Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} entry induced by rapid cytosolic alkalinization in Jurkat T-lymphocytes

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INTRODUCTION

Receptors coupling to inositol-specific phospholipase C mediate an increase in the free cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]), in response to ligand-binding. Ca\textsuperscript{2+} mobilization comprises both Ins(1,4,5)P\textsubscript{3}-mediated Ca\textsuperscript{2+} release from intracellular stores [1] and Ca\textsuperscript{2+} entry across the plasma membrane. Ca\textsuperscript{2+} entry in electrically non-excitable cells is thought to be controlled either by the filling state of internal stores [2] or by second-messenger-gated plasma-membrane Ca\textsuperscript{2+} channels [3-5]. To study the effects of Ca\textsuperscript{2+}-store depletion on Ca\textsuperscript{2+} entry without simultaneous elevation of Ins(1,4,5)P\textsubscript{3} and Ins(1,3,4,5)P\textsubscript{4}, thapsigargin (Tg), a specific inhibitor of the Ca\textsuperscript{2+}-ATPase of the endoplasmic reticulum (ER), has been successfully used [6,7].

Here we report that 4-aminopyridine (4-AP) also appears to be a useful tool to study Ca\textsuperscript{2+} entry, since 4-AP (i) induced cytosolic alkalinization, (ii) facilitated Ca\textsuperscript{2+} release from an intracellular Ca\textsuperscript{2+} pool, most likely the Ins(1,4,5)P\textsubscript{3}-sensitive pool, (iii) mediated Ca\textsuperscript{2+} entry, and (iv) did not promote synthesis of inositol polyphosphates in Jurkat T-lymphocytes.

EXPERIMENTAL

Materials

4-AP and caffeine were purchased from Sigma, Deisenhofen, Germany. Tg, Fura2/AM (acetoxyethylster), 2',7'-bis-(carboxyethyl)-5(6')-carboxyfluorescein; (BCECF)/AM, BCECF/free acid and ionomycin were from Calbiochem, Bad Soden, Germany. Ins(1,4,5)P\textsubscript{3} and Ins(1,3,4,5)P\textsubscript{4} standards were bought from Boehringer, Mannheim, Germany. Monoclonal antibody OKT3, directed against the CD3 antigen, the invariable part of the T-cell receptor/CD3-complex, was purified on Protein G-Sepharose FF (Pharmacia, Freiburg, Germany). All other chemicals were of the highest purity available.

Measurement of [Ca\textsuperscript{2+}]

[Ca\textsuperscript{2+}], [Ca\textsuperscript{2+}], was measured as described previously [9], by the protocol of Treves et al. [10]. In brief, 10\textsuperscript{7} cells were loaded with Fura2/AM, and fluorescence was recorded in batches of 1.5 \times 10\textsuperscript{6} cells by using an LS-3B fluorimeter (Perkin–Elmer) at excitation and emission wavelengths of 340 \pm 3 nm and 505 \pm 10 nm respectively. The extracellular Ca\textsuperscript{2+} concentration was adjusted either to 1 mM to monitor Ca\textsuperscript{2+} release as well as Ca\textsuperscript{2+} entry, or to 0 mM (by addition of 3 mM EGTA) to measure Ca\textsuperscript{2+} release from intracellular stores only. Maximal and minimal fluorescence for calibration of the fluorescence signal were determined by addition of ionomycin plus Ca\textsuperscript{2+} and EGTA/Tris as described previously [9,11].

Measurement of Ca\textsuperscript{2+} release in permeabilized cells

Jurkat T-cells were permeabilized and Ca\textsuperscript{2+} release was measured as detailed recently [11]. In brief, cells were permeabilized in an intracellular buffer by saponin (30 \mu g/ml) for 10 min, then washed and resuspended in intracellular buffer. A sample-con-
After uptake resuspended and measured fluorescence, dithiothreitol, NaCl, MgSO4, NaH2PO4, and KCl were added. After stabilization of the fluorimeter, was then washed twice with buffer containing NaCl, glucose, KCl, pH 7.4, and 5.5 mM phosphocreatine, and 5 mM glucose, pH 7.4. BCECF and pH were measured as described previously [13].

