Non-invasive, kinetic measurements of [^{3}H]nitrendipine binding to isolated rat myocytes by condensed phase radioluminescence

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The binding of ^{3}H-labelled drug molecules to membranes of living cells gives rise to photon emission from tryptophan residues at proteinaceous binding sites. This phenomenon, called condensed phase radioluminescence, has been used to measure non-invasively the kinetics of [^{3}H]nitrendipine binding and dissociation on the same samples of cultured beating cardiac myocytes. Signal arose only from bound drug molecules. Binding was monoexponential (τ = 5.5 min) as was dissociation (14.3 min). Preincubating cells with non-radioactive nifedipine reduced the amplitude and rate of [^{3}H]nitrendipine but not of [^{3}H]dihydroalprenolol binding. The potential uses of this phenomenon are discussed.

Condensed phase radioluminescence *Nitrendipine binding* *Binding kinetics* *Cardiac myocyte* *Dihydroalprenolol*

1. INTRODUCTION

Radioligand binding studies have revealed a great deal about drug–receptor and hormone–receptor interactions in many biological systems [1]. The method is essentially a steady-state method which is usually applied to membrane fractions prepared from tissues. The kinetics of binding can only be measured by repeated sampling of a single incubation or by incubating in parallel a pooled tissue preparation and then constructing a time course. One can never exclude the possibility that receptor modification or inactivation has occurred during the isolation of the membrane fraction. In most such experiments, radioligand is presented to both sides of the biological membrane, unlike in vivo. Vesicle preparations may be used for some tissues but these present additional problems of incomplete sealing and separating 'right-side-out' and 'inside-out' vesicles. These factors contribute to the problems of differences in potency between organ bath and isolated membrane preparations not infrequently observed.

Ideally, one wants to measure on intact, living cells hormone or drug binding and its immediate membrane effects (the transduction phase) leading to the ultimate biological response. This is particularly true when the biological response is difficult to measure; e.g., in brain tissue. An advance in the techniques available is required to address these problems experimentally.

The β-decay from a tritiated molecule may be used to excite a fluorophore in a model membrane system [2]. When the tritium and the fluorophore are in close proximity, a signal results which may be detected using single photon counting techniques. The technique, called condensed phase radioluminescence (CPR), has been used to study changes in the surface area of phospholipid vesicles and to examine the effects of fatty acids on membrane charge [2,3].

Here, we demonstrate that the binding of ^{3}H-labelled molecules to the membranes of living cells

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can give rise to photon emission from protein native to the membrane. We show that this CPR signal may be used to determine values for the half-time of [^{3}H]nitrendipine association and dissociation with a single class of binding sites on beating myocytes of 5 ± 1 min and 14.5 ± 1 min respectively. This may form the basis of a powerful tool for studying non-invasively receptors in intact tissue.

2. EXPERIMENTAL

Suspensions of beating heart cells were prepared as in [4]. The mixed fibroblast and myocyte preparation was incubated in 14 cm culture plates for 30 min at 37°C to allow most of the fibroblasts to attach. The plates were then swirled gently and the myocyte-enriched supernatant seeded into 1 cm^3 wells of a tissue culture plate (Limbo) containing a washed and heat-sterilised 1 cm^2 glass coverslip (Chance-Propper Ltd). The plates were incubated for 4 days at 37°C and the medium changed on days 2 and 4. The medium used was a complete medium containing 10% newborn calf serum and 6.2 mM D-glucose as in [4]. On day 4, each of the coverslip cultures (by now monolayers) was transferred to a clean, sterile glass scintillation vial (Packard) under stringent sterile conditions and 2 cm^3 of oxygenated medium added before capping the vials. After 1 h incubation at 37°C, the vials were packed in an insulated polystyrene container and transported by airline cabin freight to Switzerland. The cells arrived in good condition and, after an initial 1 day incubation and medium change, the cells beat as a synchronous monolayer at rates in excess of 60 min^{-1} at 20°C. CPR measurements were made after the second day in Switzerland (culture day 6).

CPR was detected using an SLM fluorimeter equipped for single photon counting. The photomultiplier was arranged to be 4 cm above a well into which the coverslip cultures and 450 mm^3 of medium was placed. The entire assembly was mounted in a light-tight box. All experiments were carried out at room temperature (about 20°C). Photons were counted for 30 s periods, averaged and recorded as counts per second (cps).

