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SHORT COMMUNICATIONS

Interaction of ionophore A23187 with a "masked" Ca^{2+} binding site in pig and pigeon red cells

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Ca^{2+}-sensitive red cell permeability to K^+ has been studied extensively in red cells from a number of species [1–6]. Two main techniques have been used to raise $[Ca^{2+}]_i$, namely (1) the calcium ionophore A23187 [7] and (2) ATP depletion procedures to inactivate the Ca^{2+} extrusion pump and allow $[Ca^{2+}]_i$ accumulation. Previous studies have indicated that in pig red cells a Ca^{2+}-sensitive K^+ permeability is evoked by A23187 in the presence of external Ca^{2+} [2] but not by ATP depletion procedures [3]. Similar findings have also been reported for pigeon red cells [8]. These results suggest that the A23187–Ca^{2+} complex may directly alter plasma membrane permeability. In this report the effects of both procedures on the plasma membrane permeability of nucleated pigeon and non-nucleated pig red cells were investigated in order to more fully characterize A23187 action.

Methods and results

Blood was washed three times by centrifugation (50 *g* for 10 min) in ice-cold saline [115 mM KCl, 20 mM NaCl, 60 mM sucrose, 0.5 mM $MgSO_4$, 5 mM 4-(2-hydropyethyl)-1-piperazine-ethanesulfonic acid (Hepes), pH 7.4, with NaOH]. High K^+ saline was used to depolarize the cells and avoid membrane potential alterations influencing ^{86}Rb (used as marker for K^+) influx rates. The washed red cells were resuspended to a 10% haematocrit (determined with a Coulter counter) in high K^+ saline and incubated at 42° (pigeon) or 37° (pig).

Cells were depleted of ATP in either 0.5 mM ethyleneglycolbis(amino-ethylether)tetra-acetate (EGTA) or Ca^{2+} (0.2, 1 or 5 mM), high K^+ saline. Pigeon red cells contain mitochondria allowing the use of 2 mM cyanide or 2 μM carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP) to deplete ATP; both procedures reduced ATP levels from millimolar to micromolar levels within 2 hr. Similar ATP levels were achieved in pig red cells by incubation with the glycolytic inhibitor iodoacetamide (5 mM) in the presence of antibiotics for 21–24 hr at 37°. ATP levels were determined using the technique of Bergmeyer [9], and total cell-associated Ca^{2+} was assessed by atomic absorption spectrophotometry.

The A23187 distribution in red cell suspensions was measured by iso-butanol extraction. Aliquots of suspension (4 ml) were incubated with 15 μM (150 μmoles/litre of cells) A23187 for 10 min at 37°, and the cells were washed twice by centrifugation, resuspended in 4 ml of saline and mixed vigorously with 4 ml of saline-saturated iso-butanol. The aqueous and organic phases were allowed to separate, and the A23187 concentration in the iso-butanol phase was determined by spectrophotometry at 385 nm excitation and 435 nm emission.

Isotope influx rates were assessed by centrifuging 0.5-ml aliquots of cell suspension with 0.5 ml silicon oil (Dow-Corning 550) and 0.5 ml of ice-cold saline in an Eppendorf 5141 centrifuge for 15 sec. Red cells passed through the oil but the saline remained above. The oil and supernatant fractions were carefully aspirated, the tip of the tube was removed, and the radioactivity in the pellet was measured. Aliquots of whole suspension were taken as totals.

Neither cell type exhibited a Ca^{2+}-sensitive ^{86}Rb permeability following ATP depletion despite increases in cell-associated Ca^{2+} of up to 2 orders of magnitude. ATP levels were reduced from 1.5–2 mM to 2–10 μM and cell-associated Ca^{2+} was raised from 20–30 μM to 0.5–5 mM (depending on the Ca^{2+} concentration in the depletion saline). Both ATP-depleted and non-depleted cells, however, showed a dramatic increase in ^{86}Rb influx when exposed to 150 μmoles A23187/litre of cells in the presence (*but not in the absence*) of external Ca^{2+} (Fig. 1). The plasma membrane permeability of pig red cells was dramatically raised ^{42}K and ^{86}Rb but not to ^{22}Na or ^{135}Cs. Pigeon red cells, on the other hand, showed an increased influx rate to all the isotopes investigated (Fig. 2). To maximize isotope influx rates, A23187 was routinely used at 150 μmoles/litre of cells after dose–response experiments had determined that similar permeabilities were evoked with lower A23187 concentrations.

