High affinity binding of [^{125}I]monoiodoapamin to isolated guinea-pig hepatocytes

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The bee venom neurotoxin apamin has been labelled with ^{125}I and its binding to isolated guinea-pig hepatocytes measured under physiological conditions. A single saturable component of [^{125}I]monoiodoapamin binding with a K_d of 350 pM and B_{max} of 0.99 fmol/mg dry wt was identified. Native apamin displaced labelled apamin with a K_d of 376 pM which is broadly in keeping with the concentrations found to inhibit K loss from guinea-pig hepatocytes. These observations, together with the binding found in other tissues, suggest that specific binding of labelled apamin is particularly associated with apamin-sensitive, Ca-activated K-channels.

Apamin *Guinea-pig hepatocyte* *Ca-activated K-channel*

1. INTRODUCTION

Ca^{2+}-dependent K^+-conductance channels are a feature of the plasma membrane of many types of cell (e.g., erythrocytes, certain neurones, intestinal smooth muscle and hepatocytes of some species). Elevation of the intracellular [Ca^{2+}] leads to an increase in potassium permeability which normally results in the loss of cell potassium and hyperpolarization of the cell membrane [1]. Apamin, a neurotoxic polypeptide isolated from bee venom, has recently been shown to block this K-conductance increase in guinea-pig hepatocytes [1], smooth muscle [2,3,4] and neuroblastoma cells [5]. Apamin can be labelled with ^{125}I; the monoiodinated derivative is biologically active and binds with high affinity to a variety of tissue homogenates [5,6], membranes [7] and synaptosomes [8].

This communication describes, for the first time, the binding of [^{125}I]monoiodoapamin to intact cells under physiological conditions. An estimate of the affinity of native apamin for its binding site has been obtained from competition studies and related to its activity in blocking the efflux of potassium from liver cells under essentially identical conditions.

2. MATERIALS AND METHODS

2.1. *Isolation of guinea-pig hepatocytes*

Hepatocytes were prepared from male Hartley guinea pigs as described in [1]. Viability assessed by Trypan blue exclusion was typically 80–90%; potassium content of the cells was 275.1 ± 4.5 mmol/kg dry wt (± SE, n = 56). Cells were incubated and experiments conducted at 37°C in Eagle's MEM (Wellcome) supplemented with 2% bovine serum albumin and 10% newborn calf serum. Cell suspensions contained about 8×10^6 cells/ml (10^6 cells = 2.5 mg dry wt).

2.2. *Preparation of [^{125}I]monoiodoapamin*

Apamin was radioiodinated and purified essentially as in [8]. The reaction mixture (monoiodo-, diiodo- and a large excess of unlabelled apamin) was separated using an SP Sephadex C25 column (12 × 0.9 cm), equilibrated with 160 mM NaCl,

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50 mM sodium phosphate buffer, pH 6.0. The column was eluted with the equilibration buffer, increasing the NaCl concentration stepwise to 300 mM after 20 ml. The later fractions of the monoiodoapamin peak were discarded to avoid contamination by native apamin [8]. Thin-layer chromatography (TLC) of the monoiodoapamin peak revealed a single radioactive spot whose R_f-value corresponded to that expected for monoiodoapamin [6]. The purity of the [^{125}I]monoiodoapamin was checked by TLC at regular intervals. Assuming 100% isotopic abundance, the spec. act. of the [^{125}I]monoiodoapamin is 2000 Ci/mmol and the concentration of the labelled compound in the fractions could accordingly be determined by measuring radioactivity in a calibrated γ counter. It was impractical to desalt the monoiodoapamin fractions from the ion exchange column. Consequently, concentrations of [^{125}I]monoiodoapamin greater than 600 pM were avoided in order to prevent large changes in sodium chloride concentration and pH, originating from the elution buffer.

2.3. *Binding studies*

In the experiments described below [^{125}I]monoiodoapamin which had become associated with cells was separated from free-labelled apamin by rapid centrifugation of the cells through di-*n*-butylphthalate (BDH, referred to subsequently as 'oil') [9]. Some extracellular fluid was carried through with the cells and studies with [^{3}H]inulin suggested that most of the 'non-inhibitable' binding of labelled apamin was attributable to this phenomenon. The terms 'inhibitable' and 'non-inhibitable' are used in preference to the more common 'specific' and 'non-specific' because the former do not assume a particular pharmacological significance of the binding.

