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

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4-Aminopyridine (4-AP), a compound usually known as a K^+ -channel inhibitor, induced rapid cytosolic alkalinization from pH 7.15 to pH 7.4, and subsequently Ca^{2+} mobilization in the T-lymphocyte cell line Jurkat. Other weak bases, such as NH_4Cl or triethanolamine, induced a smaller and/or slower increase in cytosolic pH, resulting in a lower or no detectable Ca^{2+} signal. In the presence of extracellular Ca^{2+} , 4-AP mediated a rapid and sustained increase in the free cytosolic Ca^{2+} concentration similar to that obtained by T-cell receptor-mediated stimulation. In the absence of extracellular Ca^{2+} , 4-AP transiently released Ca^{2+} from an intracellular store that is most likely identical with the agonist- and $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool of Jurkat T-cells. As possible mechanisms for Ca^{2+} release from this particular

pool as induced by 4-AP we examined (i) formation of $\text{Ins}(1,4,5)\text{P}_3$ and (ii) sensitization of the $\text{Ins}(1,4,5)\text{P}_3$ -receptor/ Ca^{2+} -release system by increasing intracellular pH. Although 4-AP did not induce formation of inositol polyphosphates, as demonstrated by h.p.l.c. analysis, in permeabilized cells the dose-response curve for $\text{Ins}(1,4,5)\text{P}_3$ was shifted to the left by changing the intracellular pH from 7.2 to 7.4. This indicated that sensitization of the $\text{Ins}(1,4,5)\text{P}_3$ -receptor/ Ca^{2+} -release system was responsible for the effects of 4-AP seen in intact cells. In conclusion, 4-AP appears a novel tool for depletion of the agonist-sensitive Ca^{2+} pool of T-cells without simultaneous formation of $\text{Ins}(1,4,5)\text{P}_3$, thereby inducing capacitative Ca^{2+} entry in these cells.

INTRODUCTION

Receptors coupling to inositide-specific phospholipase C mediate an increase in the free cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in response to ligand-binding. Ca^{2+} mobilization comprises both $\text{Ins}(1,4,5)\text{P}_3$ -mediated Ca^{2+} release from intracellular stores [1] and Ca^{2+} entry across the plasma membrane. Ca^{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^{2+} channels [3–5]. To study the effects of Ca^{2+} -store depletion on Ca^{2+} entry without simultaneous elevation of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, thapsigargin (Tg), a specific inhibitor of the Ca^{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^{2+} entry, since 4-AP (i) induced cytosolic alkalinization, (ii) facilitated Ca^{2+} release from an intracellular Ca^{2+} pool, most likely the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive pool, (iii) mediated Ca^{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 (acetoxymethylester), 2',7'-bis-(carboxyethyl)-5(6')-carboxyfluorescein; (BCECF)/AM, BCECF/free acid and ionomycin were from Calbiochem, Bad Soden, Germany. $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ 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.

Cell culture

Jurkat T-lymphocytes were cultured as described in a previous publication [8].

Measurement of $[\text{Ca}^{2+}]_i$

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

Measurement of Ca^{2+} release in permeabilized cells

Jurkat T-cells were permeabilized and Ca^{2+} release was measured as detailed recently [11]. In brief, cells were permeabilized in an intracellular buffer by saponin (30 $\mu\text{g}/\text{ml}$) for 10 min, then washed and resuspended in intracellular buffer. A sample con-

Abbreviations used: AM, acetoxymethyl ester; 4-AP, 4-aminopyridine; BCECF, 2',7'-bis-(carboxyethyl)-5(6')-carboxyfluorescein; $[\text{Ca}^{2+}]_i$, free cytosolic Ca^{2+} concentration; ER, endoplasmic reticulum; pH_i, cytosolic pH; TEA, triethanolamine; Tg, thapsigargin.