Cells were added to 450 mm^3 of medium containing 2.1 μM [^{3}H]nitrendipine (New England Nuclear), an antagonist of the slow calcium channel [5], and the CPR signal observed until equilibrium was reached. The medium was then aspirated off the cells and replaced with non-radioactive medium. The subsequent decay in the CPR signal was used to obtain the kinetics of dissociation of the bound drug. Experiments were also performed using 2.1 μM [^{3}H]dihydroalprenolol (Amersham International), a β-receptor antagonist [6], and on cells preincubated for 20 min at 37°C with medium containing 0.1 μM nifedipine, another slow channel antagonist.

3. RESULTS

Fig.1A shows that adding coverslip cultures of cells to medium containing [^{3}H]nitrendipine increases the CPR signal observed. The signal may

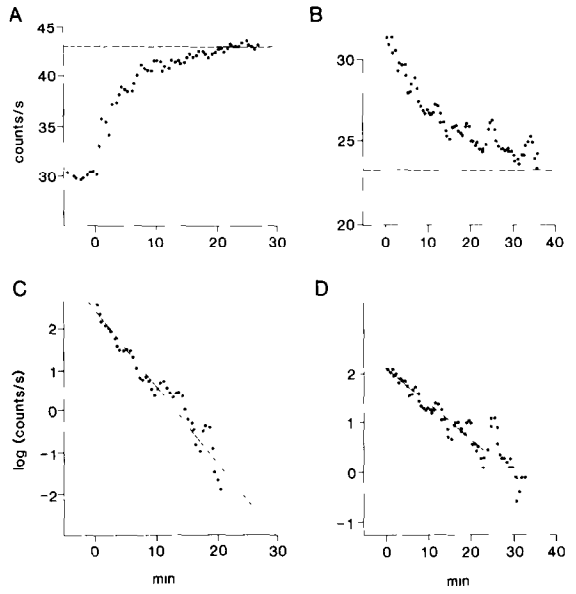

Fig.1. The association and dissociation of [^{3}H]nitrendipine with isolated myocytes. The time courses of CPR signal resulting from the association (A) and dissociation (B) of [^{3}H]nitrendipine with isolated cardiac myocytes in culture is shown. Myocytes were added at time 0. The initial signal observed before the addition of the cells is caused by the dark current of the phototube and the stopping radiation of the tritium. Semi-logarithmic transformations of the data for (C) association and (D) dissociation. (– – –) Baselines for the transformation in (A,B); (——) τ of 5.5 min (C) and 14.5 min (D).

be blocked by glass indicating that it is in the ultraviolet frequency range and therefore, in this system, arises from tryptophan. Adding cells in the absence of any tritium did not alter the background count rate. The signal must therefore arise from [^{3}H]nitrendipine binding to proteinaceous sites on the myocyte membranes. Very little signal comes from [^{3}H]nitrendipine in solution. There is also no interference from any intrinsic chemiluminescence of the cells under these conditions.

The kinetics of [^{3}H]nitrendipine binding are monoexponential (panel C). From this it may be inferred that CPR is monitoring the drug binding to a single class of proteinaceous binding sites. Theoretically, the tritium and the fluorophore must be within about 50 nm in the membrane for a CPR signal to be observed [2,3]. So if the [^{3}H]nitrendipine is also binding to non-protein binding sites in these experiments, then the contribution to the CPR signal of drug binding to these sites is negligible. Furthermore, if there were a superimposed conformational change at one protein binding site or if there were more than one major binding protein (having different kinetic constants), then the kinetics observed by CPR would be more complex.

Monoexponential kinetics of dissociation were also observed (fig.1D) when the medium on the same cells was replaced by medium containing no radioactivity. The half-time of dissociation was 14.5 ± 1 min. The cells continued to beat after a further change into maintenance medium at the end of the experiment.

Cells were pre-incubated at 37°C for 20 min with 0.1 μM nifedipine before being added to medium containing 2.1 μM [^{3}H]nitrendipine but no nifedipine. Under these circumstances a 33% lower amplitude and 70% slower rate of binding were observed. Pre-incubation with 1 μM nifedipine caused a marked reduction in the rate of [^{3}H]nitrendipine binding. The amount of nifedipine bound to the myocytes which was carried over into the sample chamber is not known, but its effect was to alter the kinetics of the CPR signal change produced by [^{3}H]nitrendipine binding. This is consistent with a competition for the single class of proteinaceous binding sites observed by CPR.