2.3.1. Kinetic studies

6 ml of incubation medium containing labelled apamin was added to 9 ml of cell suspension in a conical flask (final [^{125}I]monoiodoapamin concentration 16 pM). The cells were shaken at 37°C under a gas phase of 5% CO_2 in O_2. At fixed time intervals two 0.5-ml aliquots were removed, added to 1.5 ml Eppendorf tubes containing 0.25 ml of oil and immediately centrifuged for 15 s at 6000 × *g*. The aqueous and oil layers were removed and the bottom of the tube, containing the cell pellet, cut off and counted. The rate of association of labelled apamin with the cells was studied in this way over a period of 9.5 min. Dissociation of tracer was monitored in a second flask to which 1 μM apamin was added after 3.5 min. In a third flask 1 μM apamin was present throughout to follow non-inhibitable binding.

2.3.2. Equilibrium binding

0.2 ml of incubation medium containing a range of concentrations of labelled apamin was added to quadruplicate Eppendorf tubes containing 0.3 ml of cell suspension layered above 0.25 ml of oil. After 2 min at 37°C the tubes were centrifuged and the pellets counted as described above. Non-inhibitable binding was determined in a parallel set of tubes containing 1 μM unlabelled apamin. Free [^{125}I]monoiodoapamin concentrations have not been corrected for the small reduction due to binding (always less than 3%).

2.3.3. Competition experiments

0.3 ml of cell suspension was incubated with 0.2 ml of incubation medium containing labelled apamin (final concentration 46 pM) and varying concentrations of unlabelled apamin (final concentrations 10 pM–1 μM). After 2 min the bound labelled apamin was measured as described above.

2.4. *Potassium loss experiments*

Potassium release from hepatocytes in response to α-adrenoceptor activation was determined using a K-sensitive electrode immersed in a suspension of the cells [1]. Potassium loss in the first 30 s after drug application was expressed as a percentage of the total cell K content, evaluated for each aliquot of cells by the subsequent addition of 100 μM digitonin, which caused complete release of potassium from the cells. Apamin was applied 3 min before phenylephrine. All solutions contained 5 μM (±)-propranolol to prevent any β-actions of phenylephrine.

3. RESULTS

3.1. *Kinetics of [^{125}I]monoiodoapamin binding*

Fig.1 shows that at 37°C in physiological solution the uptake of labelled apamin from a 16 pM

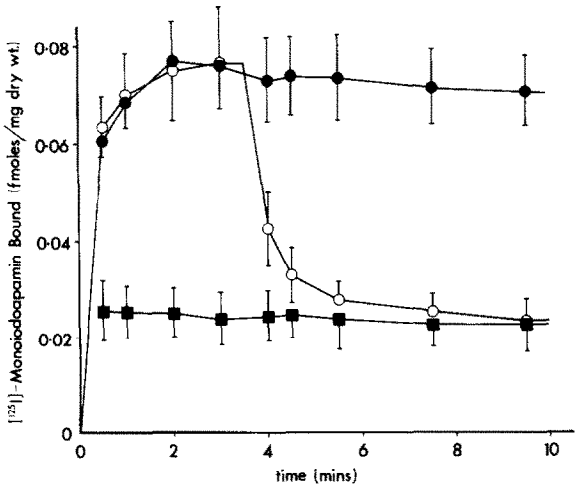

Fig.1. Binding of [^{125}I]monoiodoapamin as a function of time. Guinea-pig hepatocytes were exposed to 16 pM labelled apamin at $t = 0$. Filled circles and filled squares show the time course of total and non-inhibitable binding respectively. Open circles show the reduction in total binding, due to dissociation of labelled apamin from saturable sites, following the addition of 1 μM apamin at 3.5 min. Points are means ± SE from 3 expts, each performed in duplicate.

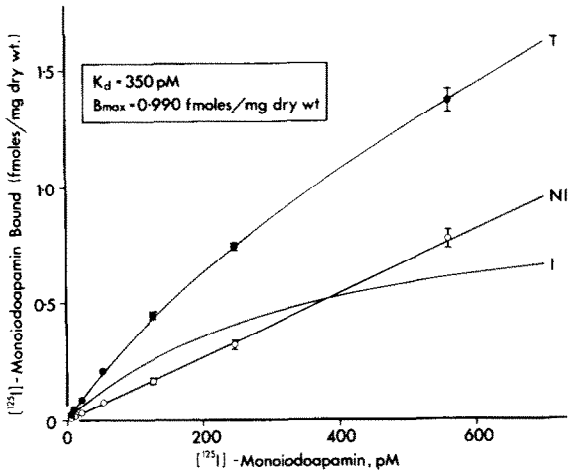

Fig.2. Binding of [^{125}I]monoiodoapamin to guinea-pig hepatocytes as a function of concentration. Points are means ± SE from 5 expts (4–8 observations/expt). The curve for total binding (T) is a least squares fit of a linear plus a hyperbolic saturable component (see text). NI indicates non-inhibitable binding. The theoretical saturable (inhibitable) component alone is represented by curve I.