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Figure 1 Correlation between 490/440 nm ratio of BCECF and pH

Intracellular buffer, containing 110 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 5 mM KH_2PO_4 , 2 mM dithiothreitol, 2 mM EGTA, 1% BSA and 20 mM Hepes was adjusted to pH values between 6.6 and 7.8, and fluorescence was measured in the presence of 1 μ M BCECF free acid. Excitation wavelengths were alternatively adjusted to 490 ± 7 nm or 440 ± 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.

taining 6.7×10^7 cells was transferred to the cuvette, and Fura2 free acid, creatine kinase, phosphocreatine and ATP were added. After uptake of Ca^{2+} into intracellular stores, stimuli were added in small volumes. Calibration was carried out at the end of each measurement by using ionomycin/ Ca^{2+} (2 μ M/1 mM) and EGTA/Tris (4 mM/40 mM) to obtain maximal and minimal fluorescence, respectively.

Measurement of cytosolic pH (pH_i)

For this, 10^7 cells were loaded with BCECF/AM (final concn. 1 μ M) 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 $MgSO_4$, 1 mM $CaCl_2$, 20 mM Hepes, 1 mM NaH_2PO_4 and 5.5 mM glucose, pH 7.4, and resuspended in 6 ml of the same buffer. A sample (1.5×10^6 cells) was then transferred to the cuvette of a Perkin-Elmer type LS-3B fluorimeter. Excitation wavelengths were adjusted alternatively to 440 ± 7 or to 490 ± 7 nm, while the emission wavelength was set to 520 ± 10 nm. Thereby, each 30 s or each 60 s a ratio between excitation at 440 nm and at 490 nm was obtained. After stabilization of the baseline, stimuli were added in small volumes. Calibration of pH_i in cell suspensions *in situ* is difficult and erroneous, since it is nearly impossible to change the buffer (and thereby the pH) without losing or damaging cells. Therefore, ratios were calibrated by external measurements using BCECF free acid (1 μ M) in intracellular buffer (composition as described above for Ca^{2+} -release experiments, including 1% BSA) titrated to different pH values between 6.6 and 7.8. The ratios plotted against pH resulted in a highly linear correlation, with correlation coefficients > 0.999 (Figure 1). By this method a resting pH_i of 7.15 ± 0.03 ($n = 27$) was obtained, which is in good agreement with recent reports for pituitary cells [12,13] and for other cell types [14].

H.p.l.c. analysis of inositol polyphosphates

Mass analysis of inositol polyphosphates was carried out by using a recently developed anion-exchange h.p.l.c./post-column complexometric dye system [15] as described by Guse and Emmrich [8,16]. Commercially available $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ were used as standards.

RESULTS

4-AP induced a rapid and sustained elevation of $[Ca^{2+}]_i$ in Jurkat T cells (Figure 2a). The effect was dose-dependent, reaching a maximal response at about 5 mM 4-AP. In contrast, NH_4Cl - and triethanolamine (TEA)-mediated Ca^{2+} 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_i (Figures 2d–2f). 4-AP induced a rapid and sustained alkalinization, from $pH\ 7.15 \pm 0.03$ ($n = 27$) to about pH 7.4 (Figure 2d). A similar rapid increase in pH_i was observed in response to NH_4Cl , although the value never exceeded pH 7.3 (Figure 2e). TEA, which did not induce a Ca^{2+} signal (Figure 2c), induced a slow but significant increase in pH_i, 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 Ca^{2+} signal.

Figure 3 (upper part) shows that the Ca^{2+} 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 Ca^{2+} entry is still unclear in T-cells, we examined (i) whether physiological stimulation by anti-CD3 antibodies would lead to an alkalinization comparable with that by 4-AP, (ii) whether 4-AP would deplete one or more of the intracellular stores, thereby leading to capacitative Ca^{2+} 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 alkalinization (Figure 3, lower part). Figure 3 also demonstrates that 4-AP-induced alkalinization preceded the Ca^{2+} signal, providing further evidence that rapid alkalinization is the trigger for the Ca^{2+} signal.