The known inhibitors of Ca^{2+}-sensitive K^+ permeability, quinidine [10] and DiS-C_2-5 [11] reduced the A23187–Ca^{2+} complex-evoked ^{86}Rb influx in red pig cells with K_i values of approximately 130 and 1.3 μM respectively. Neither inhibitor had any effect on the increased ^{86}Rb influx rate observed in pigeon red cells (Fig. 3). To determine if external Ca^{2+} was necessary for A23187 to partition into

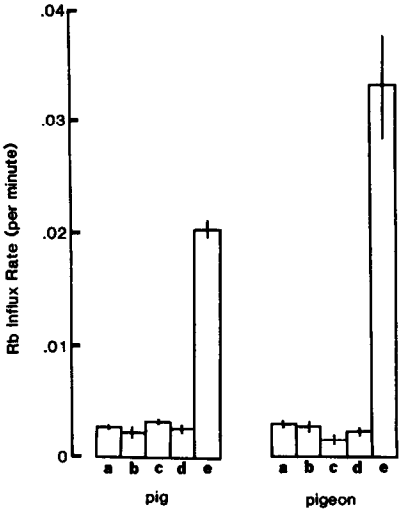

Fig. 1. Effects of ATP depletion and 150 μmoles A23187/litre of cells in the presence of 0.2 mM external Ca^{2+} on ^{86}Rb influx rates. The influx rate constants were calculated from the function: $y = -\ln[1-(\text{sample count/total count})]$ plotted against time. A least squares linear regression line was fitted by computer. The error bars are the S.D. of the regression coefficient. The conditions are: (a) control, non-depleted cells, (b) cells depleted of ATP in 0.5 mM EGTA saline, (c) cells depleted at ATP in 5 mM Ca^{2+} saline, (d) cells depleted of ATP in 1 mM Ca^{2+} + A23187 in 0.5 mM EGTA saline, and (e) cells depleted of ATP in 1 mM Ca^{2+} + A23187 in 0.2 mM Ca^{2+} saline.

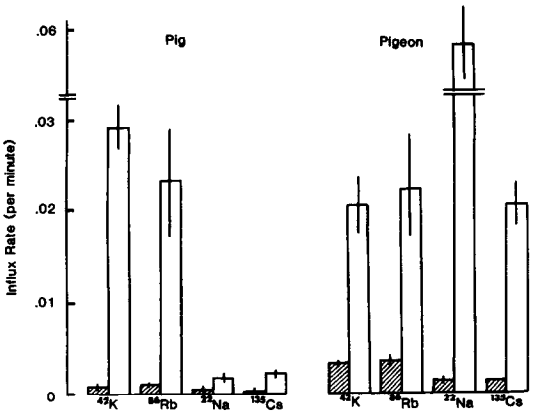

Fig. 2. Influx rates of various cations in the presence (unshaded bars) and absence (shaded bars) of 150 μmoles/litre of cells. A23187 experiments were carried out in modified saline composed of 0.2 mM Ca^{2+}, 40 mM KCl, 40 mM RbCl, 40 mM NaCl, 40 mM $CsCl_2$, 5 mM Hepes, pH 7.4, with NaOH. The errors are the S.D. of the regression line fit. The reason for the large comparative increase in ^{22}Na influx is unclear and was not investigated further in this study.

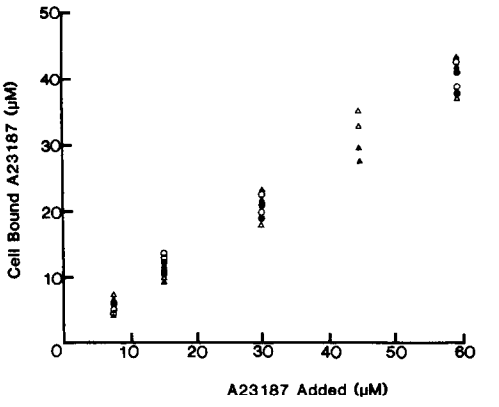

Fig. 4. Iso-butanol extractable A23187 from pig and pigeon red cells in the presence and absence of external Ca^{2+}. The points are the means of duplicate determinations. The cell-associated A23187 concentration was calculated with a previously constructed calibration curve. The conditions are: (▲) pigeon red cells, 0.5 mM EGTA saline, (△) pigeon red cells, 0.2 mM Ca^{2+} saline, (●) pig red cells, 0.5 mM EGTA saline, and (○) pig red cells, 0.2 mM Ca^{2+} saline.

