. Competition curves of various opiate compounds with ^{3}H-Sufentanil (0.5 nM) binding to filter-adsorbed membranes. Assays were carried out using the standard conditions presented under Materials and Methods and increasing concentrations of etonitazene (○——○), levorphanol (●——●), naloxone (□——□), fentanyl (△——△) and dextrorphan (▲——▲).

Comparison of drug binding properties at opiate receptor sites using filter or conventional binding assays

As shown in Table 1A, similar apparent binding affinities of classical opiate drugs (with the exception of lofentanil) for specific ^{3}H-Sufentanil binding sites were found under filter or batch incubation conditions. Moreover, K_i-values of drugs for ^{3}H-Sufentanil binding were in agreement with those found for ^{3}H-DAGO binding though the later binding assay provided an intermediate binding affinity constant for lofentanil and the lowest one for levorphanol.

Table 1B enables us to compare the binding affinity properties of several aryldiazonium derivatives and of their corresponding photolysis products, determined from competition experiments performed under different incubation conditions. With the exception of the photoactivatable fentanyl derivative, all aryldiazonium ligands displaced specific ^{3}H-DAGO or ^{3}H-Sufentanil binding at nanomolar concentrations. Previous photolysis of these aryldiazonium derivatives differently affected their binding affinities. Some of them remained unchanged while the photolysis products of the spirodecanone and etonitazene derivatives, respectively displayed a 10-fold lower affinity or became inactive.

Use of the filter procedure to study the dissociation of drug–receptor complexes

When considering technical aspects of this procedure, it might be observed that the minimal dissociation time, referred to as time 0, did not take into account the preliminary dilution-induced dissociation step (10 min at 37°) and the incubation time with ^{3}H-Sufentanil (5 min at 37°) necessary to quantitate free receptor sites.

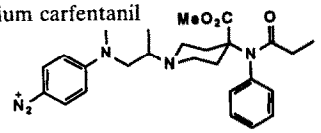
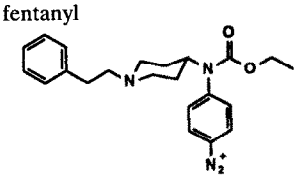
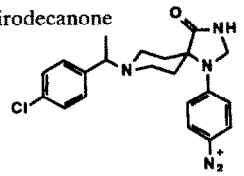
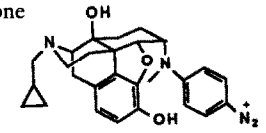
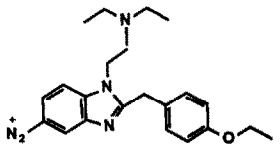
Several preliminary experiments were performed in order to test the accuracy and the reliability of this method.

(a) Whatever the length of the washing procedure (up to 60 min) and the content of the washing buffer (deprived or supplemented with 1 M NaCl), specific ^{3}H-Sufentanil binding sites were entirely recovered and showed no significant changes in their drug affinity properties.

Moreover, the favourable specific vs non-specific binding ratio, determined at 0.5 nM ^{3}H-Sufentanil, remained identical to that found for unwashed membrane material. It should be noted that special attention was paid to the elimination of salts before ^{3}H-Sufentanil binding was performed. Indeed, many opiate ligands, including ^{3}H-Sufentanil itself [22], display a reduced binding affinity constant in the presence of physiological concentrations of salts.

(b) Four opiate compounds, belonging to different chemical classes, were incubated at saturating concentrations with rat brain membranes and the resulting drug–receptor complexes were submitted to the filter-dissociation procedure (Fig. 5).

Table 1. Binding affinities of compounds for opiate receptor sites using different assay methods.

Compounds	Binding affinity constants (K_i, nM) ^{3}H-Sufentanil		^{3}H-DAGO
1A	Batch*	Filter	Batch
Lofentanil	0.04	0.86	0.28
Etonitazene	0.11	0.20	0.56
Levorphanol	1.2	1.06	0.16
Fentanyl	1.6	7.85	2.92
Naloxone	3.1	4.55	1.5
Dextrorphan	—	4375	630
1B			
Aryldiazonium carfentanil 		11 (—)	3.5 (3.9)
Aryldiazonium fentanyl 		—	80 (85)
Aryldiazonium spirodecanone 		10 (150)	10 (142)
Aryldiazonium naltrexone 		8 (—)	0.6 (0.5)
Aryldiazonium etonitazene 		1.3 (—)	9 (>1000)

Reported K_i-values for ^{3}H-DAGO binding or for ^{3}H-Sufentanil binding (using the filter-adsorbed tissue technique) were from competition experiments performed as described under Materials and Methods. Values under parentheses are K_i values of photolyzed aryldiazonium derivatives.

