cycle by 20-fold (Challiss *et al.*, 1984a,b), a finding consistent with the proposed role of cycles in improving sensitivity of the flux through the phosphofructokinase reaction (Newsholme & Crabtree, 1976). Using ^{13}C -n.m.r. it has been possible to demonstrate the existence of a glycogen/glucose-1-phosphate cycle in liver of the rat *in vivo* (Barrett & Shulman, 1991) which is consistent with the finding of significant rates of this cycle in the isolated incubated soleus muscle of the rat (Challiss *et al.*, 1987).

Sixth, other substrate cycles have also been reported in man. Hers (1976) demonstrated the existence of the glucose/glucose 6-phosphate cycle in the liver of man by following the decrease in the $^3\text{H}/^{14}\text{C}$ ratio in 2- ^3H , ^{14}C glucose in the blood of normal subjects, a change which did not occur in patients with hepatic glucose 6-phosphatase deficiency. The rate of this cycle plus that of fructose 6-phosphate/fructose biphosphate has been shown to decrease markedly in the hypothyroid state *in vivo* in man (Shulman *et al.*, 1985).

We fully appreciate the need for caution in the extrapolation of biochemical findings to the physiological situation (see Newsholme & Crabtree, 1981); indeed we believe we emphasized caution in our letter concerning the proposed glutamine cycles in skeletal muscle. In 1972 we proposed the *hypothesis* that bumble bee flight muscle fructose biphosphate was involved in a substrate cycle "for the generation of heat during short periods of rest" (Newsholme *et al.*, 1972). This hypothesis was tested by Surholt *et al.* (1991) nearly 20 years later and strong evidence in support is reported. We hope someone will take up the challenge for the glutamine cycles in muscle since, if glutamine is important for the immune system, such cycles may not be physiologically or indeed clinically unimportant. We hope it takes less than 20 years.

Except perhaps for the work on thermogenesis of Surholt *et al.* (1991), Bahr *et al.* (1990) and Wolfe *et al.* (1987a) these findings do not prove a particular role for substrate cycles. The evidence that they play a role in control is still circumstantial, e.g. the rates of cycles are increased under conditions when increased sensitivity is known to be required, and mathematical models support this role. The same pattern of circumstantial evidence is used to support the view that interconversion cycles (covalent modification) play a role in improving sensitivity in control (Koshland *et al.*, 1982) but no *direct* quantitative physiological tests for this improvement have ever been carried out.

We accept that detailed knowledge of all the reactants of the processes of glutamine transport are required for calculation of the equilibrium nature. Our calculations and other evidence support the view that both processes are non-equilibrium [see p. 104 of Newsholme & Crabtree (1976) for division between equilibrium and non-equilibrium reactions, or see Rolleston (1972)] and, therefore, cannot be catalysed by the *same* transporter. Whether both processes provide the basis for a translocation cycle is still, however, hypothetical, as I believe we were at pains to point out in our original letter.

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Agonist-evoked Ca^{2+} entry in human platelets

In a recent report, Garcia-Sancho and colleagues asserted that agonist-induced Ca^{2+} influx into human platelets is secondary to the emptying of intracellular Ca^{2+} stores [1]. This hypothesis is based on the findings that thrombin-evoked Mn^{2+} entry lags behind the release of Ca^{2+} from intracellular stores, and that inhibitors of cytochrome *P*-450, effective in reducing store-related Mn^{2+} entry in other cell types [2,3], also inhibit agonist-evoked Mn^{2+} entry in platelets. Our own work supports the existence of a store-regulated Ca^{2+} entry pathway in platelets. However, our data do not (as incorrectly stated by Garcia-Sancho and coworkers) support the notion that such an entry pathway can account for the full response to agonists other than ADP. Additionally, our work indicates that caution is needed when interpreting data obtained using Mn^{2+} as a tracer for Ca^{2+} entry, and in the use of cytochrome *P*-450 inhibitors.

We have shown using stopped-flow fluorimetry that ADP evokes a biphasic elevation in $[\text{Ca}^{2+}]_i$ [4]. Both phases of $[\text{Ca}^{2+}]_i$ rise are associated with Mn^{2+} and therefore, we assume, Ca^{2+} entry. The first phase of entry commences without measurable delay and appears to be conducted by an ADP-receptor-operated non-selective cation channel which we have identified electrophysiologically [5,6]. ADP-evoked currents are conducted not

only by Na^+ , but also by Ba^{2+} , indicating (contrary to the comment by Garcia-Sancho's group) that the channel is permeable to divalent cations, almost certainly including Ca^{2+} . The delayed phase of ADP-evoked entry coincides with, and appears to be caused by, the release of Ca^{2+} from intracellular stores [4]. We have also suggested that such an entry pathway could contribute to the responses evoked by thrombin and other agonists [7]. In addition, we have recently shown that inhibitors of the endomembrane Ca^{2+} -ATPase, 2,5-di(t-butyl)-1,4-benzohydroquinone and thapsigargin, which deplete the intracellular Ca^{2+} store, promote Ca^{2+} and Mn^{2+} entry in platelets [8]. These results support the existence of a store-regulated Ca^{2+} entry pathway in these cells, but other evidence indicates that such a mechanism cannot alone account for agonist-evoked Ca^{2+} influx.