As for the second possibility, we studied the effects of 4-AP on the four intracellular Ca^{2+} pools which we have defined recently in Jurkat T-lymphocytes [11]. To exclude effects of Ca^{2+} entry, EGTA was added to chelate extracellular Ca^{2+} . Under such conditions, 4-AP induced a transient increase, which in its amplitude (peak $[Ca^{2+}]_i$) and kinetics resembled the OKT3-induced Ca^{2+} signal (Figure 4a). Moreover, addition of OKT3 on top of the 4-AP-induced Ca^{2+} signal did not mediate further Ca^{2+} release (Figure 4a), and vice versa (Figure 4b). We have recently shown that the $Ins(1,4,5)P_3$ -sensitive Ca^{2+} 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 Ca^{2+} release from the agonist/ $Ins(1,4,5)P_3$ -sensitive pool (pool I) of Jurkat T-cells [11]. In contrast, there was no interference with Ca^{2+} release from the agonist/ $Ins(1,4,5)P_3$ -insensitive and Tg-sensitive Ca^{2+} pool (pool II [11]), since addition of Tg after 4-AP mediated further Ca^{2+} release (results not shown) in a similar way to that shown for OKT3 and Tg [11]. Likewise, Ca^{2+} release from another Ca^{2+} 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 Ca^{2+} entry, 4-AP was added in the presence of extracellular Ca^{2+} either on top of the OKT3-mediated Ca^{2+} signal or 10 min after OKT3. 4-AP did not influence the OKT3-mediated signal when added

Figure 2 Increases in $[Ca^{2+}]_i$ and pH_i induced by weak bases

Jurkat T-cells were loaded with either Fura2/AM (a–c) or BCECF/AM (d–f), and $[Ca^{2+}]_i$ or pH_i was measured as described in the Experimental section. Arrows indicate addition of 4-AP (5 mM), NH_4Cl (5 mM) or TEA (5 mM). In each panel results from three independent experiments are displayed.

after 3 min (results not shown), but induced a further stimulatory effect when added after 10 min (Figure 5a). In contrast, 4-AP-mediated Ca^{2+} signalling was not influenced when OKT3 was added either 3 min (results not shown) or 10 min later (Figure 5b). However, in some experiments a small further increase, as induced by OKT3 after 10 min, was observed.

Next we tested whether cytosolic alkalization as induced by 4-AP in intact cells would have a direct effect on $Ins(1,4,5)P_3$ -mediated Ca^{2+} 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 $Ins(1,4,5)P_3$ (100 nM) released significantly more Ca^{2+} at pH 7.4 as compared with pH 7.2. At 300 nM $Ins(1,4,5)P_3$ the difference between pH 7.2 and pH 7.4 was abolished. Further increasing the pH to 7.6 inhibited $Ins(1,4,5)P_3$ -mediated Ca^{2+} release (Table 1). However, 4-AP itself had no effect on Ca^{2+} 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 Ca^{2+} in response to an intracellular increase of $Ins(1,4,5)P_3$ ([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 Ca^{2+} efflux from the $Ins(1,4,5)P_3$ -sensitive store. To our surprise, in intact Jurkat cells 4-AP did not block, but induced, a Ca^{2+} signal quite similar to the one observed in response to physiological stimulation. This Ca^{2+} signal appears to be initiated by sensitization of the $Ins(1,4,5)P_3$ -receptor/ Ca^{2+} channel by cytosolic alkalization, a subsequent transient Ca^{2+} release from an intracellular com-

Figure 3 $[Ca^{2+}]_i$ and pH_i in response to 4-AP or to physiological stimulation by anti-CD3 antibody OKT3

Jurkat T-cells were loaded with either Fura2/AM or BCECF/AM, and $[Ca^{2+}]_i$ or pH_i was measured as described in the Experimental section. At time point '0' either OKT3 (10 μ g/ml: $\circ$, ∇) or 4-AP (5 mM: $\bullet$, $\blacktriangledown$) was added. Results from one typical experiment out of five are displayed.