**Measurement of cytosolic pH (pH)**

For this, 10⁶ cells were loaded with BCECF/AM (final concn. 1 μM) in RPMI 1640 containing 10% (v/v) newborn-calf serum for 20 min at 37 °C. After centrifugation (600 g, 2 min), the cells were resuspended in 5 ml of fresh prewarmed RPMI 1640 containing 10% newborn-calf serum and incubated for 30 min at 37 °C. Then the cells were washed twice with buffer containing 140 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM CaCl2, 20 mM HEPES, 1 mM Na2HPO4 and 5.5 mM glucose, pH 7.4, and resuspended in 6 ml of the same buffer. A sample (1.5 x 10⁶ cells) was then transferred to the cuvette of a Perkin–Elmer type LS-3B fluorimeter. Excitation wavelengths were adjusted alternately to 440 ± 7 nm or 420 ± 7 nm, while the emission wavelength was set to 520 ± 10 nm. A typical calibration curve with a correlation coefficient of 0.9997 is displayed.

**RESULTS**

4-AP induced a rapid and sustained elevation of [Ca2⁺], in Jurkat T cells (Figure 2a). The effect was dose-dependent, reaching a maximal response at about 5 mM 4-AP. In contrast, NH4Cl- and triethanolamine (TEA)-mediated Ca2⁺ signals were smaller or nearly undetectable (Figures 2b and 2c). To explain the effects of 4-AP and the other weak bases mechanistically, we examined their effect on pH (Figures 2d–2f). 4-AP induced a rapid and sustained alkalization, from pH 7.15 ± 0.03 (n = 27) to about pH 7.4 (Figure 2d). A similar rapid increase in pH was observed in response to NH4Cl, although the value never exceeded pH 7.3 (Figure 2e). TEA, which did not induce a Ca2⁺ signal (Figure 2c), induced a slow but significant increase in pH, to about pH 7.3 (range pH 7.26–7.33). These data may indicate that a rapid increase in the intracellular pH to at least 7.4 as induced by 4-AP may serve as trigger for the Ca2⁺ signal.

Figure 3 (upper part) shows that the Ca2⁺ signals induced by 4-AP and by stimulation of the T-cell receptor/CD3-complex are of similar magnitude and kinetics, raising the question whether similar mechanisms are involved. Since the exact physiological mechanism of the regulation of Ca2⁺ entry is still unclear in T-cells, we examined (i) whether physiological stimulation by anti-CD3 antibodies would lead to an alkalization comparable with that by 4-AP, (ii) whether 4-AP would deplete one or more of the intracellular stores, thereby leading to capacitative Ca2⁺ entry, or (iii) whether 4-AP would induce formation of inositol phosphates. The first possibility could be ruled out since physiological stimulation of T-cells via the T-cell receptor/CD3-complex did not induce significant alkalization (Figure 3, lower part). Figure 3 also demonstrates that 4-AP-induced alkalization preceded the Ca2⁺ signal, providing further evidence that rapid alkalization is the trigger for the Ca2⁺ signal.