solution is largely complete in 2 min, the half-time being about 20 s. Dissociation, initiated by adding an excess of native apamin, had a similar time course. These observations validate the use of a 2 min incubation in the experiments described in 2.3.2 and 2.3.3 and stress the importance of a rapid technique for isolating bound from free ligand.

3.2. *Equilibrium binding of [^{125}I]monoiodoapamin to guinea-pig hepatocytes*

Fig.2 shows the combined results of 5 experiments. 'Non-inhibitable' binding increased linearly with the concentration of labelled apamin over the range 5–600 pM. When this component was subtracted from the values for 'total' binding a saturable 'inhibitable' component was identifiable. A non-linear least squares computer fit [10] of the data was performed for total binding according to equation (1):

$$B = B_{max}\frac{[L]}{K_L + [L]} + m[L] \quad (1)$$

where:

B = the amount of labelled apamin bound;
B_{max} = the total binding capacity of the inhibitable component;
$[L]$ = the concentration of labelled apamin;
K_L = its equilibrium dissociation constant.

The coefficient for non-inhibitable binding, m, was constrained to be the slope of the fitted linear regression line (1.36 μl/mg dry wt) obtained directly from the data for non-inhibitable binding. With the data weighted inversely with variance, the values giving the best fit were: K_L = 350 pM and B_{max} = 0.99 fmol/mg dry wt. Since concentrations above 600 pM could not be used (see section 2) these estimates are relatively imprecise (approximate SD's based on the variance of residuals being 60 pM and 0.15 fmol/mg dry wt, respectively).

3.3. *Competition between apamin and [^{125}I]-monoiodoapamin*

The results of 3 experiments in which the binding of 46 pM labelled apamin was inhibited by native apamin are shown in fig.3. The inhibition observed is consistent with simple competition at a single class of binding site and the data has accordingly been subjected to a non-linear least squares fit to equation (2):

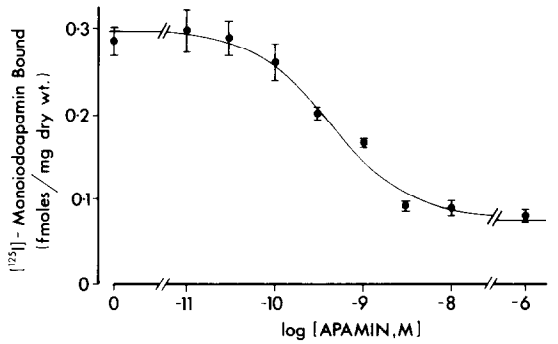

Fig.3. Inhibition of [^{125}I]monoiodoapamin binding to guinea-pig hepatocytes by native apamin. The concentration of labelled apamin was 46 pM. The curve is drawn according to equation (2) using the least squares estimates for K_U, B_0 and B_∞ (see text). Points are means ± SE from 3 expts (5 observations/expt).

$$B = B_\infty + (B_0 - B_\infty)\frac{1 + [L]/K_L}{1 + [L]/K_L + [U]/K_U} \qquad (2)$$

where:

B = the amount of labelled apamin bound in the presence of unlabelled apamin at concentration $[U]$;

B_0 and B_∞ = the amounts bound in the absence, or in the presence of an excess, of unlabelled apamin, respectively;

$[L]$ = the fixed concentration of labelled apamin (46 pM);

K_L and K_U = the equilibrium dissociation constants for labelled and unlabelled apamin, respectively.

K_L was given the value 350 pM (from the results of fig.2). With points weighted inversely with their variance, the fitted value for K_U was 376 pM (approximate SD = 83 pM).

3.4. *Inhibition of potassium loss from hepatocytes by apamin*

As has previously been demonstrated [1,11], α-adrenoceptor agonists cause a loss of potassium from guinea-pig liver cells. Increasing concentrations of apamin produced a progressive depression of the dose–response curve to phenylephrine (fig.4), the fractional block being relatively independent of the agonist concentration; 50% inhibition of the initial rate of potassium loss was obtained with about 1 nM apamin.