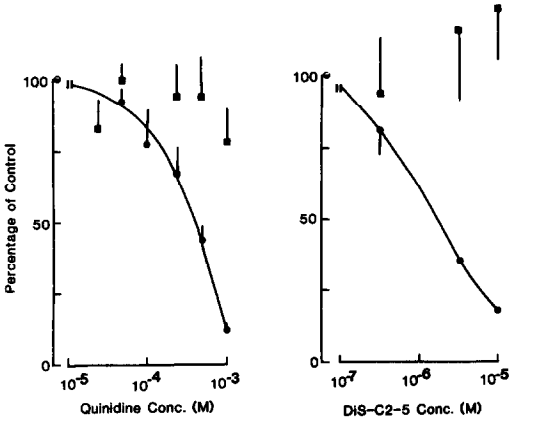

Fig. 3. Effects of the Ca^{2+}-sensitive K^+ permeability inhibitors quinidine and DiS-C_2-5 on the ^{86}Rb influx rate constant in pig (●) and pigeon (■) red cells. The results are expressed as percentage of control (no inhibitor present). The errors are the S.D. of the regression coefficient expressed as percentage of the control influx rate. The K_i of the pig red cell inhibition curves was determined with Dixon plots and is stated in the text.

the red cell plasma membrane, cell-associated A23187 concentrations were assessed by iso-butanol extraction in the presence and absence of extracellular Ca^{2+} (Fig. 4). The extractable A23187 concentration was unchanged, within the resolution of the technique, in Ca^{2+} or Ca^{2+}-free saline.

Discussion

The finding that ATP depletion, resulting in vastly increased cell-associated Ca^{2+}, was ineffective at evoking an increase in ^{86}Rb permeability suggests that the A23187–Ca^{2+} complex-induced effects in these cell types are not due solely to the ionophore raising $[Ca^{2+}]_i$ and activating a Ca^{2+}-sensitive K^+ permeability. Previous workers have shown that A23187 does not act as a monovalent cation ionophore in either bulk solvent systems or red blood cells [12–14]. These data, in conjunction with the findings presented in this report showing that extracellular Ca^{2+} is essential for the induction of any monovalent cation fluxes by A23187, indicate that ^{86}Rb and ^{22}Na are not transported through the plasma membrane by the ionophore.

An attractive explanation for the findings reported is that A23187 may "unmask" a Ca^{2+} binding site (or sites) associated with Ca^{2+}-sensitive ion channel which under ATP depletion conditions alone cannot be occupied. This model would be consistent with the findings of Klausner *et al.* [15] who postulate that A23187 binds to both lipids and proteins and may well have a particular affinity for, and cause the reorganization of, lipid protein interface. This interface may be the site of the normally "masked" Ca^{2+} binding site which may then be exposed for binding and lead to activation of the Ca^{2+}-sensitive channel. An alternative, but similar, explanation may be that A23187 accesses a particular intracellular plasma membrane associated compartment which is in contact with the Ca^{2+}-sensitive channel binding site and which cannot be accessed by ATP depletion procedures. It was not possible, however, with the techniques used in this study to differentiate between these two possibilities. The Ca^{2+}-sensitive channels once activated appear to be similar to previously described Ca^{2+}-sensitive K^+ channels in pig red cells. In pigeon red cells, however, the "unmasked" Ca^{2+}-sensitive channels are less specific, allowing a variety of cations to pass through the plasma membrane.

In summary, ATP depletion in pig and pigeon red cells resulted in substantial increases in cell-associated Ca^{2+} but failed to elicit an increase in the ^{86}Rb influx rate. A23187 in the presence of external Ca^{2+}, however, evoked large increases in the rate of ^{86}Rb influx. In pig red cells, the A23187–Ca^{2+} complex elicited permeability increase was selective for ^{86}Rb and ^{42}K but in pigeon red cells it caused a general increase in plasma membrane cation permeability. The increase in ^{86}Rb influx rate in pig red cells was blocked by inhibitors of the Ca^{2+}-sensitive K^+ permeability but in pigeon red cells the inhibitors were ineffective. A possible explanation for these results may be that in pig red cells A23187 "unmasks" an otherwise hidden Ca^{2+} binding site allowing activation of the Ca^{2+}-sensitive K^+ channel. In pigeon red cells A23187 could play a similar role but may expose a Ca^{2+} binding site associated with a less specific calcium-sensitive ion channel which is not blocked by Ca^{2+}-sensitive K^+ permeability inhibitors.