As for the second possibility, we studied the effects of 4-AP on the four intracellular Ca2⁺ pools which we have defined recently in Jurkat T-lymphocytes [11]. To exclude effects of Ca2⁺ entry, EGTA was added to chelate extracellular Ca2⁺. Under such conditions, 4-AP induced a transient increase, which in its amplitude (peak [Ca2⁺]) and kinetics resembled the OKT3-induced Ca2⁺ signal (Figure 4a). Moreover, addition of OKT3 on the top of the 4-AP-induced Ca2⁺ signal did not mediate further Ca2⁺ release (Figure 4a), and vice versa (Figure 4b). We have recently shown that the Ins(1,4,5)P3-sensitive Ca2⁺ pool of Jurkat cells is a sub-compartment of the ER [11]. Accordingly, previous addition of Tg, which completely depleted the ER, abolished any effect of 4-AP (Figure 4c), indicating that 4-AP induced Ca2⁺ release from the agonist/Ins(1,4,5)P3-sensitive pool (pool I) of Jurkat T-cells [11]. In contrast, there was no interference with Ca2⁺ release from the agonist/Ins(1,4,5)P3-sensitive and Tgsensitive Ca2⁺ pool (pool II [11]), since addition of Tg after 4-AP mediated further Ca2⁺ release (results not shown) in a similar way to that shown for OKT3 and Tg [11]. Likewise, Ca2⁺ release from another Ca2⁺ pool sensitive to caffeine (pool III [11]) was still observed after previous addition of 4-AP (results not shown).

To clarify whether there is an additional effect of 4-AP on Ca2⁺ entry, 4-AP was added in the presence of extracellular Ca2⁺ either on top of the OKT3-mediated Ca2⁺ signal or 10 min after OKT3. 4-AP did not influence the OKT3-mediated signal when added.
Jurkat T-cells

Ca²⁺ mediated effect when added after

Figure 2 Increases added either after

3 min

4-AP in

by

NH₄Cl (5 mM)

(5 Ca²⁺ mediated

Ca²⁺ more concentrations of

by

alkalinization

Ins(1,4,5)P₃ abolished. Further

Next we tested whether cytosolic alkalinization as induced by

4-AP in intact cells would have a direct effect on Ins(1,4,5)P₃-mediated Ca²⁺ release in permeabilized cells. After permeabilization by saponin, the intracellular pH was adjusted by addition of buffer to pH 7.2, 7.4 or 7.6. Table 1 demonstrates that low concentrations of Ins(1,4,5)P₃ (100 nM) released significantly more Ca²⁺ at pH 7.4 as compared with pH 7.2. At 300 nM Ins(1,4,5)P₃ the difference between pH 7.2 and pH 7.4 was abolished. Further increasing the pH to 7.6 inhibited Ins(1,4,5)P₃-mediated Ca²⁺ release (Table 1). However, 4-AP itself had no effect on Ca²⁺ release when added to permeabilized cell preparations (results not shown).

To exclude an indirect effect of 4-AP via formation of inositol polyphosphates, h.p.l.c. analysis was carried out after stimulation of Jurkat T-lymphocytes using 4-AP. 4-AP did not induce increased levels of inositol polyphosphates as compared with control samples (Table 2).

**DISCUSSION**

Recent data indicated a role for K⁺ channels in counter-movement of K⁺ ions into intracellular compartments that release Ca²⁺ in response to an intracellular increase of Ins(1,4,5)P₃ ([17]; reviewed in [18]). Therefore we intended to use 4-AP, a K⁺-channel inhibitor, to block selectively K⁺ channels in Jurkat T-cells, thereby inhibiting indirectly Ca²⁺ efflux from the Ins(1,4,5)P₃-sensitive store. To our surprise, in intact Jurkat cells 4-AP did not block, but induced, a Ca²⁺ signal quite similar to the one observed in response to physiological stimulation. This Ca²⁺ signal appears to be initiated by sensitization of the Ins(1,4,5)P₃-receptor/Ca²⁺ channel by cytosolic alkalinization, a subsequent transient Ca²⁺ release from an intracellular com-
Jurkat T-cells were loaded with either Fura2/AM or BCECF/AM, and [Ca\textsuperscript{2+}]\textsubscript{i} or pH\textsubscript{r} was measured as described in the Experimental section. At time point '0' either OKT3 (10 \mu g/ml: O, ▽) or 4-AP (5 mM: ●, ▼) was added. Results from one typical experiment out of five are displayed.

