Rapid Report

4-Aminomethylbenzoic acid is a non-translocated competitive inhibitor of the epithelial peptide transporter PepT1

D. Meredith, C. A. R. Boyd, J. R. Bronk *, P. D. Bailey †, K. M. Morgan †, I. D. Collier † and C. S. Temple

*Department of Human Anatomy, University of Oxford, South Parks Road, Oxford OX1 3QX, *Department of Biology, University of York, Heslington YO1 5DD and †Department of Chemistry, Heriot-Watt University, Riccarton, Edinburgh EH14 4AS, UK*

1. 4-Aminomethylbenzoic acid, a molecule which mimics the spatial configuration of a dipeptide, competitively inhibits peptide influx in both *Xenopus laevis* oocytes expressing rabbit PepT1 and through PepT1 in rat renal brush border membrane vesicles.

2. This molecule is not translocated through PepT1 as measured both by direct HPLC analysis in PepT1-expressing oocytes and indirectly by its failure to trans-stimulate labelled peptide efflux through PepT1 in oocytes and in renal membrane vesicles.

3. However 4-aminomethylbenzoic acid does reverse trans-stimulation through expressed PepT1 of labelled peptide efflux induced by unlabelled peptide. Quantitatively this reversal is compatible with 4-aminomethylbenzoic acid competitively binding to the external surface of PepT1.

4. 4-Aminomethylbenzoic acid (the first molecule discovered to be a non-translocated competitive inhibitor of proton-coupled oligopeptide transport) and its derivatives may thus be particularly useful as experimental tools.

Since the discovery of the natural plant product phlorizin (Nakazawa, 1922) as a highly specific, non-transported, inhibitor of glucose absorption in kidney and small intestine, it has been of great value in elucidating the many different physiological roles of sodium-coupled glucose transport (for example in lung and choroid plexus, see Hediger & Rhoads, 1994). In the field of peptide transport no such non-transported inhibitor has yet been described, and the absence of such a reagent may explain the relatively slow progress made in investigating the function (Matthews, 1991; Fei *et al.* 1998) of peptide transporters, e.g. within the central nervous system. We report here that 4-aminomethylbenzoic acid (4-AMBA) is such an inhibitor and suggest that it may form the basis for the design of synthetic high-affinity ligands for the PepT1 family (analogous to phlorizin for the sodium–glucose cotransporter SGLT). This finding is also of interest because of the very close chemical similarity between 4-AMBA, a competitive inhibitor which is not translocated, and 4-aminophenylacetic acid (4-APAA) which recently has been shown to be a substrate for translocation by epithelial peptide transporters (Temple *et al.* 1998).

METHODS

4-Aminomethylbenzoic acid (4-AMBA), 4-aminophenylacetic acid (4-APAA) and D-Phe-L-Gln were synthesized in-house using standard techniques. 4-AMBA and 4-APAA are also available commercially (Sigma-Aldrich, Poole, UK). [4-^{3}H-D-Phe]-L-Gln was custom synthesized by Zeneca (Northwich, Cheshire, UK). All other reagents were of research grade and obtained as previously described (Temple *et al.* 1998).

All animal procedures were carried out under Home Office guidelines. Brush border membrane vesicles were obtained from rat renal cortex using standard methods (Biber *et al.* 1981). Renal tissue was removed from rats under pentobarbitone (Sagatal) anaesthesia (30 mg, I.P.), and the animal killed by cervical dislocation. Flux measurements through PepT1 (Temple & Boyd, 1998) were determined by rapid filtration, under initial rate conditions at 37 °C as described previously (Temple *et al.* 1995, 1996). *Xenopus laevis* were anaesthetized with 0·1 % w/v MS-222 (Sigma, Poole, UK) prior to ovariectomy. Oocyte expression of rabbit PepT1 cRNA was as previously described (Fei *et al.* 1994; Meredith *et al.* 1996) and influx and efflux experiments were performed at room temperature as described in Temple *et al.* 1998. HPLC analysis of 4-AMBA and 4-APAA was determined using a modified method of Temple *et al.* 1996. Oocytes were suspended in

