Regulation of cADP-ribose-induced Ca\(^{2+}\) Release by Mg\(^{2+}\) and Inorganic Phosphate*

(Received for publication, April 15, 1996, and in revised form, July 8, 1996)

Andreas H. Guse§, Cristina P. da Silva††, Karin Weber‡, Gloria A. Ashamuli, Barry V. L. Potter***, and Georg W. Mayr‡‡

From the ˚University of Hamburg, Institute of Physiological Chemistry, Department of Enzyme Chemistry, Grindelallee 117, D-20146 Hamburg, Germany and University of Bath, School of Pharmacy and Pharmacology, Claveron Down, Bath BA2 7AY, United Kingdom

cADP-ribose (cADPr) has recently been shown to release Ca\(^{2+}\) from an intracellular store of permeabilized T lymphocyte cell lines (Guse, A. H., da Silva, C. P., Emmrich, F., Ashamu, G. A., Potter, B. V. L., and Mayr, G. W. (1995) J. Immunol. 155, 3353–3359). Using permeabilized Jurkat and HPB-ALL T lymphocytes, the effects of varying concentrations of inorganic phosphate and Mg\(^{2+}\) on cADPr-induced Ca\(^{2+}\) release were investigated. cADPr-induced Ca\(^{2+}\) release was dependent on the concentration of inorganic phosphate, showing very low Ca\(^{2+}\) release activity between 0.5 and 2 mM inorganic phosphate. At 4 to 5 mM inorganic phosphate, the cADPr-induced Ca\(^{2+}\) release was much more pronounced, reaching maximal values at 10 mM inorganic phosphate. The underlying mechanism for this stimulatory effect was an increased loading of the cADPr-sensitive Ca\(^{2+}\) store, which was demonstrated by enhanced reseques-tration of Ca\(^{2+}\) selectively into the cADPr-sensitive Ca\(^{2+}\) store. The free Mg\(^{2+}\) concentration also influenced cADPr-induced Ca\(^{2+}\) release in permeabilized cells; at 0 and 8.58 mM the release was nearly completely abolished, whereas at 1.06 mM maximal Ca\(^{2+}\) release by cADPr was observed. High performance liquid chromatographic analysis of exogenously added cADPr revealed that the catabolism of cADPr at varying Mg\(^{2+}\) and P\(_i\) concentrations had only minor relevance for the modulatory effects observed.

To correlate the effects of inorganic phosphate and Mg\(^{2+}\) on cADPr-induced Ca\(^{2+}\) release observed in the permeabilized cell preparations, measurements of these ions in intact Jurkat T lymphocytes were carried out. Intact Jurkat T cells stimulated via the T cell receptor-CD3 complex did not respond with significant elevation of the free intracellular Mg\(^{2+}\) concentration. In contrast, stimulation via the T cell receptor-CD3 complex resulted in an increase in the intracellular inorganic phosphate concentration. These data indicate a role for the intracellular inorganic phosphate concentration in the regulation of cADPr-mediated Ca\(^{2+}\) release in T lymphocytes.

Ca\(^{2+}\) release from intracellular stores is a major event trans-ducing extracellular signals into living cells. In addition to the well characterized d-myo-inositol-1,4,5-trisphosphate (Ins(1,4,5)P\(_3\)) system for Ca\(^{2+}\) release from intracellular stores (for review, see Berridge (1993) and Bootman and Berridge (1995)), a second, probably second messenger-controlled, Ca\(^{2+}\) release system consisting of cADP-ribose (cADPr), specific binding proteins for cADPr (Walseth et al., 1993), and ryanodine receptors (RyRs) exists and may be involved in receptor-operated Ca\(^{2+}\) signaling in living cells (for review, see Galiano and White (1994) and Lee (1994)).

cADPr-induced Ca\(^{2+}\) release has been shown: (i) to work independently of Ins(1,4,5)P\(_3\)-induced Ca\(^{2+}\) release (Dargie et al., 1990); (ii) to be enhanced by caffeine (Lee, 1993); (iii) to be inhibited by 8-NH\(_2\)-cADPr, 8-bromo-cADPr, ruthenium red, ryanodine, and procaine (Galian et al., 1991; Walseth and Lee, 1993; Guse et al., 1995); (iv) to release Ca\(^{2+}\) from the nuclear envelope, indicating a potential mechanism for elevation of intranuclear [Ca\(^{2+}\)] during cell activation (Gerasimenko et al., 1995); and (v) to be dependent on the presence of calmodulin (Lee et al., 1994).