Stopped-flow fluorimetry with fura-2-loaded platelets (shown itself to be without effect on the cells [9]) clearly indicates that not only ADP, but also thrombin, platelet activating factor, vasopressin and the thromboxane A_2 analogue, U46619, all evoke rises in $[\text{Ca}^{2+}]_i$ which occur with shorter delays in onset when Ca^{2+} is present externally than when it is absent [10]. A similar, slower timecourse of agonist-evoked release of Ca^{2+} from intracellular stores is also revealed when external Ni^{2+} is used to block Ca^{2+} entry. These data indicate, contrary to the statement by Garcia-Sancho and colleagues, that the onset of Ca^{2+} influx precedes release from intracellular stores. Our stopped-flow results *do*, however, indicate that the Mn^{2+} influx evoked by thrombin [7] and vasopressin lags behind internal release. We have suggested that this delayed Mn^{2+} entry might proceed via a store-regulated pathway, an idea supported by the recent work of Garcia-Sancho's group [1]. However, the finding that the rise in $[\text{Ca}^{2+}]_i$ evoked by agonists other than ADP in the presence of external Ca^{2+} precedes internal release, whilst detectable Mn^{2+} entry lags behind, remains to be explained.

It could be that the early phase of entry evoked by thrombin and similar agonists proceeds via a pathway selective for Ca^{2+} over Mn^{2+} , and distinct from that conducting the later stages of agonist-evoked entry [7]. In rat parotid salivary acinar cells, agonists have been shown to evoke Ca^{2+} , but little [11] or no [12] Mn^{2+} entry. Thus it appears that one or more of the Ca^{2+} influx pathways in this cell type are poorly permeable to Mn^{2+} . The same might be true of some, but not all, Ca^{2+} entry routes in other cells, including platelets. This suggests that it may be misleading to assume that Mn^{2+} is a true surrogate for Ca^{2+} in all cases when tracing divalent cation entry. It follows that even complete inhibition of agonist-evoked Mn^{2+} entry, such as that reported with inhibitors of cytochrome *P*-450 by Garcia-Sancho's group [1], may not indicate complete inhibition of Ca^{2+} entry, since some might proceed via a pathway impermeable to Mn^{2+} . As the effects of these inhibitors on agonist-evoked rises in $[\text{Ca}^{2+}]_i$ in the presence of external Ca^{2+} are not reported in their paper, it is difficult to judge how important any store-regulated or cytochrome-dependent entry pathway is in platelets.

In our hands, the cytochrome *P*-450 inhibitors, econazole and miconazole, at a concentration of $3\text{ }\mu\text{M}$, were only found to inhibit the Mn^{2+} entry evoked by thrombin (1 unit/ml) by 10% [8]. The Mn^{2+} entry evoked by ADP and PAF were similarly less markedly inhibited than found by Garcia-Sancho and colleagues [1]. We were unable to use the imidazole antimycotics at higher concentrations since the inhibitors themselves elevated $[\text{Ca}^{2+}]_i$, indicating non-specific effects and cautioning their use. Indeed, in the recent report from Garcia-Sancho's group [1], it appears that basal $[\text{Ca}^{2+}]_i$ may be elevated and rising in econazole-treated cells (see their Fig. 8). We cannot explain the difference in potency of econazole found between our experiments and those of Garcia-Sancho's group. However, we note that our cells are

Fig. 1. Effect of econazole on ADP-evoked rise in $[\text{Ca}^{2+}]_i$ and Mn^{2+} influx

Fura-2-loaded platelets were prepared as previously described [10] and resuspended in medium of composition 145 mM-NaCl, 5 mM-KCl, 1 mM- MgCl_2 , 10 mM-NaHepes, pH 7.4 at 37°C . fura-2 fluorescence was recorded in a Cairn Spectrophotometer at excitation wavelengths of 360 nm (a) or 340 and 380 nm (b). Emission was collected at 500 nm. The 340/380 nm fluorescence ratio was calibrated in terms of $[\text{Ca}^{2+}]_i$ as previously described [7]. External Ca^{2+} was 1 mM in all experiments. In (a), 1 mM- Mn^{2+} was added 25 s before the agonist. ADP ($40\text{ }\mu\text{M}$) was added as indicated by the arrows. Traces on the right of each panel were obtained from platelets pretreated for 5 min before agonist addition with $3\text{ }\mu\text{M}$ -econazole. Traces on the left show control results from cells after incubation with the vehicle (dimethyl sulphoxide) alone. Each interval on the abscissa represents 1 min.

loaded with fura-2 in citrate plasma whilst they load in a washed suspension.