* K_i-values for inhibition of ^{3}H-Sufentanil binding to rat brain membranes in solution were taken from Ref. 22.

Figure 5A shows that complete reversal of fentanyl– or of naloxone–receptor complexes was achieved within 10–15 min. Etonitazene provided a half-time of dissociation of 40 min while lofentanil remained firmly bound to the receptor sites during the same period of time.

However, when the washing buffer was supplemented with 1 M NaCl (Fig. 5B), the dissociation rate of etonitazene increased ($t_{1/2}$ = 15 min) as well as that of lofentanil ($t_{1/2}$ = 35 min).

(c) Figure 6 makes it possible to compare the dissociation profiles of three aryldiazonium derivatives using the filter dissociation procedure in the absence (Fig. 6A–C) or in the presence of 1 M NaCl (Fig. 6D).

As shown in Fig. 6A, aryldiazonium carfentanil displayed the slowest dissociation rate while the spirodecanone and etonitazene derivatives developed much more labile complexes with the receptor sites.

Photolysis products of these three compounds, even used at higher concentrations, were entirely and rapidly dissociated from the specific ^{3}H-Sufentanil binding sites (Fig. 6B). Such a phenomenon was particularly clear for the carfentanil photolysis product. It should be noted too that at time zero, 30% of the specific ^{3}H-Sufentanil binding sites were

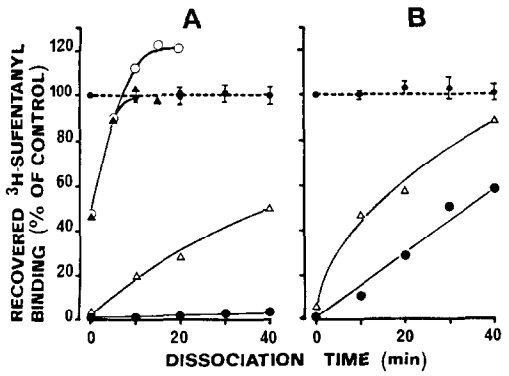


Fig. 5. Drug–receptor dissociation curves assessed using the filter–adsorbed tissue technique. As stated under Materials and Methods, membranes were incubated for 20 min at 37° with 10^{-6} M naloxone (○——○), 10^{-6} M fentanyl (▲——▲), 10^{-7} M etonitazene (△——△) or 10^{-7} M lofentanil (●——●) or without any added drugs (control experiment, ●-●-●-). After a 4-fold dilution, the incubation media were layered on filters and submitted to increasing dissociation periods of time using Tris–EDTA buffer deprived (A) or supplemented (B) with 1 M NaCl. Then ^{3}H-Sufentanil binding proceeded as described. Points are mean values of at least 3 independent experiments performed in triplicate where 100% represented 1065 ± 23 (minus NaCl) and 1056 ± 20 (plus 1 M NaCl) dpm per filter (control mean values ± SEM, N = 11).

occupied by the photolyzed product of etonitazene, suggesting that, as was the case for its native congener, it displayed a higher affinity in the ^{3}H-Sufentanil binding assay than in the ^{3}H-DAGO one.

Figure 6C shows that the formation of long lasting complexes between opiate receptor sites and aryldiazonium carfentanil was partially or totally prevented by the presence of 10^{-5} M naloxone depending on whether membranes were incubated with an excess (3.10^{-7} M) or with lower (10^{-7} M) concentrations of this carfentanil derivative.

The presence of 1 M NaCl during the filter-washing procedure (Fig. 6D) considerably improved the dissociation of high concentrations of aryldiazonium carfentanil from the specific ^{3}H-Sufentanil binding sites. Such an increase in dissociation efficacy by using a high salt concentration has already been shown for etonitazene and lofentanil (Fig. 5B). Conversely, aryldiazonium spirodecanone was insensitive to this experimental condition (Figs. 6A and 6D).