partment, which most likely is the $Ins(1,4,5)P_3$ -sensitive Ca^{2+} store, followed by Ca^{2+} entry secondary to depletion of this Ca^{2+} pool. Since the Ca^{2+} signals in response to 4-AP and to physiological stimulation were rather similar, we tested whether physiological stimulation would lead to comparable cytosolic alkalinization and thereby to sensitization of the $Ins(1,4,5)P_3$ receptor. However, a significant alkalinization in response to anti-CD3 antibody OKT3 was not observed, so that this possible mechanism for sustained Ca^{2+} entry can be excluded in T cells. In contrast, in human foreskin fibroblasts cytosolic alkalinization in response to mitogens such as fetal-calf serum and epidermal growth factor was observed [19]. Therefore, alkalinization as induced by these mitogens may contribute to the length and magnitude of the Ca^{2+} signal in some cell types.

Despite these results, 4-AP appears to be a useful tool for selective depletion of the agonist/ $Ins(1,4,5)P_3$ -sensitive Ca^{2+} pool, without affecting other intracellular Ca^{2+} pools, e.g. the $Ins(1,4,5)P_3$ -insensitive/Tg-sensitive Ca^{2+} pools or the caffeine-sensitive Ca^{2+} pool of T-cells [11]. Our results of alkalinization-induced sensitization of the $Ins(1,4,5)P_3$ receptor/ Ca^{2+} -release channel are compatible with pH-dependent binding of $[^3H]Ins(1,4,5)P_3$ to its receptor from cerebellum ([20]; reviewed in [18]). Similarly, a pH-dependence of $Ins(1,4,5)P_3$ -induced Ca^{2+} release was observed (reviewed in [18]). However, in our hands pH values > 7.4 rather decreased the magnitude of the $Ins(1,4,5)P_3$ -mediated Ca^{2+} release. A possible explanation may be an inhibitory effect of $pH_i > 7.4$ on the transmembrane pore formed by the C-terminal domain of the $Ins(1,4,5)P_3$ receptor/ Ca^{2+} channel, whereas binding of $Ins(1,4,5)P_3$ to its binding site in the N-terminal domain is increased.

In pituitary lactotrophs, it was shown by combined measurements of pH_i and $[Ca^{2+}]_i$ that an increase in pH_i induced a Ca^{2+} signal [12]. Although in the report by Zorec et al. [12] the mechanism by which weak bases induce increases in $[Ca^{2+}]_i$ was not further examined, they could show clearly by simultaneous measurement of pH_i and $[Ca^{2+}]_i$ that the rise in pH_i preceded the increase in $[Ca^{2+}]_i$. Although our results and those of Zorec et al.

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

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

[12] suggest a mechanism dependent on cytosolic alkalinization, in a recent report it was demonstrated that 4-AP inhibited the Ca^{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^{2+} -ATPase, probably a Ca^{2+} -ATPase specific for the agonist/ $Ins(1,4,5)P_3$ -sensitive Ca^{2+} pool, and

Figure 5 Effects of subsequent additions on OKT3- or 4-AP-induced Ca^{2+} signals

Jurkat T-cells were loaded with Fura2/AM, and $[Ca^{2+}]_i$ was measured in the presence of extracellular Ca^{2+} as described in the Experimental section. In (a) the cells were first stimulated by OKT3 (10 μ g/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 μ M). Representative tracings from three independent experiments are displayed.

Table 1 Sensitization of $Ins(1,4,5)P_3$ -induced Ca^{2+} release by increasing pH

Jurkat T-cells were permeabilized and $[Ca^{2+}]_i$ was measured as detailed in the Experimental section. The pH of the intracellular buffer used was adjusted to pH 7.2, 7.4 or 7.6. Values shown are Ca^{2+} release (as difference between the basal $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ after addition of $Ins(1,4,5)P_3$) versus $Ins(1,4,5)P_3$ concentration. Data are presented as means $\pm$ 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 nM Ca^{2+} was measured: e.g. at pH 7.2 the relative signal was $100 \pm 3.1\%$, at pH 7.4 $100 \pm 6.7\%$ and at pH 7.6 $95 \pm 2.7\%$.