3% v/v perchloric acid (35 μl), centrifuged for 10 min at 14000 g; 30 μl of 1 M KOH was added to 30 μl of supernatant and samples freeze–thawed and recentrifuged; 20 μl of resulting supernatant was injected onto a 250 mm C18-RP column (Merck). Elution was carried out using methanol–H_2O (25% for 4-AMBA and 2% for 4-APAA) mobile phase buffered with 21 mM KH_2PO_4 at a flow rate of 1 ml min^{-1} and sample detected by UV absorbance at 236 nm (Lister *et al.* 1995). The level of sensitivity of the assay is some 7-fold lower for 4-AMBA compared with 4-APAA (integrated area under the curve for 1 nmol of 4-AMBA is 453 compared with 3351 for 4-APAA).

The molecular modelling of 4-AMBA and 4-APAA (on a Power Macintosh G3) was performed using CambridgeSoft Chem3D Pro version 3.5.1 and was controlled by a custom Applescript (K. M. Morgan). This generated random starting points and governed the fine tuning of minimizations in order to obtain global minimum energy conformations.

Kinetic calculations

Inhibition constants (and apparent inhibition constants) for influx were calculated from standard Michaelis–Menten kinetics as described previously (Temple *et al.* 1995). Efflux rate constants were calculated as described by Temple *et al.* (1996). Kinetics describing efflux of labelled substrate (S) from oocytes into a

medium containing external analogue (substrate, A) and increasing concentration of external unlabelled inhibitor (I) is shown:

$$V_{IA} = \frac{V_S[S]/K_S + V_{SA}[S][A]/K_{SA}K_A}{1 + [A]/K_A + [I]/K_I}, \tag{1}$$

where V_S, K_S, V_{SA}, K_{SA}, K_A and K_I are also defined (Deves & Boyd, 1989).

RESULTS

Figure 1 shows experiments using both rabbit PepT1 expressed in *Xenopus laevis* oocytes (Fig. 1*A*) and native epithelial membranes (rat renal cortex brush border membrane vesicles (BBMV), Fig. 1*B*). The results in both systems show that 4-aminomethylbenzoic acid (4-AMBA) is a competitive inhibitor of labelled peptide influx. In each case 4-AMBA inhibits in a concentration-dependent fashion with data well described by a single system. Additionally, in the presence of external unlabelled substrate the K_i (inhibition constant) is shifted to the right. Quantitatively these results are compatible with predictions for simple competitive inhibition since the concentrations of unlabelled substrate employed (tracer and 1·0 mM in oocytes; tracer
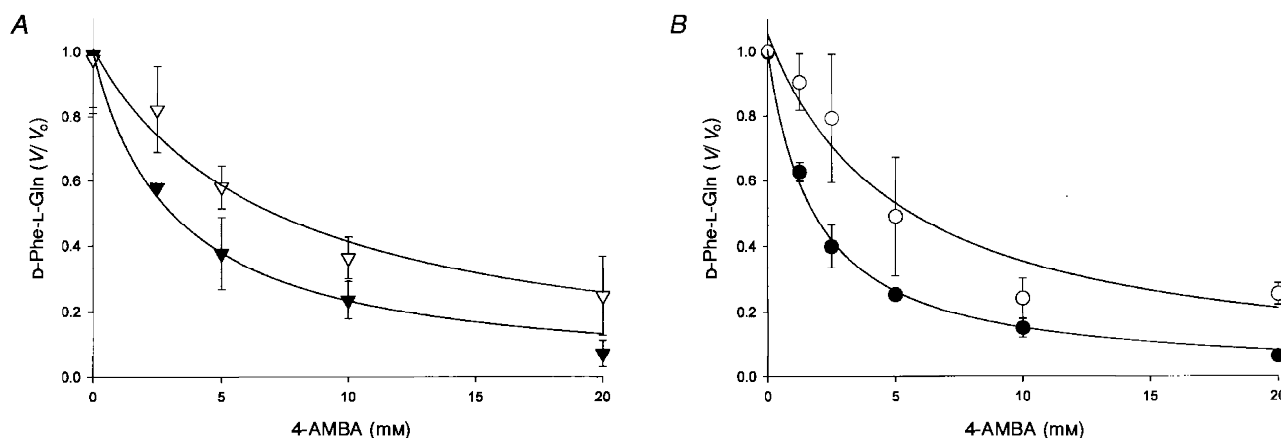

