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Characterization of altered calcium signalling in T lymphocytes from patients with rheumatoid arthritis (RA)

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SUMMARY

Abnormal function of peripheral blood T lymphocytes is characteristic of RA; diminished proliferation and secretion of cytokines following *in vitro* mitogen stimulation are observed. We have investigated the calcium flux initiating T cell activation in rheumatoid peripheral blood mononuclear cells (PBMC) to determine whether abnormalities in signalling are also present. We have found that both phytohaemagglutinin (PHA-P)- and anti-CD3-stimulated calcium fluxes were much reduced in the patients' PBMC compared with controls, with a mean six-fold difference ($P < 0{\cdot}01$) in rate of Ca^{2+} flux with PHA-P stimulation. When purified T cells were examined with PHA and CD3 stimulation, a reduction in the peak and plateau $[Ca^{2+}]_i$ was observed in RA T cells, but the rate of rise of $[Ca^{2+}]_i$ was only reduced in those cells stimulated with PHA. These results suggest that alterations in the initiating signal may underlie the functional T cell abnormalities associated with RA, and that there may be an additional extrinsic influence from non-T cells in the PBMC population.

Keywords rheumatoid arthritis T lymphocyte calcium signalling phytohaemagglutinin indo-1

INTRODUCTION

In RA a number of immune abnormalities have been observed which particularly affect T lymphocyte function. Both synovial and peripheral blood T cells appear to be functionally deficient, as manifested by cutaneous anergy and depressed *in vitro* responses to recall antigens [1], poor responses to mitogens [2–4], poor suppressor [5] and helper function [6], and depressed autologous mixed lymphocyte responses [7,8].

Many of these effects could be explained by the abnormal lymphokine production, i.e. depressed IL-2 production [9–11] and interferon-gamma (IFN-γ) secretion [12], observed with RA T cells. Such a suggestion is reinforced by the observation that the deficient *in vitro* transformation seen with rheumatoid T cells can be, at least partially, corrected by the addition of exogenous IL-2 [1,10]. Our previous studies indicate that the deficient response of RA T cells is not due to an inadequacy in accessory signals [13], or to a lack of IL-2-producing cells [14], suggesting that there is an intrinsic change in the T lymphocyte. Since both IL-2 and IFN-γ production are decreased, and similar gene activation pathways are used to control their synthesis, then a common defect in the early activation process could help to explain both the deficient lymphokine production and consequent poor proliferative response seen in RA.

Activation of human T cells, and the subsequent secretion of cytokines, has been intensively studied, and two major complementary pathways, involving the CD3 and CD2 complexes, have been described [15]. Activation of either complex, using lectins or MoAbs, causes a rise in cytoplasmic Ca^{2+} which is derived from both extracellular sources and from stores in the endoplasmic reticulum [15,16]. The initial stimulation leads to the generation of inositol-1,4,5-trisphosphate (IP_3) which binds to intracellular membranes [17], causing mobilization of intracellular stores of Ca^{2+}. The exact mechanism by which Ca^{2+} entry across the plasma membrane is initiated is not yet clear [18]. Current opinion favours the capacitance entry hypothesis [19,20] proposed by Putney. Depletion of intracellular stores and concurrent release of an unidentified calcium influx factor (CIF) [21,22] from the endoplasmic reticulum activates transmembrane Ca^{2+} flux through specific calcium channels. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) elevation contributes to the now classical gene activation pathways involving protein kinase C [23,24], translocation of cytoplasmic factors into the nucleus and subsequent gene transcription, cytokine synthesis and cell proliferation.

In order to determine whether the diminished cytokine secretion and proliferation observed with RA T cells was associated with an alteration in the initial activation signal, we investigated the changes in cytoplasmic $[Ca^{2+}]_i$ occurring in RA peripheral blood mononuclear cells (PBMC) following stimulation *in vitro*. The results indicate that the Ca^{2+} flux induced via the CD2 pathway was diminished in RA PBMC, and it was confirmed that both the CD2 and CD3 pathways are affected using purified

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T lymphocytes. An alteration in an early signalling event may be an important factor underlying immune abnormalities seen in this disease. Possible mechanisms underlying this phenomenon are discussed.

MATERIALS AND METHODS

All chemicals were of analytical reagent grade and were purchased from FSA (Loughborough, UK), unless stated otherwise.