[^{3}H]Dihydroalprenolol was used to compare the

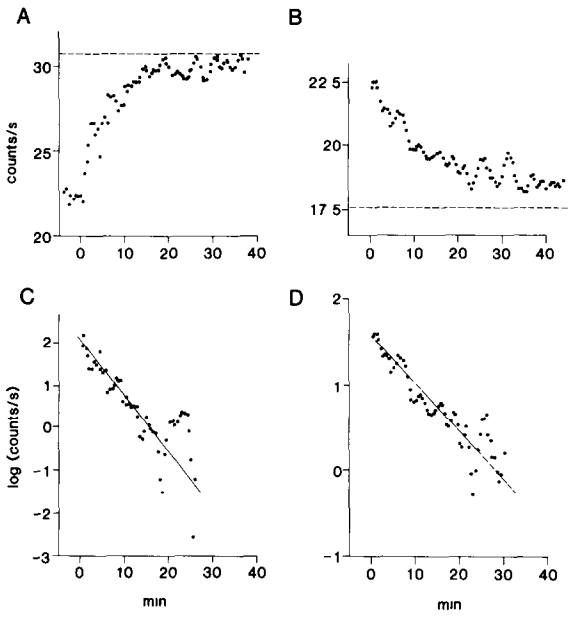

Fig.2. The association and dissociation of [^{3}H]dihydroalprenolol with isolated myocytes. The time course of the CPR signal resulting from the association (A) and dissociation (B) of [^{3}H]dihydroalprenolol are shown. The medium was replaced at time 0. Semilogarithmic transformations of the data for association and dissociation are shown (C,D), assuming the baselines indicated at (– – –) in (A,B); (——) τ of 6.8 min (C) and τ = 14.8 min (D).

effects of the calcium slow channel antagonists above with an antagonist of the β-receptor. The binding and dissociation kinetics for [^{3}H]dihydroalprenolol were both monoexponential with half times of 6 ± 1 min and 14.8 ± 1 min, respectively (fig.2). Pre-incubation of the cells with nifedipine had no effect on these parameters. This indicates that CPR is applicable to systems in addition to the calcium slow channel. It also shows that nifedipine does not exert its effect in the nitrendipine system other than at the binding site.

4. DISCUSSION

The results demonstrate that CPR can be used to measure the kinetics associated with binding of drug molecules to intact, living cells. The CPR signal observed when [^{3}H]nitrendipine binds appears to arise from a single class of proteinaceous binding sites at which nifedipine and nitrendipine

compete. This is likely to be the dihydropyridine binding site of the calcium slow channel [5], but a contribution of displaceable binding (with similar kinetics) to other proteins cannot be ruled out. A formal pharmacological evaluation of the binding site is in train. The sensitivity of the measurement is sufficiently high to permit the determination of the kinetics of association and dissociation on a single sample.

The rate of dissociation of [^{3}H]nitrendipine is similar to values previously obtained by classical techniques [5]. The advantage of CPR is that the measurements were made non-invasively on intact, viable cells. This overcomes the major problem of classical binding studies, where inactivation or modification of receptors may occur during the isolation of membrane fractions. Furthermore, CPR obviates the measurement of binding to non-viable cells as dead cells would have already detached from the monolayer and not been included in the sample chamber. CPR only measures bound ^{3}H-labelled drug molecules and so no separation of bound and free ligand is required. This means that there is no error introduced into the measurements by the use of rapid separation techniques. This advantage may ultimately permit analysis using CPR of binding kinetics of the order of 10 s.

These experiments demonstrate that CPR has a sensitivity comparable to other luminescence techniques and has adequate sensitivity to be useful in addressing biologically relevant problems in native membranes. An improvement of a factor of 10 in drug concentration may be possible using modified apparatus. The multiplicity of protein molecules in natural membranes gives many potential fluorophores, but the energy transfer processes involved and the specificity of drug binding are such that almost all of the signal probably arises from specific binding sites. Even so, several different tryptophan residues within the binding protein probably emit photons. They do so against a background caused only by the stopping radiation in the medium [2,3]. This forms the limitation of sensitivity of the technique and may only be reduced by decreasing the volume of the supernatant. The sensitivity of the measurement then depends solely on the statistics of counting.

As in all fluorescence measurements, an external calibration is required to relate the signal intensity to the actual amount of material bound and thus obtain a K_D for binding. Given this limitation, more information can be obtained by CPR and more quickly than by classical binding studies. For example, the aggregation of drug–receptor complexes could be measured in intact cells [2,3]. As tritiated agonists, partial agonists and antagonists for the same receptor may be used, and the interaction of several partial agonists or antagonists with one ^{3}H-labelled agonist may be measured, CPR may eventually be used to map receptors and illustrate structure–activity relationships on living cells.

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