Figure 1. Fractional inhibition of labelled D-Phe-L-Gln (0·42 μM) influx

A, fractional inhibition (V/V_0) of labelled D-Phe-L-Gln (0·42 μM) influx into PepT1-expressing *Xenopus laevis* oocytes by 4-AMBA (0–20 mM) when $pH_o = 5·5$. Continuous lines represent the best fit of data to Michaelis–Menten kinetics for a single system in the absence (▼) and presence (▽) of unlabelled D-Phe-L-Gln (1·0 mM). Calculated inhibition constants (K_i) are 3·1 ± 0·4 and 6·9 ± 1·2 mM, respectively. Data has been corrected for non-mediated transport by subtracting D-Phe-L-Gln flux seen in H_2O-injected control oocytes. Labelled D-Phe-L-Gln flux in the absence of 4-AMBA has been normalized to 1. Absolute fluxes in the absence and presence of unlabelled D-Phe-L-Gln are 194 ± 36 and 118 ± 18 fmol oocyte^{-1} h^{-1}, respectively. Data points are means ± s.e.m. ($n = 5$). *B*, fractional inhibition of labelled D-Phe-L-Gln (0·34 μM) influx into rat renal BBMV by 4-AMBA (0–20 mM) under equilibrium exchange conditions: $pH_o = 5·5$, $pH_i = 7·4$, $4\text{-AMBA}_o = 4\text{-AMBA}_i$ and membrane potential clamped inside negative. Continuous lines represent the best fit of data to Michaelis–Menten kinetics for a single system in the absence (●) and presence (○) of unlabelled D-Phe-L-Gln (0·21 mM). Calculated inhibition constants (K_i) are 1·8 ± 0·1 and 5·1 ± 1·3 mM, respectively. Data has been corrected for non-mediated transport by subtracting labelled D-Phe-L-Gln flux seen in the presence of a saturating concentration of unlabelled peptide (10 mM). Labelled D-Phe-L-Gln flux in the absence of 4-AMBA (V_0) has been normalized to 1. Absolute fluxes in the absence and presence of unlabelled D-Phe-L-Gln are 854 ± 227 and 464 ± 208 fmol (mg protein)$^{-1}$ s^{-1}, respectively. Data points are means ± s.e.m. ($n = 3–6$).

and 0·21 mM in renal vesicles) were chosen on the basis of previous work (Meredith *et al.* 1998) which showed that for these peptides these values are the approximate K_m values in the two systems. For a competitive inhibitor the Michaelis–Menten equation predicts that the K_i observed in the presence of unlabelled substrate at this concentration therefore should be double the K_i observed in its absence. For both assays, within the limits of experimental error, this is what is observed (the K_i in oocytes is increased from 3·1 ± 0·4 to 6·9 ± 1·2 mM while the analogous K_i values in BBMV are 1·8 ± 0·1 and 5·1 ± 1·3 mM). (Note that all affinity constants measured are smaller in renal BBMV (by approximately 2- to 5-fold) when compared with those measured in the *Xenopus* oocyte expression system. We

attribute this relatively small difference to the different experimental conditions between the two assay systems (electrochemical driving force and temperature) and not to the involvement of the higher affinity isoform of the peptide transporter PepT2, which previously we have shown (using D-Phe-L-Gln) not to be functionally present in this preparation (Temple & Boyd, 1998).