Study population

Fifteen patients with classical or definite RA [25] who were receiving a single non-steroidal anti-inflammatory drug only were studied. Patients on steroids or any other disease-modifying anti-rheumatic drug were excluded. Disease activity was assessed by clinical indices (number of swollen joints, tender joint score and early morning stiffness), as well as laboratory markers of inflammation—the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). Patients and healthy controls were matched for age and sex with no statistically significant difference between the two groups.

Cell isolation

PBMC preparations were isolated on Ficoll–Paque (Pharmacia Ltd, Milton Keynes, UK) density gradients from 20 ml of peripheral blood from each patient and a matched control. In some experiments adherent cells (mostly monocytes and some *in vivo* pre-activated lymphocytes) were depleted from patient and control samples by two 1-h incubations at 37°C in plastic dishes. Later experiments were performed using unfractionated cells in order to retain the high cell numbers required for the spectrofluorometric assessment of $[Ca^{2+}]_i$.

In those experiments where purified T cell populations were analysed, PBMC underwent an initial adherence step before further depletion of non-T cells. This was carried out by a negative selection technique using MoAbs and magnetic beads. The cells were incubated for 30 min on ice with MoAbs to CD19, 16 and 14 to deplete B cells, natural killer (NK) cells and monocytes/macrophages, respectively. These labelled cells were removed with anti-mouse IgG magnetic beads. This negative selection technique avoids unwanted activation of T cells and routinely results in a 90–95% purity of cells when analysed by flow cytometry.

Cell loading with intracellular dye

PBMC were suspended in HEPES-buffered balanced salt solution [26] at 10^7 cells/ml and loaded with fura2 AM (Molecular Probes (Calbiochem Novabiochem (UK) Ltd, Nottingham, UK)) using the method described by Malgaroli *et al.* [26]. Cells were incubated at 15°C for 30 min with the fura2 AM, to give a final intracellular concentration of 1–10 μM fura2. After washing, PBMC were resuspended in the same HEPES-buffered medium, at $2{\cdot}5 \times 10^6$ cells/ml, and kept on ice until use, in order to minimize leakage of the dye. Cells were routinely examined within 2 h of dye loading.

For studies on purified T cells, the fluorescent dye indo-1 was used instead. This dye has the advantage of less intracellular compartmentalization, being more temperature stable and having less dye leakage. T cells at $2{\cdot}5 \times 10^6$/ml were incubated for 40 min at 37°C with 3 μg/ml indo-1. The cells were warmed to 37°C in a water bath before analysis in the fluorimeter.

Spectrofluorimetry

Fluorescence measurements were performed at 37°C using a temperature-controlled, stirred cuvette in a Perkin Elmer LS-50B spectrofluorimeter. In contrast to fura2 (which has dual excitation properties), indo-1 is a dye with a dual emmission spectrum. Using computer software provided with the fluorimeter, for fura2 the excitation wavelength was driven between 340 nm (absorption maximum of the Ca–fura2 complex) and 361 nm (the isobestic wavelength) [26]. For indo-1, excitation was at 355 nm with the emission of each spectrum recorded at 405 nm (Ca^{2+} bound dye) and 495 nm (Ca^{2+} free dye). Calibration values were obtained from ionomycin (5·6 μM)-treated cells [26], thus allowing assessment of R_{max} (the ratio of fluorescence intensities of the dye in the Ca^{2+} bound and Ca^{2+} free states at saturating amounts of Ca^{2+}). R_{min} (the ratio of fluorescence intensities of the dye in the Ca^{2+} bound and Ca^{2+} free states at limiting concentrations of Ca^{2+}) was obtained by use of the Ca^{2+} chelator EGTA (pH 8·7, final concentration 12 mM). The calibration procedure indicated that there was no difference in fura2 or indo-1 loading of patient and control cells (data not shown). Absolute $[Ca^{2+}]_i$ levels were calculated using these calibration data and the equation $[Ca^{2+}]_i = Kd.(R - R_{min})/(R_{max} - R).Sf_2/Sb_2$ [27], where Kd is the effective dissociation constant (250 nM for indo-1 and 240 nM for fura2), and Sf2/Sb2 is the ratio of the 495 nm fluorescence intensity of indo-1-loaded cells at minimum and maximum saturating Ca^{2+}, respectively.