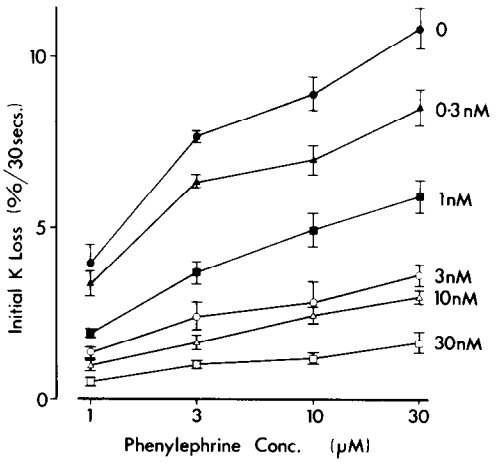

Fig.4. Inhibition of K release from guinea-pig hepatocytes in response to (−)-phenylephrine by apamin. Potassium loss is expressed as the % of cell K lost during the first 30 s of exposure to phenylephrine. Apamin was added 3 min before phenylephrine at the concentrations shown on the right. Means of 4–9 observations ± SE.

3.5. *Binding of [^{125}I]monoiodoapamin to human erythrocytes and rat hepatocytes*

The binding of labelled apamin to erythrocytes and rat hepatocytes was investigated under identical conditions to those described for guinea-pig hepatocytes. Human red blood cells showed no significant inhibitable binding at concentrations up to 500 pM. Similarly in 4 experiments with rat hepatocytes significant inhibitable binding could not be detected at concentrations of 9, 115 and 500 pM; e.g., with 115 pM labelled apamin inhibitable binding was 0.0067 ± 0.0041 (SE) fmol/mg dry wt, representing only 5% of the total binding, which compares with 0.245 ± 0.014 fmol/mg dry wt inhibitable binding to guinea-pig hepatocytes (61% of total) at the same concentration.

4. DISCUSSION

The present results demonstrate a saturable component of [^{125}I]monoiodoapamin binding to guinea-pig hepatocytes. The rapid attainment of equilibrium and rapid dissociation (fig.1) suggests that binding primarily occurs at the level of the cell membrane and that there is negligible intracellular uptake of the toxin. The lack of inhibitable bin-

ding in rat liver cells also argues against the presence of an active uptake mechanism. The estimated K_d for inhibitable binding, 350 pM, lies between the values reported for preparations from neuroblastoma cells, 71 pM [5], and guinea-pig colon, 700 pM [7] (binding studies in physiological medium but at 0°C). Much lower values are reported for binding studies in solutions of low ionic strength [5,7,8]. Our competition experiments showed native apamin to have a similar affinity (K_d = 376 pM) to the iodinated derivative. The density of binding sites was estimated to be 0.99 fmol/mg dry wt of cells, corresponding to about 1500 sites/cell.

Concern has been expressed over the stability of [^{125}I]monoiodoapamin at 37°C [6,7]. However, as fig.1 shows, binding is well maintained after 10 min incubation at this temperature, suggesting that degradation is not a problem with relatively short incubations.

The results with other tissues suggest that the inhibitable binding is directly related to the presence of apamin-sensitive K-channels. Firstly, we were unable to demonstrate significant inhibitable binding to rat hepatocytes, which do not appear to possess Ca-activated K-channels. However, a recent report [12] describes exceptionally high affinity binding (K_d = 0.135 pM) of ^{3}H-labelled apamin to rat liver plasma membranes, suggesting there may be a binding component either too small to be detected or absent in whole cells under the present experimental conditions. Secondly, intestinal smooth muscle, in which α-mediated relaxation is inhibited by apamin [2], also binds monoiodoapamin with high affinity ([7], and our own unpublished observations). Finally, red blood cells are particularly interesting in that their Ca-dependent K-conductance is not blocked by apamin [1], and in keeping with this observation we also have failed to detect any inhibitable binding of labelled apamin to these cells.

The relatively simple and indirect measure of inhibition of K-conductance provided by the present experiments (see fig.4) suggests that, after 3 min, 1 nM apamin has probably inactivated at least 50% of the K-channels. A K_d of 376 pM, as suggested by the competition studies, would result in 73% of the sites being occupied at this concentration. Thus, if we assume that binding site occupancy corresponds to channel block, it appears that the measured K_d is consistent with the degree of block that we observe.

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