Figure 2 shows directly that 4-AMBA (2 mM) is not transported by PepT1; the very small endogenous signal which is detected at the same elution time as 4-AMBA is found identically both in H_2O-injected and in PepT1-expressing oocytes (Fig. 2*Ac* and *d*). PepT1-mediated transport therefore is not different from zero (Fig. 2*B*). As a positive control, Fig. 2*Aa* also shows that the HPLC assay readily
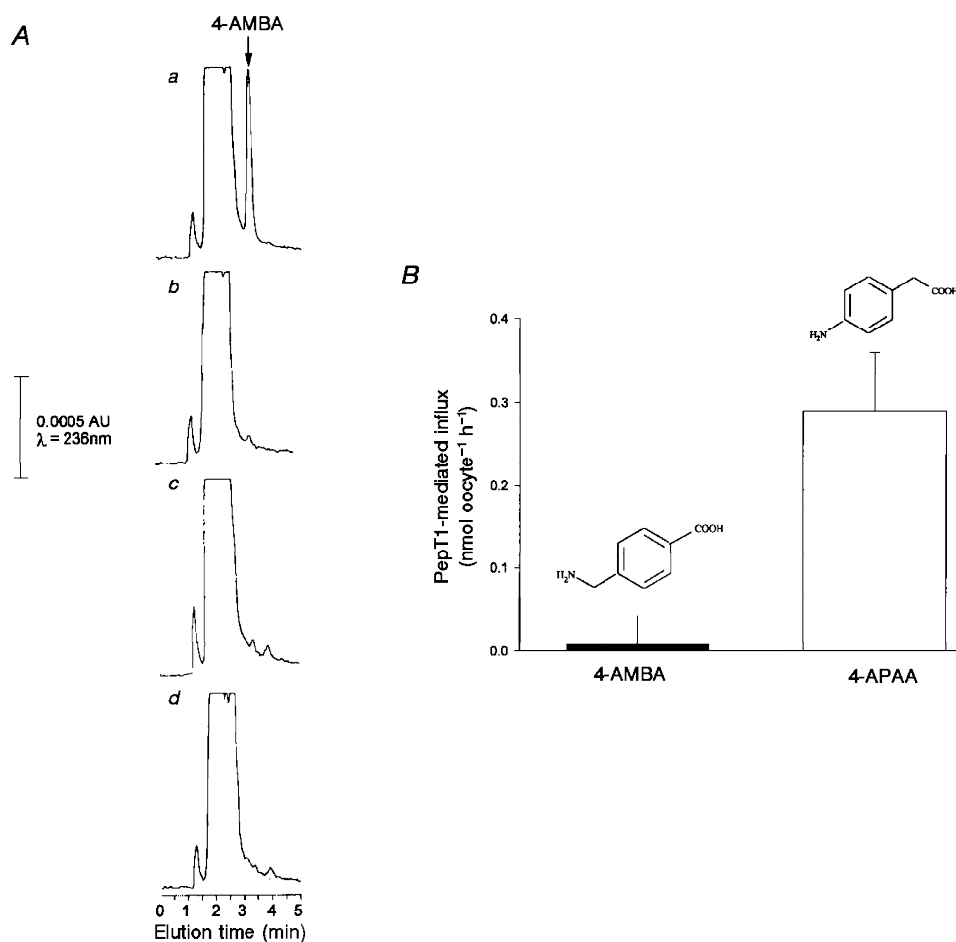

Figure 2

A, representative HPLC traces for (*a*) oocyte extract spiked with 0·1 mM 4-AMBA (20 μl was injected onto the column and thus the peak is equivalent to 2 nmol), (*b*) oocyte extract, (*c*) H_2O-injected control oocyte extract after 1 h incubation with 2 mM 4-AMBA and (*d*) PepT1-injected oocyte extract after 1 h incubation with 2 mM 4-AMBA. HPLC was performed as described in Methods. 4-AMBA was detected by UV absorbance at maximum sensitivity where full scale is equivalent to 0·001 absorbance units (AU). *B*, PepT1-mediated flux of 4-aminomethylbenzoic acid (4-AMBA, 2 mM) was not different from zero as detected by HPLC; flux of 4-aminophenylacetic acid (4-APAA, 2 mM) is shown for comparison. Fluxes were measured after 1 h incubation at $pH_o = 5·5$ into oocytes injected with PepT1 cRNA and H_2O-injected controls. Data shown has been subtracted from that seen in H_2O-injected controls. Data points are means ± S.E.M. (*n* = 5 from a representative oocyte preparation).