A baseline intracellular calcium value was recorded for 60 s, after which 5 μg/ml of PHA-P (Sigma, Poole, UK) or 2·5 μg/ml of the anti-CD3 MoAb OKT3 (Ortho Diagnostics, High Wycombe, UK) were added to PBMC to induce a calcium response, as previously described [28,29]. Preliminary experiments indicated that these were the optimal concentrations for the batches of these agents used (data not shown). Most of these experiments were carried out on PBMC, but purified T lymphocytes were used in a smaller number of experiments. Dose–response curves to assess the suboptimal concentrations of PHA (20 μg/ml) and OKT3 (0·21 μg/ml) for the batches of these reagents used to stimulate the purified T cells were carried out. It was necessary to cross-link the OKT3 with a goat anti-mouse antibody to induce a calcium signal in the purified T cells: this was added 100 s after the OKT3. Four parameters were derived to describe the Ca^{2+} flux: the initial $[Ca^{2+}]_i$ before stimulation; the rate of increase over the first 30 s after the onset of $[Ca^{2+}]_i$ elevation; the maximum peak $[Ca^{2+}]_i$ level achieved over the period of measurement; and the rise in $[Ca^{2+}]_i$ from initial to peak level.

Statistical analysis

Statistical comparison of sets of results was done using a Mann–Whitney rank test for non-parametric data, and correlations between parameters were performed using regression analysis.

RESULTS

The adherence step during cell preparation made no difference to the extent of the Ca^{2+} flux in either the patient or control groups. Furthermore, our previous studies using the same individuals investigated here [13] have shown that the absolute number and proportion of $CD3^+$ cells within the PBMC preparations were not different between this group of patients with RA (65%) and controls (63%). For this reason, unfractionated cells were used in order to maintain a high cell number.

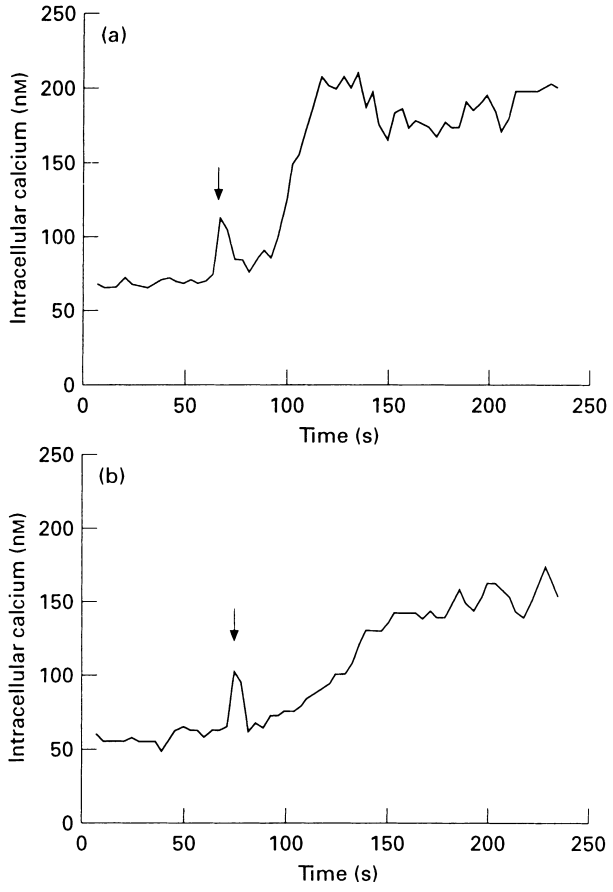

Fig. 1. Phytohaemagglutinin (PHA-P)-stimulated calcium flux in peripheral blood mononuclear cells (PBMC). PBMC were isolated from peripheral blood and, after loading with fura2, the baseline $[Ca^{2+}]_i$ was monitored in the fluorimeter for 1 min using a cell suspension containing $2{\cdot}5 \times 10^6$ cells/ml. Cells were then stimulated with PHA-P (5 μg/ml) (arrow). (a) Cytoplasmic $[Ca^{2+}]_i$ in control cells. (b) Cells from patient with RA.