The endogenous levels of cADPr have been determined by radioimmunoassay, HPLC, and/or bioassays in different tissues and cells (Walseth et al., 1991; Takahashi et al., 1995; Kuenmerle and Makhlouf, 1995; Guse et al., 1995); however, so far, receptor-mediated formation of cADPr has been described only for cholecystokinin octapeptide-stimulated longitudinal muscle (Kuenmerle and Makhlouf, 1995), posing the question of whether cADPr in general is a second messenger, transducing signals in response to binding of extracellular agonists. Another possibility would be indirect regulation either of the amount of cADPr or its Ca\(^{2+}\) release activity. The former has been published for sea urchin eggs, in which the endogenous levels of cADPr may be controlled by a cGMP-dependent process (Galione et al., 1993). Recently, evidence of the second possibility has been presented. Chini et al. (1995) demonstrated that spermine and related polyamines, in physiological concentrations, inhibited the cADPr- but not the Ins(1,4,5)P\(_3\)-induced Ca\(^{2+}\) release in sea urchin egg homogenates.

The majority of work on cADPr has been performed in the sea urchin egg system; however, there is increasing evidence that also in T lymphocytes cADPr is involved in intracellular Ca\(^{2+}\) signaling. We have recently demonstrated that cADPr released Ca\(^{2+}\) from a non-thapsigargin-sensitive intracellular

---

*This work was supported in part by Deutsche Forschungsgemeinschaft Grant Gu 360/2-1 (to A. H. G. and G. W. M.) and grants from the Medical Research Council and the British-German ARC Programme (to B. V. L. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by Junta Nacional de Investigación Científica e Tecnológica (Portugal) Grant Praxis XXI-BPD/4228/94.

** A Lister Institute Research Professor.

¶ To whom correspondence should be addressed. Tel.: 49-40-4123-4276; Fax: 49-40-4123-4275; E-mail: guse@uke.uni-hamburg.de.

†† The abbreviations used are: Ins(1,4,5)P\(_3\), d-myo-inositol-1,4,5-trisphosphate; cADPr, cADP-ribose; HPLC, high performance liquid chromatography; [Mg\(^{2+}\)]\(_{free}\), free Mg\(^{2+}\) concentration; [Mg\(^{2+}\)]\(_{nuc}\), free intracellular Mg\(^{2+}\) concentration; [P\(_i\)]\(_{free}\), inorganic phosphate concentration; [P\(_i\)]\(_{nuc}\), intracellular inorganic phosphate concentration; RyR, ryanodine receptor; TCR-CD3, T cell receptor-CD3 complex.
store of T lymphocyte cell lines (Guse et al., 1995). Moreover, Bourguignon et al. (1995) showed that a fraction of light density membrane vesicles of mouse T-lymphoma cells bound [3H]ryanodine with high affinity and released Ca2+ in response to cADPr. These authors also demonstrated the presence of RyRns in T-lymphoma cells by Western blot analysis. Evidence of the expression of brain type RyR in human Jurkat T cells was also presented by Hakamata et al. (1994). However, HPLC analysis of endogenous cADPr did not reveal any T cell receptor-CD3 complex (TCR-CD3)-induced elevation of this compound (Guse et al., 1995).