detects this molecule in an oocyte extract spiked with 2 nmol of 4-AMBA. The limit of detection of 4-AMBA is 0·05 nmol; it is noteworthy that even with 15 mM 4-AMBA, no PepT1-mediated transport into oocytes could be detected (data not shown). Furthermore 4-aminophenylacetic acid (4-APAA, shown previously by Temple *et al.* 1998 to be a substrate for PepT1) was, as expected, readily and reproducibly transported in these experiments, which were run in parallel on cells from identical batches of oocytes as those in which transport of 4-AMBA was not detected (Fig. 2*B*). When both the limit of detection of 4-AMBA (0·05 nmol) and the measured transport rate of 4-APAA (0·3 nmol oocyte^{-1} h^{-1} at a substrate concentration of 2 mM) are taken into account then the 'permeability' (V_{max}/K_m) of 4-APAA may be shown to be at least 44-fold greater than that for 4-AMBA.

In keeping with the result shown in Fig. 2, 4-AMBA does not trans-stimulate labelled peptide efflux from either renal membranes (Fig. 3*A*) or PepT1-expressing oocytes (Fig. 3*B*). In contrast, confirming previous results (Temple *et al.* 1996, 1998), the presence of external unlabelled dipeptide does indeed markedly trans-stimulate in both systems. Taken together the results shown in Figs 2 and 3 show that 4-AMBA, although it is a competitive inhibitor of peptide transport, is not a substrate for translocation by this transporter.

The conclusion that 4-AMBA binds, but does not trans-locate, is supported by further efflux experiments carried out on PepT1-expressing *Xenopus* oocytes. Cells were micro-injected with labelled peptide and efflux determined with and without external substrate (0, 1·2 and 20 mM D-Phe-L-Gln) as

a function of external 4-AMBA concentration (0–24 mM). The efflux rate constants with no 4-AMBA present (Fig. 4*A*) are consistent with the predicted effect from Michaelis–Menten kinetics: increasing substrate concentration from 0 to 1·2 mM (i.e. 2 × K_i) D-Phe-L-Gln results in approximately 6 % of the maximal rate of efflux (the maximal rate being that seen with saturating (20 mM) external peptide). The lines fitted to the data in Fig. 4*A* are the best fits to eqn (1) for a model where there is a translocated substrate (*A*, in this case D-Phe-L-Gln) and a non-translocated inhibitor (*I*, 4-AMBA). When there is no *A* present, increasing concentrations of *I* have no effect on the efflux rate constant, since under the conditions of the experiment no measurable efflux occurs. In the presence of 1·2 mM *A*, increasing concentrations of *I* are able to inhibit the trans-stimulation seen in the absence of *I*. The ability of *I* to inhibit the trans-stimulation is reduced when the concentration of *A* is increased to saturating concentrations (20 mM), as would be predicted for a substrate and inhibitor binding at the same site (i.e. simple competitive binding).

Figure 4*B* shows data from the same experiment transformed to show the fractional inhibition of efflux seen on increasing the 4-AMBA concentration in the presence of either 1·2 or 20 mM D-Phe-L-Gln. The data are fitted with Michaelis–Menten kinetics for a single system, analogous to that used in Fig. 1. The calculated apparent half-inhibition constants for *I* (4-AMBA) are 6·8 and 37 mM at analogue *A* (D-Phe-L-Gln) concentrations of 1·2 and 20 mM, respectively. Further application of the Michaelis–Menten equation then allows an indirect calculation of the substrate K_m. Increasing *A* 16·7-fold (from 1·2 to 20 mM) increases the measured apparent K_i
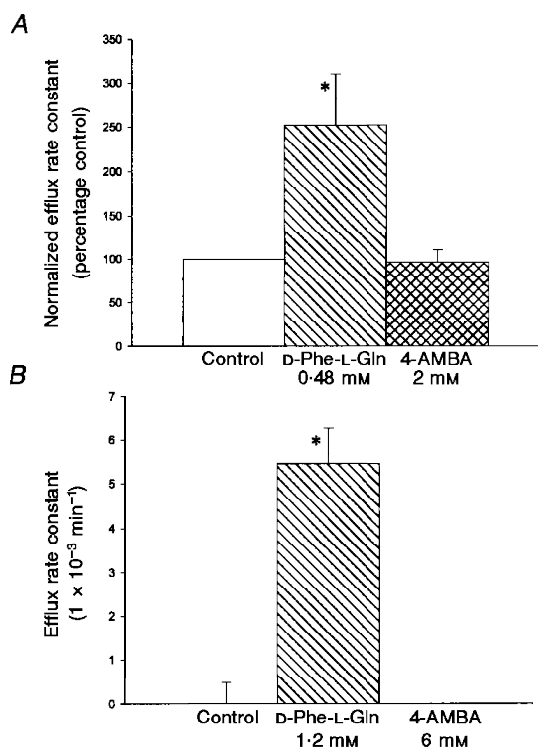