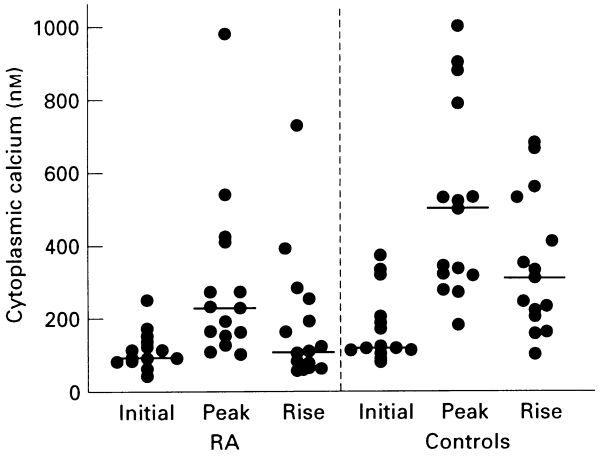

Fig. 2. Comparison of initial, peak and rise in cytoplasmic calcium in peripheral blood mononuclear cells (PBMC). Initial, peak and rise in $[Ca^{2+}]_i$ for each of the rheumatoid cells ($n = 15$) and control cells ($n = 14$) were calculated. Analysis using the Mann–Whitney test indicated that rheumatoid cells showed a lower (median = 90 nM) initial level as well as a lower peak $[Ca^{2+}]_i$ (median = 225 nM; $P < 0{\cdot}01$) and rise in $[Ca^{2+}]_i$ (median = 105 nM; $P < 0{\cdot}05$). Median values are indicated by the horizontal bars.

The control cells gave the typical response observed by many groups [28–30], of an initial rapid increase in cytoplasmic calcium after a delay of 20–30 s from the addition of the stimulus. The rapid increase occurred over a period of about 30 s and was followed by a decline to an equilibrium calcium concentration, which was then sustained for the period of observation (Fig. 1a). In the case of the cells from patients with RA, however, this picture was not observed. The initial rapid increase was largely absent in the PBMC, and there was a slower rise following addition of the PHA-P (Fig. 1b).

When the results from all the rheumatoid and control PBMC were compared it was found that the median resting $[Ca^{2+}]_i$ for the control group was 115 nM while that for RA patients was 90 nM (not significant) (Fig. 2). However, RA peak $[Ca^{2+}]_i$ (median 225 nM) was significantly lower than that of the control group (median 500 nM) ($P < 0{\cdot}01$). There was also a significant difference ($P < 0{\cdot}05$) between the rise in $[Ca^{2+}]_i$ in samples from RA (median 105 nM) and control (median 310 nM). For the RA PBMC this peak value was only reached several minutes after the addition of the stimulus.

The most striking difference between groups was the gradient of the $[Ca^{2+}]_i$ rise: in the rheumatoid group the median value was 1·44 nM/s, while the control group was almost six-fold higher at 8·6 nM/s ($P < 0{\cdot}01$) (Fig. 3a).

To determine if these altered responses might reflect the activity of the disease, RA patients were divided into two groups according to disease activity as determined from laboratory markers of inflammation, the ESR and CRP. Comparison of the less active group (median CRP = 11; $n = 6$) with the active group (median CRP = 23; $n = 9$) showed that the respective peak $[Ca^{2+}]_i$ levels were 270 nM and 160 nM, a difference which was significant ($P < 0{\cdot}05$) (Fig. 3b). The gradient of the Ca^{2+} flux was also significantly different ($P < 0{\cdot}05$) with the values being 2·78 nM/s and 1·22 nM/s, respectively. The parameters measured for the less active group (peak $[Ca^{2+}]_i$ and gradient) remained statistically different from the control group ($P < 0{\cdot}01$) (Fig. 3a). No correlation between age, disease duration or individual clinical indices of disease activity and the calcium parameters was observed.

Several pathways are thought to be involved in the physiological activation of T lymphocytes, among them the CD2 and CD3 clusters. The experiments described thus far on PBMC made use of PHA-P, which is thought to act via CD2 [29]. We were interested to determine whether the difference we observed also affected signalling via CD3, as this pathway is better characterized. In addition, we also wished to eliminate any effect of other cells on T cell signalling and so in a limited number of experiments we stimulated purified T cells with both PHA-P and the anti-CD3 antibody, OKT3.

PHA-P stimulation of purified T cells from a normal control and a patient with RA (Fig. 4) shows a reduction in peak $[Ca^{2+}]_i$ (260 *versus* 205 nM, respectively) and sustained $[Ca^{2+}]_i$ (230 *versus* 205 nM, respectively) achieved. The rate of rise of $[Ca^{2+}]_i$ measured over the first 30 s of $[Ca^{2+}]_i$ rise was also reduced (1·9 nM/s *versus* 0·83 nM/s for RA cells). Stimulation via CD3 also showed a pattern similar to that seen with PHA, with a reduction in peak and sustained $[Ca^{2+}]_i$ (Fig. 5), but the decreased rate of rise observed with PBMC and also with PHA-stimulated T cells was not seen with the dose of OKT3 used here.