$Ins(1,4,5)P_3$ added (nM)	Ca^{2+} release (nM)		
	pH 7.2	pH 7.4	pH 7.6
40	12.2 ± 6.7	12.8 ± 3.9	5.3 ± 4.0
100	19.8 ± 5.4	51.0 ± 12.0	23.8 ± 9.6
300	98.0 ± 33.0	89.3 ± 18.0	26.1 ± 9.2

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

Jurkat T-lymphocytes (4×10^8 cells per sample) were stimulated with buffer (Control), 5 mM 4-AP or 10 μ g/ml OKT3 for 3 min. $HClO_4$ 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 $\pm$ S.D. ($n = 3$).

Addition	Inositol polyphosphate formed (pmol/ 10^9 cells)		
	$Ins(1,4,5)P_3$	$Ins(1,3,4,5)P_4$	$Ins(1,3,4,6)P_4$
Control	54 ± 17	118 ± 3	< 20
4-AP	54 ± 6	83 ± 35	< 20
OKT3	84 ± 8	5353 ± 45	351 ± 14

thereby contributes by a second mechanism to the effects observed. However, the fact that 4-AP did not mediate Ca^{2+} release in permeabilized T-cells does not support such a mechanism.

In the presence of extracellular Ca^{2+} , 4-AP mediated a rapid and sustained increase in $[Ca^{2+}]_i$. Therefore, our data indicate that Ca^{2+} entry in Jurkat T-cells can be experimentally induced by cytosolic-alkalinization-dependent depletion of the $Ins(1,4,5)P_3$ -sensitive Ca^{2+} pool. This finding is in accordance with a previous publication describing Ca^{2+} 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' Ca^{2+} entry [2]. However, the precise mechanism of 'capacitative' Ca^{2+} entry remains unclear. Involved in such a mechanism might be a so-far undefined intracellular diffusible messenger that is generated or released upon Ca^{2+} -store depletion, and that induces opening of plasma-membrane Ca^{2+} channels. Evidence for the existence of such a messenger has been presented recently [23]. In the light of our results, depletion of the $Ins(1,4,5)P_3$ -sensitive Ca^{2+} pool by cytosolic alkalinization, 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 Ca^{2+} 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 $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} . In contrast, addition of 4-AP on top of the OKT3-mediated Ca^{2+} 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 $[Ca^{2+}]_i$. 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 $[Ca^{2+}]_i$ induced by OKT3 was observed. These data may indeed indicate that at least two mechanisms are involved in T-cell receptor/CD3-mediated Ca^{2+} entry. The first mechanism is based on transient $Ins(1,4,5)P_3$ generation [11] and perhaps capacitative Ca^{2+} entry secondary to depletion of the $Ins(1,4,5)P_3$ -sensitive Ca^{2+} store. Since $Ins(1,4,5)P_3$ levels are increased 3 min after stimulation, a further sensitization of the $Ins(1,4,5)P_3$ receptor by cytosolic alkalinization, as induced by 4-AP, does not further increase the Ca^{2+} signal. The second, more sustained and so far unknown, mechanism appears to be independent of the action of $Ins(1,4,5)P_3$, since sensitization of the $Ins(1,4,5)P_3$ receptor by 4-AP after 10 min, when the levels of $Ins(1,4,5)P_3$ are low again [11], re-activates the first system. Similar results were obtained by using Tg instead of 4-AP [24]. On the other hand, the 4-AP-induced cytosolic alkalinization and Ca^{2+} signal are still observed after 10 min (Figures 2a and 2d), indicating that both the $Ins(1,4,5)P_3$ -dependent and the unknown, $Ins(1,4,5)P_3$ -independent, Ca^{2+} -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 Ca^{2+} pool of Jurkat T-cells [11] may be involved in the unknown Ca^{2+} -entry mechanism discussed above (A. H. Guse, unpublished work).

In conclusion, we show that cytosolic alkalinization induces Ca^{2+} signals in Jurkat T-cells and present weak bases such as 4-AP as novel tools to deplete selectively the $Ins(1,4,5)P_3$ -sensitive Ca^{2+} pool of Jurkat T-cells without formation of inositol polyphosphates.

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