Importantly, we found that at concentrations which reduced ADP-evoked Mn^{2+} entry by 25% (determined from extent of quench 20 s after agonist addition; Fig. 1a), econazole was without detectable effect on ADP-evoked rises in $[\text{Ca}^{2+}]_i$ (Fig. 1b). This result leads us to question seriously the significance of inhibited Mn^{2+} entry when attempting to assess the effects of econazole on Ca^{2+} signal generation. Further, we found that econazole ($3\text{ }\mu\text{M}$) did not alter the early timecourses of ADP-evoked rises in $[\text{Ca}^{2+}]_i$ or ADP-evoked Mn^{2+} entry as resolved by stopped-flow fluorimetry. Similar results were obtained with thrombin and PAF (not shown). Our data thus suggest that any role for a cytochrome *P*-450-dependent divalent cation entry pathway is likely to be in longer term Ca^{2+} homeostasis, such as store refilling, rather than in Ca^{2+} signal generation.

In summary, our work supports the existence of a store-regulated Ca^{2+} entry pathway in human platelets, and suggests a possible role for cytochrome *P*-450 in mediating this in part. However, our data are not compatible with the suggestion that agonists other than ADP evoke Ca^{2+} entry only via such a store-

dependent mechanism. Further work is needed to elucidate at least one additional pathway for Ca^{2+} entry, which may be Mn^{2+} impermeant, and which stopped-flow fluorimetry indicates is activated prior to the release of Ca^{2+} from intracellular stores by agonists such as thrombin.

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Agonist-evoked Ca^{2+} entry in human platelets: a reply

Sage *et al.* [1] claim that agonists such as thrombin or PAF evoke Ca^{2+} entry in platelets by mechanisms not dependent on the emptying of the intracellular Ca^{2+} stores and report that the effects of the cytochrome *P*-450 inhibitors econazole and miconazole on agonist-evoked entry of Ca^{2+} or Mn^{2+} are only marginal. This controverts our recent proposal that agonist-induced Ca^{2+} entry in platelets is secondary to the emptying of intracellular Ca^{2+} stores and that a cytochrome *P*-450 may be involved in coupling the stores to the plasma membrane channels [2].

On the basis of data obtained with stop-flow fluorimetry Sage *et al.* [3] had proposed that agonist-induced Ca^{2+} entry may take place by “three different mechanisms...: (i) a close coupling of ADP receptors to Ca^{2+} entry..., for which Mn^{2+} is an effective substitute; (ii) an early phase of thrombin-stimulated entry, possibly activated by diffusible second messengers, that passes Mn^{2+} only poorly; (iii) a later phase of thrombin-evoked entry that is promoted by emptying of the dischargeable intracellular Ca^{2+} pool”. Mn^{2+} is also an effective substitute for mechanism (iii), which was proposed to apply also for a delayed (200 ms lag) response to ADP [4].

Non-selective cation channels, identified later in platelet membrane patches [5], were proposed to mediate mechanism (i). We

do not question that this channel *can* be permeated by Ca^{2+} , as stated by Sage *et al.* [1], but feel that “it is not clear whether it could explain the Ca^{2+} influx observed in intact cells” [2]. For example, Penner *et al.* [6] described a similar cation channel activated by agonists in mast cells, but they felt it could not explain the agonist-induced Ca^{2+} influx observed in intact cells. This view proved to be correct, as Hoth & Penner [7] found later a different channel (which, incidentally, was activated by emptying the Ca^{2+} stores) that better fitted this role.

The experimental support for the involvement of mechanism (ii) in thrombin-induced Ca^{2+} entry was the observation that the lag for the $[\text{Ca}^{2+}]_i$ increase induced by thrombin was 26 % longer in Ca^{2+} -free medium than in Ca^{2+} -containing medium [3,8]. This compares to a 1000–2000 % increase of the lag time observed for ADP on Ca^{2+} removal [3,8]. The delay observed for thrombin on

Fig. 1. Effects of cytochrome *P*-450 inhibitors on agonist-induced Ca^{2+} entry in human platelets

(a) Effects of econazole on the increase of $[\text{Ca}^{2+}]_i$ of fura-2-loaded human platelets stimulated with thrombin in the presence and in the absence of external Ca^{2+} (1 mM- CaCl_2 or 1 mM- CaCl_2 + 2 mM-EGTA). Econazole (10 μM; Eco) was added 2 min before thrombin (1 unit/ml; Thr). $[\text{Ca}^{2+}]_i$ was estimated from the ratio of the fluorescence excited at 340 and at 380 nm. The difference in peak height of the control responses (with no added drug) measured in the presence or absence of external Ca^{2+} is attributed to Ca^{2+} influx. (b) Typical raw fluorescence traces (with scale converted to $[\text{Ca}^{2+}]_i$) of quin2-loaded platelets stimulated with ADP (20 μM) in the presence or in the absence of external Ca^{2+} (1 mM- CaCl_2 or 1 mM-EGTA). SK&F 96365 (at the concentrations shown) was added 2 min before ADP. Reproduced from Merritt *et al.* [3]. (c) Comparison of the structures of SK&F 96365 and econazole.