Figure 3. 4-AMBA in contrast to unlabelled dipeptide

4-AMBA, in contrast to unlabelled dipeptide, does not stimulate efflux of labelled tracer dipeptide from either (*A*) rat renal BBMV (pH$_o$ = pH$_i$ = 5·5 and membrane potential is clamped to zero) or (*B*) PepT1-expressing *Xenopus* oocytes (pH$_o$ = 5·5). Concentration of substrate is 2 × K_i for substrate cis-inhibition of labelled dipeptide influx. Efflux rate constants were calculated as in Methods. *A*, efflux rate constants are normalized to the control (0·086 s^{-1}). Data points are means ± S.E.M. (triplicate estimates from 4 vesicle preparations) * Data significantly different from control (*P* < 0·05, unpaired Student's *t* test). *B*, no efflux was measurable from H$_2$O-injected control oocytes (data not shown). Data points are means ± S.E.M. (5 oocytes from a representative preparation). * Data significantly different from control (*P* < 0·001, unpaired Student's *t* test).

for 4-AMBA 5·4-fold predicting a true K_m for D-Phe-L-Gln of 0·4 mM, a value not dissimilar from that determined directly (0·6 mM, Meredith *et al.* 1998). Thus the findings shown in Fig. 4 provide strong quantitative support for the idea that 4-AMBA binds to, but is not translocated by, PepT1.

DISCUSSION

The cloning from small intestinal mRNA of the gene product (PepT1) responsible for epithelial peptide transport (Fei *et al.* 1994) marked a watershed in investigation of this process. Although much effort is currently directed towards understanding how this transporter functions (see Daniel, 1997), investigation of its role in normal physiology has long been hampered by the absence of a 'good' inhibitor. In this paper we report results suggesting that it will be possible to design a high affinity inhibitor based on peptide mimic chemistry using 4-AMBA as a starting point. It seems likely that the affinity of this molecule for the transporter (4-AMBA having a K_i in the low millimolar range) will be increased by chemical modification, e.g. by incorporating an additional hydrophobic moiety in the region of the aromatic ring (cf. Daniel *et al.* 1992).

Possible reasons underlying differences in the way in which 4-AMBA and 4-APAA are handled are intriguing. The two molecules (cf. Fig. 2) initially appear structurally similar yet differ sufficiently for the one (4-APAA) both to bind and be translocated rapidly by the transporter whereas the other (4-AMBA) binds with higher affinity yet is not translocated. The small differences in the N (amine) to C (carboxy) spacing (0·643 nm and 0·645 nm for 4-APAA and 4-AMBA, respectively) suggest that this spacing is not an important factor here. However one chemical feature that does distinguish these two molecules is the nature of the N-terminus. As 4-AMBA is not an aromatic amine the N-terminus can be protonated more easily within the physiological range (in contrast to 4-APAA). Thus at pH 5·5 more 4-AMBA will be in the cationic species, whereas 4-APAA is predominantly neutral. It is of note that cationic dipeptides are generally poorer substrates than neutral or anionic ones (Steele *et al.* 1997). As with the work of Deves & Krupka on the choline transporter (Deves & Krupka, 1979, see Stein, 1990) systematic studies with a series of such analogues should help resolve what are the structural features of PepT substrates that respectively determine binding and translocation. It is interesting that the classical carrier theory developed for analysis of efflux in other well-studied model systems such as erythrocytes is directly applicable to labelled peptide efflux in PepT1-expressing *Xenopus* oocytes. Specifically the data shown in Fig. 4 has been modelled on the basis of theory for efflux of labelled substrate into a medium containing both unlabelled substrate and a non-transported inhibitor. The excellent fit of the data to the model (Fig. 4*B*) and the general applicability of the constants derived from the model so that those derived in fitting one set of experimental conditions (1·2 mM external substrate) readily apply to those observed under other conditions (20 mM external substrate) suggest that the applicability of carrier kinetics to this physiologically important cotransporter is robust.