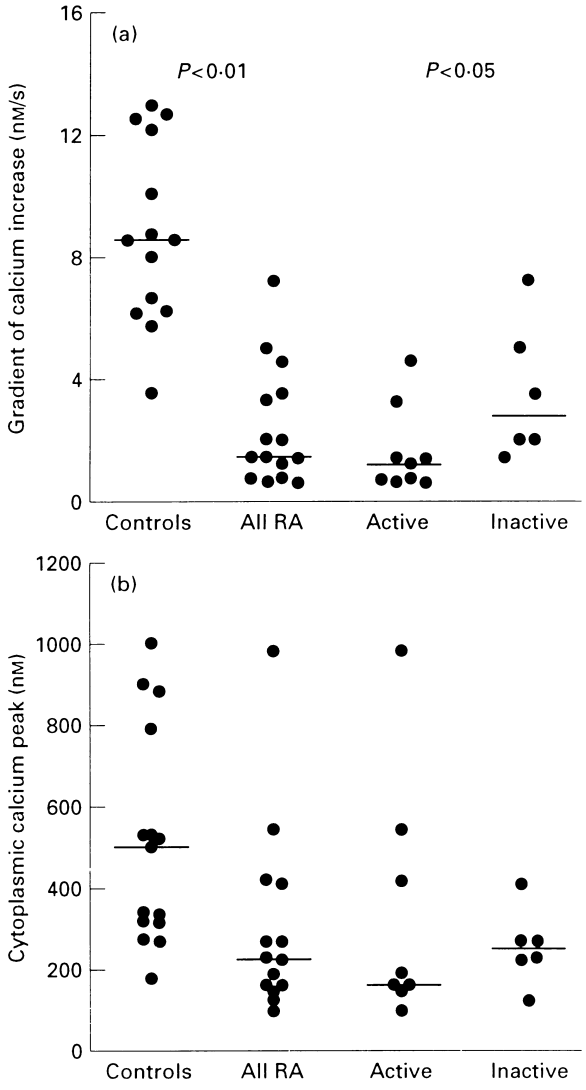

Fig. 3. Comparison of rate of calcium influx into rheumatoid and control peripheral blood mononuclear cells (PBMC) after stimulation with phytohaemagglutinin (PHA-P). (a) The rate at which the PHA-P-stimulated rise in $[Ca^{2+}]_i$ occurred over the initial 30-s period after the onset of $[Ca^{2+}]_i$ elevation was calculated and plotted for rheumatoid cells and controls. Analysis using Mann–Whitney test showed a highly significant difference ($P < 0{\cdot}01$) between controls (median = 8·6 nM/s) and rheumatoid cells (1·44 nM/s). There was also a significant difference between values for active and inactive rheumatoid patients ($P < 0{\cdot}05$), with the median values being 1·22 nM/s and 2·78 nM/s, respectively. (b) Comparison of peak calcium flux in PBMC from active and inactive RA. RA patients were divided depending on their disease activity—active group (median C-reactive protein (CRP) = 23) and inactive (median CRP = 6). The difference between peak $[Ca^{2+}]_i$ in the two groups was significant ($P < 0{\cdot}05$).

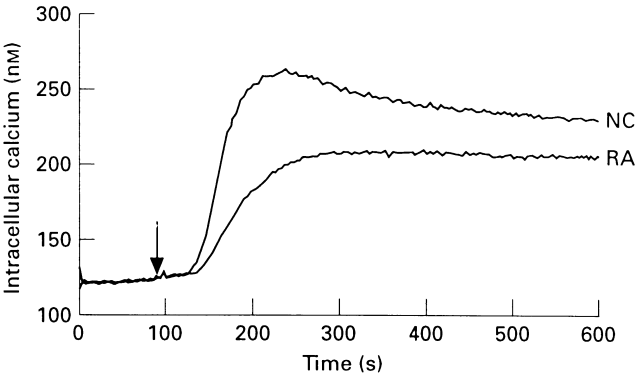

Fig. 4. Phytohaemagglutinin (PHA-P)-induced calcium flux in purified T cells. T cells were purified by negative selection (see Materials and Methods). Ninety to 95% purity of T cell populations was obtained (assessed by flow cytometry). PHA-P was added where indicated (arrow) at 20 μg/ml (suboptimal concentration for batch used). Upper trace from age- and sex-matched normal control (NC), lower trace from patient with RA. Representative of three experiments. Peak $[Ca^{2+}]_i$ 260 nM (control), 205 nM (patient); plateau $[Ca^{2+}]_i$ 230 nM (control), 205 nM (patient).