Figure 3  [Ca\textsuperscript{2+}]\textsubscript{i} and pH\textsubscript{r} in response to 4-AP or to physiological stimulation by anti-CD3 antibody OKT3

Figure 4  Release from intracellular Ca\textsuperscript{2+} pools by 4-AP

Cells were loaded with Fura2/AM, transferred to the cuvette of the fluorimeter, and [Ca\textsuperscript{2+}]\textsubscript{i} was measured at 340 nm (excitation) and 505 nm (emission). Before addition of drugs EGTA (3 mM) was added to chelate extracellular Ca\textsuperscript{2+}. In (a) addition of 4-AP (5 mM) resulted in transient Ca\textsuperscript{2+} release. Subsequent addition of OKT3 (10 \mu g/ml) on top of the signal did not induce further release. Likewise, (b) demonstrates that after addition of OKT3 (10 \mu g/ml) no further release by 4-AP (5 mM) could be achieved. In (c) the ER was depleted by addition of Tg (1 \mu M); subsequent Ca\textsuperscript{2+} release by 4-AP was abolished. IM and Ca\textsuperscript{2+} respectively indicate addition of ionomycin (2 \mu M) and Ca\textsuperscript{2+} (5 mM). Representative tracings from three to four independent experiments are displayed.

portment, which most likely is the Ins(1,4,5)P\textsubscript{3}-sensitive Ca\textsuperscript{2+} store, followed by Ca\textsuperscript{2+} entry secondary to depletion of this Ca\textsuperscript{2+} pool. Since the Ca\textsuperscript{2+} signals in response to 4-AP and to physiological stimulation were rather similar, we tested whether physiological stimulation would lead to comparable cytosolic alkalization and thereby to sensitization of the Ins(1,4,5)P\textsubscript{3} receptor. However, a significant alkalization in response to anti-CD3 antibody OKT3 was not observed, so that this possible mechanism for sustained Ca\textsuperscript{2+} entry can be excluded in T cells. In contrast, in human foreskin fibroblasts cytosolic alkalization in response to mitogens such as fetal-calf serum and epidermal growth factor was observed [19]. Therefore, alkalization as induced by these mitogens may contribute to the length and magnitude of the Ca\textsuperscript{2+} signal in some cell types.

Despite these results, 4-AP appears to be a useful tool for selective depletion of the agonist/Ins(1,4,5)P\textsubscript{3}-sensitive Ca\textsuperscript{2+} pool, without affecting other intracellular Ca\textsuperscript{2+} pools, e.g. the Ins(1,4,5)P\textsubscript{3}-insensitive/Tg-sensitive Ca\textsuperscript{2+} pools or the caffeine-sensitive Ca\textsuperscript{2+} pool of T-cells [11]. Our results of alkalization-induced sensitization of the Ins(1,4,5)P\textsubscript{3} receptor/Ca\textsuperscript{2+}-release channel are compatible with pH-dependent binding of \textsuperscript{3}H]Ins(1,4,5)P\textsubscript{3} to its receptor from cerebellum ([20]; reviewed in [18]). Similarly, a pH-dependence of Ins(1,4,5)P\textsubscript{3}-induced Ca\textsuperscript{2+} release was observed (reviewed in [18]). However, in our hands pH values > 7.4 rather decreased the magnitude of the Ins(1,4,5)P\textsubscript{3}-mediated Ca\textsuperscript{2+} release. A possible explanation may be an inhibitory effect of pH\textsubscript{r} > 7.4 on the transmembrane pore formed by the C-terminal domain of the Ins(1,4,5)P\textsubscript{3} receptor/ Ca\textsuperscript{2+} channel, whereas binding of Ins(1,4,5)P\textsubscript{3} to its binding site in the N-terminal domain is increased.