Comparison of the efficacy of the filter-dissociation procedure to that of the more conventional dilution–centrifugation method

As shown in Fig. 7 (upper panel), repeated dilution and centrifugation steps resulted in a progressive loss of specific ^{3}H-DAGO binding sites. However, providing the dissociation procedure was only repeated twice, the specific ^{3}H-DAGO binding capacity (fmol/mg protein) remained fairly stable (92% of that found for control membranes).

Classical opiate compounds (naloxone and fentanyl) which were easily dissociated by the filter procedure (Fig. 5A), aryldiazonium derivatives and their photolysis products, required at least two repeated dilution–centrifugation steps to be removed from the opiate receptor sites (Fig. 7, middle and lower panels). Under such conditions, specific ^{3}H-DAGO binding sites became entirely free of cold drugs except of aryldiazonium carfentanil which still occupied about 90% of the receptor sites. Conversely, its photolyzed product, incubated at the same saturating concentration and exhibiting the same binding affinity for specific ^{3}H-DAGO binding sites (Table 1B), was entirely removed.

Table 2 enables us to compare the dissociation

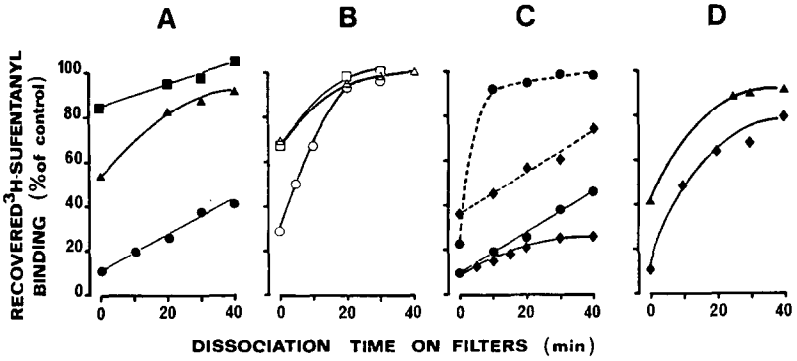


Fig. 6. Dissociation profiles of various aryldiazonium derivatives from filter-adsorbed opiate binding sites. The whole procedure followed the standard conditions described under Methods and in the legend of Fig. 5. Before starting the dissociation procedure on filters where the washing buffer was deprived (A, B, C) or supplemented with (D) 1 M NaCl, the association step of the drugs to the receptor sites followed the conditions: (A) native carfentanil (●——●, 10^{-7} M), spirodecanone (▲——▲, 10^{-6} M) and etonitazene (■——■, 10^{-7} M) aryldiazonium derivatives were used at the indicated concentrations; (B) the photolysis products of the three former aryldiazonium derivatives (identical but open symbols) were incubated at three-fold higher concentrations than those indicated in A; (C) membranes were incubated with 10^{-7} M (●——●) or 3.10^{-7} M (◆——◆) of the native aryldiazonium carfentanil derivative in the absence (solid lines) or presence (dashed lines) of 10^{-5} M naloxone; (D) dissociation of the carfentanil (◆——◆, 3.10^{-7} M) and of the spirodecanone (▲——▲, 10^{-6} M) aryldiazonium derivatives was carried out in the presence of 1 M NaCl.

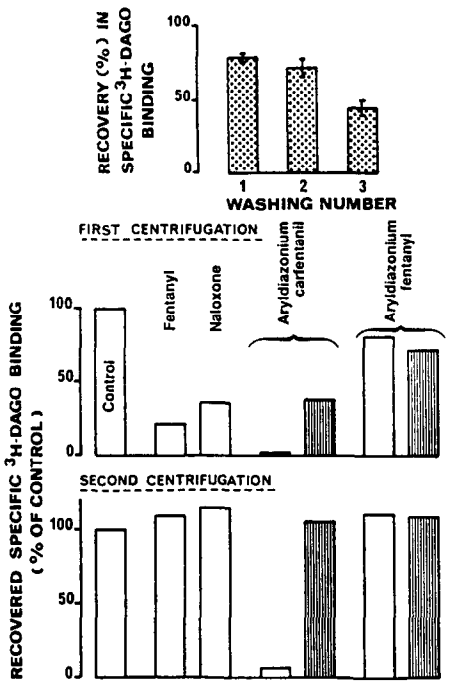


Fig. 7. Dissociation profiles of various opiate compounds using the dilution–centrifugation procedure. Membranes were incubated in the absence of cold drug (upper panel) or in the presence of 10^{-6} M fentanyl or naloxone, 3.10^{-7} M aryldiazonium carfentanil or 3.10^{-6} M aryldiazonium-fentanyl and submitted to repetitive dilution–centrifugation steps as described in Materials and Methods. Aryldiazonium derivatives were used at the same concentration whether they had been previously photolyzed (dashed histograms) or not. Data are presented as mean values ± SEM (N = 4, upper panel) or as mean values of two independent experiments (lower panels).