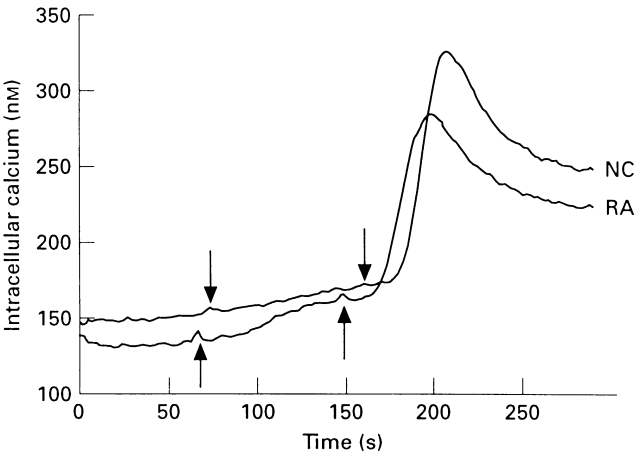

Fig. 5. OKT3-induced calcium flux in purified T cells. As for Fig. 4, except OKT3 (0·21 μg/ml) added at first arrow and cross-linking rabbit anti-mouse added 100 s later (second arrow). Peak 320 nM (control), 280 nM (patient); plateau 250 nM (control), 220 nM (patient). The time to onset of elevation in $[Ca^{2+}]_i$ after cross-linking of OKT3 is the same in patient and control samples. Representative of three experiments.

DISCUSSION

A number of methodologies are available to study intracellular Ca^{2+} signalling in immune cells. Single-cell imaging has been used successfully to study changes in $[Ca^{2+}]_i$ in neutrophils from patients with RA [31]. Flow cytometry has demonstrated changes in $[Ca^{2+}]_i$ in RA lymphocytes [32], but this method is most useful for qualitative data, since precise calibration is not possible [33]. Spectrofluorimetry offers certain advantages over other methods of $[Ca^{2+}]_i$ assessment. The relative ease of calibration with this technique allows quantification of the $[Ca^{2+}]_i$ and also allows analysis of a larger number of samples, as technical difficulties are less. For these reasons spectrofluorimetry was used in this study to quantify the differences seen in $[Ca^{2+}]_i$ RA T cells.

The results presented show a clear difference between RA and normal PBMC in the characteristics of Ca^{2+} flux induced following both PHA-P and anti-CD3 stimulation of the cells. This phenomenon has also been shown to be present in purified T cell populations. In RA, the intracellular response following CD2 activation, using PHA-P, was greatly reduced, and the rapid rise of $[Ca^{2+}]_i$ previously described [16] in normal T cells was largely absent. There was little evidence of the usual rapid flux in the RA PBMC and the maximum level was reached only after several minutes. However, once the peak level was reached, the

$[Ca^{2+}]_i$ stabilized at the new level; in this respect the pattern was similar to that seen with control cells.

Resting $[Ca^{2+}]_i$ found in the RA PBMC (median 90 nM) was lower than in the control cells (median 115 nM). In addition, the final $[Ca^{2+}]_i$ reached was significantly lower in the RA PBMC. However, the greatest difference with RA PBMC was the initial rate of Ca^{2+} flux following PHA-P stimulation. The median value for the patient group was almost six-fold lower than controls; there was also a relationship between laboratory markers of disease activity and the magnitude of the change in $[Ca^{2+}]_i$.