Figure 4

Concentration dependence of 4-AMBA (0–24 mM) inhibition of tracer D-Phe-L-Gln efflux from *Xenopus* oocytes expressing PepT1; experiments carried out at three different concentrations of external (trans) unlabelled D-Phe-L-Gln; 0 (●), 1·2 mM (▼) and 20 mM (■). *A*, continuous lines represent best fit of data to kinetics describing efflux of a labelled substrate into a medium containing unlabelled external substrate and increasing concentration of external unlabelled inhibitor (Deves & Boyd, 1989; see eqn (1) in Methods). Calculated constants are: $V_S = 44{\cdot}47 \times 10^{-3}\ \mathrm{min}^{-1}$, $K_S = 0{\cdot}09$ mM, $V_{SA} = 195 \times 10^{-3}\ \mathrm{min}^{-1}$, $K_{SA} = 0{\cdot}01$ mM. Data points are means ± S.E.M. ($n = 10$ from 2 oocyte preparations). *B*, data from *A* above normalized to show fractional inhibition. The intercepts on the y-axis from the curves fitted to the data from Fig. 4*A* are defined as unity. Continuous lines represent the best fit of the data to Michaelis–Menten kinetics for inhibition of a single system. Calculated apparent half-inhibition constants are 6·8 and 37 mM in the presence of 1·2 and 20 mM unlabelled external D-Phe-L-Gln, respectively.
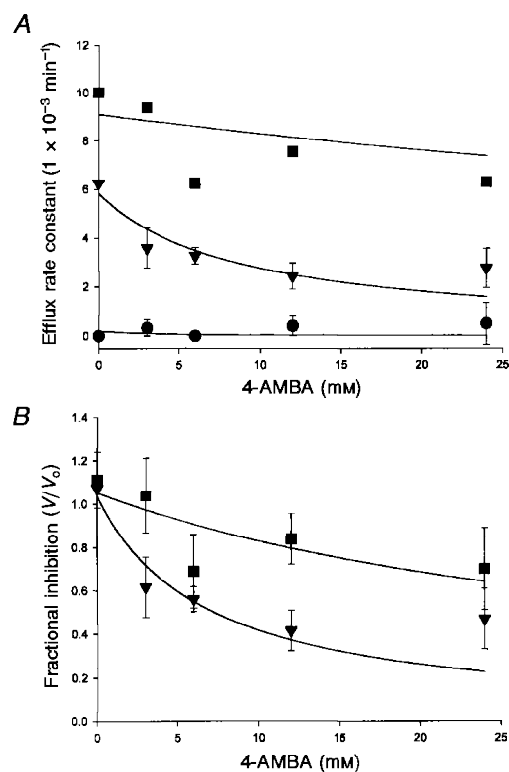
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Corresponding author

C. A. R. Boyd: Department of Human Anatomy, University of Oxford, South Parks Road, Oxford OX1 3QX, UK.

Email: richard.boyd@anat.ox.ac.uk

Authors' present addresses

D. Meredith: Department of Biochemistry, School of Medical Sciences, University Walk, Bristol BS8 1TD, UK.

C. S. Temple: Roche Discovery Welwyn, Broadwater Road, Welwyn Garden City, Hertfordshire AL7 3AY, UK.