efficiency of the dilution–centrifugation and filter dissociation procedures. Compounds belonging to the first group were removed from the receptor sites after two dilution–centrifugation steps or after short-time washings on filters, even in the absence of salts.

They corresponded to compounds known [21, 22] for their rapid dissociation kinetics (naloxone, naltrexone, fentanyl), to aryldiazonium fentanyl and to most of the photolyzed aryldiazonium derivatives.

Only two of them (photolyzed naltrexone and spirodecanone derivatives) displayed a slower dissociation rate as assessed from the results obtained through the centrifugation method. The filter-dissociation procedure permitted a successful reversal of such drug–receptor complexes.

The third group of compounds included lofentanil and etonitazene as well as all of the native aryldiazonium derivatives (with the exception of fentanyl aryldiazonium). They remained firmly bound to opiate receptor sites even after two dilution–centrifugation steps but the use of the filtration technique considerably improved the recovery in free receptor sites. Moreover, the addition of 1 M NaCl to the washing buffer markedly enhanced the efficacy of this dissociation procedure (except for the spirodecanone aryldiazonium derivative).

DISCUSSION

Most of the aryldiazonium derivatives we synthesized displayed nanomolar binding affinities for μ-opioid receptor sites labelled by ^{3}H-DAGO. Surprisingly, among these potential photoaffinity probes, aryldiazonium carfentanil seemed to behave as a good affinity label [17]. This situation could be explained by a fast reaction occurring between a well-positioned diazonium group and cysteinyl residues [17] of the receptor binding site [26].

In order to reinforce this possibility, we had to verify the real covalent attachment of this unlabelled probe to the receptor sites, thus to test its irreversibility under strongly dissociating conditions.

An alternative to the conventional dilution–centrifugation procedure was the use of a filter-dissociation technique, previously described [20, 21] as a method for measuring the dissociation rate of unlabelled ligands from their receptor sites.

Such a filter procedure needs a safe investigation of a ^{3}H-ligand binding to filter-adsorbed membranes. Our experimental conditions allowed specific ^{3}H-Sufentanil binding to fulfil the following criteria: (a) favourable specific vs non-specific binding ratio; (b) linear relationship between specific ^{3}H-Sufentanil binding and the amount of adsorbed membranes; (c) fast association rate and binding equilibrium reached within 5 min at 37°. The association rate constant was very close to that ($k_1 = 6.6\ 10^8\ M^{-1}.min^{-1}$) found under classical incubation conditions [22]; (d) saturability with K_d and B_{max} binding parameters similar to those found under filter [21] or batch [22] ^{3}H-Sufentanil binding conditions; (e) stereoselectivity and drug affinity properties in agreement with the μ-opioid nature of specific ^{3}H-Sufentanil binding to membrane binding sites in solution [22].

It is noteworthy to recall here that only apparent K_i-values of drugs may be estimated from competition experiments performed on filters. Indeed, a short incubation time (5 min), combined to the use of high affinity and slowly dissociating compounds like lofentanil [18, 19, 21, this paper] and etonitazene (Table 1A and Fig. 5) or conversely to the use of fast dissociating drugs like fentanyl [21, 22, this paper] may lead to non-equilibrium binding conditions for these unlabelled compounds [20, 21].

A number of other experiments clearly showed the superiority of the filter-dissociation technique over the dilution-centrifugation one. The capacity as well as the drug affinity properties of specific ^{3}H-Sufentanil binding sites remained unchanged whatever the length (up to 1 hr) or the content of the washing buffer (plus or minus 1 M NaCl). Moreover, the filter technique was more rapid and allowed numerous samples to be treated simultaneously. In addition to this, a number of compounds, resistant to repeated dilution–centrifugation steps, were more readily displaced from their binding sites when using the filter procedure and especially in the presence of 1 M NaCl. Such a phenomenon was particularly striking for lofentanil, known for its quasi-irreversible binding properties [18, 19, 21] and for aryldiazonium etonitazene and carfentanil derivatives.