We have shown previously that the proportion of T cells within PBMC preparations from this group of patients does not differ from normals [13]. The results therefore suggest that there is a change in intracellular signalling in RA T lymphocytes, since these are the responding cells within the PBMC preparations. Indeed, flow cytometric analysis has recently confirmed the observation that the T cell population is affected [32]. Not all lymphocytes will respond to mitogenic stimulation, and the study by Allen *et al.* [32] demonstrated that when only the responding population of T cells is considered, the $[Ca^{2+}]_i$ response to PHA and CD3 stimulation is still depressed in RA T cells. The experiments reported here add supportive data to the involvement of the T cell population with a reduced peak and plateau response obtained on stimulation of purified RA T cells. Interestingly, the rate of rise of $[Ca^{2+}]_i$ in the purified T cell population was reduced with PHA stimulation, but was not diminished by CD3 ligation. It therefore seems that the non-T cells within the PBMC population have a powerful influence on the rate of elevation of $[Ca^{2+}]_i$ in T lymphocytes, which is less depressed when purified lymphocytes are used.

Ca^{2+} signalling occurs at an early stage in the activation pathway and so could contribute to the deficient IL-2 production, reported from *in vitro* studies on rheumatoid T cells, and to the other functional abnormalities [11]. The importance of a Ca^{2+} flux in T cell activation is now well accepted, but the relative importance in gene activation of the various parameters of the flux, for instance the rate of influx, the initial $[Ca^{2+}]_i$ and the final cytoplasmic $[Ca^{2+}]_i$, has not been fully addressed. It has been demonstrated that the initial transient 'spike' of Ca^{2+} flux is not required for successful proliferation of T cells, whereas the sustained rise, attained through ingress of Ca^{2+} across the plasma membrane, is necessary [28,34].

The pattern of responses in rheumatoid PBMC suggests that Ca^{2+} influx across the plasma membrane occurs slowly (Fig. 1), but it is not possible to comment fully on the release of Ca^{2+} from endoplasmic reticulum stores. Initial reports on the rise in T cell Ca^{2+} after stimulation had suggested that the initial peak seen was mainly due to release of Ca^{2+} from intracellular stores [28]. Our own observations, and those of other groups [35–37], demonstrate that this store of Ca^{2+} contributes only a small fraction to the initial Ca^{2+} elevation. Several investigators have tried to identify the pathway that initiates Ca^{2+} influx across the plasma membrane [18–22], but the exact mechanism remains unclear.

Interestingly, reports of Ca^{2+} signalling in neutrophils from both peripheral blood and synovial fluid from patients with RA suggest that there is an increase in the amount of Ca^{2+} released from intracellular stores on activation [31]. Transmembrane Ca^{2+} flux seems to be unaffected. The authors suggest that possible mechanisms for this phenomenon may relate to previous activation of these neutrophils resulting in an increased uptake of cytosolic Ca^{2+} into IP_3-sensitive stores, and a reduction in Ca^{2+} buffering capacity of the cells. The Ca^{2+} signalling pathways in RA neutrophils may have been altered so that the release of inflammatory mediators is exacerbated, therefore providing a possible link with the abnormality that is seen in RA lymphocytes.

Physical damage to cell surface structures may occur as an outcome of chronic or acute inflammation. The high numbers of phagocytes infiltrating the joint could, through the release of reactive oxygen species and proteases, lead to alterations of the surface proteins responsible for transducing the activation signals. Such defects could accumulate given the relatively long lifetime of a T lymphocyte. Our previous studies indicate that hydrogen peroxide can alter ligand/receptor interaction on activated T cells [38], while addition of anti-oxidants in cultures partially restores the deficient proliferation and IL-2 production observed with rheumatoid T cells [14]. Furthermore, preliminary studies in this department (S. P. Young and W. G. Naylor, unpublished observation) have shown that exposure of T cells to exogenous hydrogen peroxide can reduce the subsequent Ca^{2+} flux in these cells. It remains to be seen whether such a mechanism is of physiological relevance in explaining the observed T cell defects.

It therefore appears that there are both intrinsic and extrinsic effects on RA T cells. There is a reduction in the peak and plateau levels of T cell $[Ca^{2+}]_i$ as well as an extrinsic effect exerted by non-T cells which results in a greater reduction in the rate of rise of $[Ca^{2+}]_i$ than occurs in purified T lymphocyte populations. It remains unclear why this reduction in T cell signalling occurs. Surface expression of CD2/3 is unaltered in RA T cells compared with normal controls, so possible defects in early signalling events, abnormalities in the endoplasmic reticulum calcium store or surface membrane changes could be involved in the diminished Ca^{2+} flux. These intracellular signalling pathways and the interaction and effects of antigen-presenting cells on T cells are presently being investigated.

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