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The stereoselective uptake of ibuprofen enantiomers into adipose tissue

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Ibuprofen belongs to the chemical class of 2-arylpropionic acids which is a large and important group of non-steroidal anti-inflammatory drugs. These drugs are asymmetric and with the exception of naproxen, are administered as an equal mixture of the two enantiomers, i.e. the racemates. In *in vitro* tests, anti-prostaglandin synthesis activity of these drugs has been found to reside in the *S*-enantiomers. This contrasts with the observation that frequently, although not universally, the anti-inflammatory activities of the enantiomers were similar *in vivo* [1]. Consequently, an *in vivo* pathway of stereoselective inversion was invoked [2] and subsequently this hypothesis was confirmed for several of these drugs, including ibuprofen [3–6], benoxaprofen [7], cicloprofen [8, 9] and clidanac [10]. The mechanism of this inversion appears to be as follows: the *R*-enantiomer is stereospecifically converted to its coenzyme A thioester, while the *S*-enantiomer is not a substrate for this ligase. This thioester is racemized by a non-stereoselective racemase, and is then hydrolysed to release the *S*-enantiomer [11].

Coenzyme A thioesters of xenobiotic carboxylic acids, including 2-arylpropionic acids, can replace the natural fatty acids in triacylglycerols to form "hybrid" triglycerides [12, 13]. It is reasonable to assume that the xenobiotic must first be activated by formation of its coenzyme A thioester. We hypothesised that if the proposed mechanism of inversion was correct then uptake of 2-arylpropionic acids into adipose tissue should occur stereoselectively [14]. Additionally, as inversion is stereospecific for *R*-ibuprofen, administered *S*-ibuprofen should not form coenzyme A thioesters and consequently should be unavailable for incorporation into triglycerides [15]. The present study was undertaken to investigate this hypothesis.

Materials and methods

R, *S* and *RS*-ibuprofen were supplied by Boots U.K. *S*-2-octanol (>99% optical purity) was purchased from Sigma.

Groups of 6 male Wistar rats were treated with either *RS*-ibuprofen, *R*-ibuprofen or *S*-ibuprofen (20 mg/kg, i.p.) twice daily for 7 days. Four rats from each group were sacrificed 20 hr after the last dose and perinephric fat collected. The remaining two rats were sacrificed at 116 hr and fat similarly collected. Aliquots (50 mg) of fat were fractionated by thin layer chromatography using silica gel plates (Kieselgel $60F_{254}$, Merck) and petroleum ether/diethyl ether/acetic acid (20:80:1) solvent system. The band corresponding to the triglycerides was scraped off the plate and the silica eluted with chloroform/methanol (2:1, 10 ml). This extract was taken to dryness under a stream of nitrogen and with gentle heating (40°). The triglycerides were hydrolysed with alcoholic KOH (1.0 ml, 50% MeOH/50% 10 N KOH, heated at 75° for 90 min) and, following acidification, extraction into hexane (2 × 10 ml) and evaporation to dryness, the residue was esterified with *S*-2-octanol as described previously [5]. Samples were assayed by methane chemical ionization mass spectrometry using single ion monitoring to detect each derivatised enantiomer of ibuprofen following gas chromatographic separation [17]. Results were calculated against similarly treated ibuprofen standards (2–100 μg/g). Results for standards demonstrated that under these conditions there was no racemization during sample preparation.

The optical purity of the administered enantiomers was checked by the HPLC procedure described previously [5].

Results

The *R*-enantiomer was found to contain a maximum of 4.3% *S*-enantiomer and similarly, the *S*-enantiomer contained 4.3% *R*-enantiomer.

Following chronic treatment of rats with *R*-ibuprofen, both *R* and *S*-ibuprofen were incorporated into adipose tissue with uptake being stereoselective for the *R*-enantiomer. In contrast, there was very little drug detected in fat after similar treatment with the active *S*-enantiomer (Fig. 1). These concentrations following administration of the *S*-