Therefore, the previous hypothesis [17] for a stable affinity labelling of μ-opioid receptor sites by aryl-

Table 2. Comparison of the dissociation efficacy of three methods in removing opiate compounds from their membrane-bound binding sites

Compounds (M)	Centrifugation	Filter drug dissociation procedure			
		−NaCl		+1 M NaCl	
	^{3}H-DAGO binding recovery (%)	Time (min)	^{3}H-Sufentanil binding recovery (%)	Time (min)	^{3}H-Sufentanil binding recovery (%)
Rapidly dissociating compounds					
Naloxone (10^{-6} M)	114	20	121	—	—
Fentanyl (10^{-6} M)	109	20	101	—	—
Naltrexone (10^{-6} M)	—	20	104	—	—
Bremazocine (10^{-6} M)	—	20	103	—	—
Aryldiazonium-fentanyl (3.10^{-6} M)	111 ± 3	—	—	—	—
Photolyzed aryldiazonium-					
fentanyl (3.10^{-7} M)	108 ± 5	—	—	—	—
carfentanil (3.10^{-7} M)	90 ± 15	30	96	—	—
etonitazene (3.10^{-7} M)	111	40	111 ± 10	—	—
Intermediate compounds					
Photolyzed aryldiazonium-					
naltrexone (10^{-7} M)	65	30	105	—	—
spirodecanone (3.10^{-6} M)	84	30	97	40	100
Slowly dissociating compounds					
Lofentanil (10^{-7} M)	0	40	3	40	56
Etonitazene (10^{-7} M)	—	40	50	40	88
Aryldiazonium-					
carfentanil (10^{-7} M)	20	40	47 ± 6	40	89 ± 5
naltrexone (10^{-7} M)	36 ± 3	30	70 ± 4	—	—
spirodecanone (10^{-6} M)	38	40	92 ± 6	40	91 ± 6

Three groups of compounds were defined according to their apparent dissociation rate and to the increasing efficacy of the washing procedures. The listed drug concentrations were those of the incubation media during the association step. Centrifugation data were from twice-repeated dilution–centrifugation steps. Time-values corresponded to the overall duration of the dissociation procedure or to the time required for complete dissociation of drug–receptor complexes to be observed.

When three or more independent experiments (performed in duplicate) were made, mean values ± SEM are presented.

diazonium carfentanil, already prevented by 10^{-5} M naloxone, was completely ruled out.

One important point remained: why do aryldiazonium salts form such long-lasting complexes with μ-opioid receptor sites? The extent of this phenomenon and its sensitivity to ionic strength variations depended upon the aryldiazonium ligand which was considered (Table 2) and probably reflected differences in the driving forces involved in the formation of these complexes.

Nevertheless, this particular feature for the binding of aryldiazonium ligands was not encountered with either their photolysis products (Table 2) or with their corresponding azido-derivatives (unpublished results). This suggested that the diazonium entity was *per se* responsible for this strong interaction, either through charge-transfer complexes or through formation of covalent azo-adducts with some nucleophilic residues (-OH, -SH, $-NH_2$) of the receptor site which were reversed under our dissociating conditions.

The last hypothesis is strengthened by the fact that photolysis of membranes preincubated with aryldiazonium carfentanil (data not shown) did not modify the slow dissociation rate of the drug–receptor complexes already shown here (without UV irradiation) while much faster reversible complexes occurred when the photolysis product of aryldiazonium carfentanil interacted with the receptor sites. These results indicated, too, that no significant photolabelling of μ-opioid receptors occurred.

In conclusion, ^{3}H-Sufentanil binding to filter-adsorbed rat brain membranes is an efficient tool for obtaining rapid information concerning the μ-opioid binding component of drugs as well as their dissociation rates from the receptor sites.

Furthermore, the filter dissociation technique makes it possible to discriminate between long-lasting ligand–receptor complexes and stable covalent labelling of μ-opioid receptor sites, either by potential affinity or photoaffinity probes. In that sense, this method may also be considered as a preliminary alternative to the detection of irreversible complexes between μ-opioid receptors and specific radioligands by using gel electrophoresis under denaturating conditions.

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