In pituitary lactotrophs, it was shown by combined measurements of pH\textsubscript{r} and [Ca\textsuperscript{2+}], that an increase in pH\textsubscript{r} induced a Ca\textsuperscript{2+} signal [12]. Although in the report by Zorec et al. [12] the mechanism by which weak bases induce increases in [Ca\textsuperscript{2+}], was not further examined, they could show clearly by simultaneous measurement of pH\textsubscript{r} and [Ca\textsuperscript{2+}], that the rise in pH\textsubscript{r} preceded the increase in [Ca\textsuperscript{2+}]. Although our results and those of Zorec et al. [12] suggest a mechanism dependent on cytosolic alkalization, in a recent report it was demonstrated that 4-AP inhibited the Ca\textsuperscript{2+}-ATPase of mammalian sarcoplasmic reticulum in the same concentration range as described here [21]. Since in non-muscle cells the ER is the organelle equivalent to the sarcoplasmic reticulum of muscle, the possibility remains that 4-AP also in Jurkat T-cells inhibits a Ca\textsuperscript{2+}-ATPase, probably a Ca\textsuperscript{2+}-ATPase specific for the agonist/Ins(1,4,5)P\textsubscript{3}-sensitive Ca\textsuperscript{2+} pool, and
Figure 5  Effects of subsequent additions on OKT3- or 4-AP-induced Ca²⁺ signals

Jurkat T-cells were loaded with Fura2/AM, and [Ca²⁺]i was measured in the presence of extracellular Ca²⁺ as described in the Experimental section. In (a) the cells were first stimulated by OKT3 (10 µg/ml) and in (b) by 4-AP (5 mM). A subsequent stimulus, either 4-AP (a) or OKT3 (b), was added 10 min later. IM indicates addition of ionomycin (2 µM). Representative tracings from three independent experiments are displayed.

Table 1  Sensitization of Ins(1,4,5)P₃-induced Ca²⁺ release by increasing pH

Jurkat T-cells were permeabilized and [Ca²⁺]i was measured as detailed in the Experimental section. The pH of the intracellular buffer was adjusted to pH 7.2, 7.4 or 7.6. Values shown are Ca²⁺ release (as difference between the basal [Ca²⁺]i and [Ca²⁺]) after addition of Ins(1,4,5)P₃ versus Ins(1,4,5)P₃ concentration. Data are presented as means ± S.D. (n = 4–7). Raw data were corrected for small pH-dependent changes in the fluorescence intensity of Fura2. Such changes were observed when the fluorescence signal in response to 125 mM Ca²⁺ was measured: e.g. at pH 7.2 the relative signal was 100 ± 3.1%, at pH 7.4 100 ± 6.7% and at pH 7.6 95 ± 2.7%.

<table>
<thead>
<tr>
<th>Ins(1,4,5)P₃ added (nM)</th>
<th>Ca²⁺ release (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.2</td>
</tr>
<tr>
<td>40</td>
<td>12.2 ± 6.7</td>
</tr>
<tr>
<td>100</td>
<td>19.0 ± 5.4</td>
</tr>
<tr>
<td>300</td>
<td>98.0 ± 33.0</td>
</tr>
</tbody>
</table>

Table 2  Effect of 4-AP on inositol polyphosphate formation in Jurkat T-lymphocytes

Jurkat T-lymphocytes (4 × 10⁶ cells per sample) were stimulated with buffer (Control), 5 mM 4-AP or 10 µg/ml OKT3 for 3 min. HClO₄ extracts of samples were processed and analysed for inositol polyphosphates by anion-exchange h.p.l.c./post-column complexometry as described previously [8,16]. Data are presented as means ± S.D. (n = 3).

<table>
<thead>
<tr>
<th>Addition</th>
<th>Ins(1,4,5)P₃</th>
<th>Ins(1,3,4,5)P₄</th>
<th>Ins(1,3,4,6)P₄</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>54 ± 17</td>
<td>118 ± 3</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>4-AP</td>
<td>54 ± 5</td>
<td>83 ± 35</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>OKT3</td>
<td>84 ± 8</td>
<td>535 ± 45</td>
<td>351 ± 14</td>
</tr>
</tbody>
</table>

Effect of cytosolic alkalization on free cytosolic [Ca²⁺] thereby contributes by a second mechanism to the effects observed. However, the fact that 4-AP did not mediate Ca²⁺ release in permeabilized T-cells does not support such a mechanism.

In the presence of extracellular Ca²⁺, 4-AP mediated a rapid and sustained increase in [Ca²⁺]. Therefore, our data indicate that Ca²⁺ entry in Jurkat T-cells can be experimentally induced by cytosolic-alkalization-dependent depletion of the Ins(1,4,5)P₃-sensitive Ca²⁺ pool. This finding is in accordance with a previous publication describing Ca²⁺ entry induced in a T-cell clone after artificial depletion of the ER by Tg [22]. These data are compatible with the model of ‘capacitative’ Ca²⁺ entry [2]. However, the precise mechanism of ‘capacitative’ Ca²⁺ entry remains unclear. Involved in such a mechanism might be a so-far undefined intracellular diffusible messenger that is generated or released upon Ca²⁺-store depletion, and that induces opening of plasma-membrane Ca²⁺ channels. Evidence for the existence of such a messenger has been presented recently [23]. In the light of our results, depletion of the Ins(1,4,5)P₃-sensitive Ca²⁺ pool by cytosolic alkalization, it would be interesting to see whether this postulated messenger is also observed after stimulation of T-cells with 4-AP.

Recent data from Orrenius’ group [24] indicate that more than one mechanism regulates Ca²⁺ entry in T-cells. Our results support such a view (Figure 5). As shown in [24] for the agonists OKT3 and Tg, we demonstrate that 4-AP, when added 10 min after OKT3, induced a further increase of [Ca²⁺]; in the presence of extracellular Ca²⁺. In contrast, addition of 4-AP on top of the OKT3-mediated Ca²⁺ signal (after 3 min) did not promote any further increase. However, Chow et al. [24] also reported that addition of OKT3 10 min after Tg induced a further, although small, increase in [Ca²⁺]. When 4-AP was used instead of Tg as the first stimulus, a similar result was obtained only in a few of our experiments, but mostly no further increase in [Ca²⁺] induced by OKT3 was observed. These data may indeed indicate that at least two mechanisms are involved in T-cell receptor/CD3-mediated Ca²⁺ entry. The first mechanism is based on transient Ins(1,4,5)P₃ generation [11] and perhaps capacitative Ca²⁺ entry secondary to depletion of the Ins(1,4,5)P₃-sensitive Ca²⁺ store. Since Ins(1,4,5)P₃ levels are increased 3 min after stimulation, a further sensitization of the Ins(1,4,5)P₃ receptor by cytosolic alkalization, as induced by 4-AP, does not further increase the Ca²⁺ signal. The second, more sustained and so far unknown, mechanism appears to be independent of the action of Ins(1,4,5)P₃, since sensitization of the Ins(1,4,5)P₃ receptor by 4-AP after 10 min, when the levels of Ins(1,4,5)P₃ are low again [11], reactivates the first system. Similar results were obtained by using Tg instead of 4-AP [24]. On the other hand, the 4-AP-induced cytosolic alkalization and Ca²⁺ signal are still observed after 10 min (Figures 2a and 2d), indicating that both the Ins(1,4,5)P₃-dependent and the unknown, Ins(1,4,5)P₃-independent, Ca²⁺-entry mechanisms are activated. This would explain the lack of effect of addition of OKT3 10 min after 4-AP (Figure 5b). Preliminary results from our laboratory indicate that the caffeine-sensitive Ca²⁺ pool of Jurkat T-cells [11] may be involved in the unknown Ca²⁺-entry mechanism discussed above (A. H. Guse, unpublished work).

In conclusion, we show that cytosolic alkalization induces Ca²⁺ signals in Jurkat T-cells and present weak bases such as 4-AP as novel tools to delete selectively the Ins(1,4,5)P₃-sensitive Ca²⁺ pool of Jurkat T-cells without formation of inositol polyphosphates.

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