We report here that: (i) cADPr-induced Ca2+ release in permeabilized Jurkat T lymphocytes is modulated by the concentration of P and Mg2+; and (ii) the stimulatory effect of high [P] on cADPr-induced Ca2+ release was most likely due to enhanced loading of the cADPr-sensitive intracellular Ca2+ store. In contrast, HPLC analysis revealed that the catabolism of cADPr at varying Mg2+ and P concentrations had a minor relevance for the modulatory effects observed. Importantly, the intracellular free Mg2+ ([Mg2+]i) was not altered by stimulation of intact mag.Fura-2-loaded Jurkat T cells, whereas stimulation of the TCR-CD3 resulted in a marked increase in the intracellular P concentration.

**EXPERIMENTAL PROCEDURES**

**Materials**—cADPr was prepared enzymatically from β-NAD+ (see below). Saponin, β-NAD+, adenosine, ADP, malachite green, Tergitol NP-10, ATP, and phosphocreatine were obtained from Sigma. Creatine kinase was purchased from Boehringer Mannheim. Fura2-free acid and ionomycin were from Calbiochem. Mag-Fura2/AM and 4-bromo-A23187 were obtained from Molecular Probes Europe (Leiden, The Netherlands). Ammonium heptamolybdate and trisodium citrate were from Merck (Darmstadt, Germany). Buffers used in Ca2+ release experiments or for HPLC were prepared using water that was first doubly distilled and then further purified using a MilliQ system (Waters-Millipore, Eschborn, Germany).

**Preparation of cADPr**—cADPr was synthesized from NAD+ in a broadly similar fashion to that reported (Ashamu et al., 1995). Thus, 1.5 mm β-NAD+ in 2.5 ml of HEPES buffer, pH 6.8, was incubated with 10 μl of crude ADP cyclase from *Aplysia californica* (Lee and Aarhus, 1991) at room temperature for approximately 10 min. This conversion was monitored by ion-exchange HPLC, and when cyclization was judged to be complete, the cADPr was purified by ion-exchange chromatography on Sepharose Q Fast Flow using a gradient of triethylammonium bicarbonate. cADPr was used as its triethylammonium salt. cADPr exhibited satisfactory 1H and 31P NMR spectra and HPLC data and was quantified by UV spectroscopy.

**Cell Culture**—Jurkat and HPB-ALL T lymphocytes were cultured as described in an earlier report (Guse and Emmrich, 1991). Ca2+ Release Experiments in Permeabilized Cells—Permeabilized cells were prepared, and the Ca2+ concentration was measured as described (Guse et al., 1992). In brief, cells were permeabilized in the presence of saponin (30 μg/ml) for 17.5 min (Jurkat) or 10 min (HPB-ALL) in an intracellular buffer (20 mM HEPES, 110 mM KCl, 2 mM MgCl2, 5 mM KH2PO4, 10 mM NaCl, pH 7.25 at 37 °C). The total concentrations of KH2PO4 and MgCl2 were different where indicated. To maintain the same osmolality, the concentration of KCl was changed inversely; e.g., when KHPO4 was added at 10 mM (instead of 5 mM), KCl was reduced to 105 mM (instead of 110 mM). The proportion of free [Mg2+]i depends on the concentration of total (added) Mg2+ and Mg2+-complexing ligands (ATP and creatine phosphate; for concentrations, see below) as well as on the pH of the solution. Therefore, the free [Mg2+]i was calculated using TOT2FREE software (generously supplied by R. Thieleczek, Institut f. Physiologische Chemie Bochum, Germany), which is based on the mathematics described by Fabiato (1991), e.g., at [Mg2+]i = 2 mM, [ATP] = 1 mM, and [creatine phosphate] = 20 mM, pH 7.2, the free Mg2+ concentration of the solution was calculated to be 1.07 mM. An aliquot containing 3 × 106 cells was transferred to a cuvette, and fluorescence was measured in a Hitachi F-2000 fluorometer (Colora, Lorch, Germany) with wavelength settings alternating between 340 ± 2.5 and 380 ± 2.5 nm (excitation ratio mode) and 510 ± 5 nm (emission) at 37 °C in the presence of Fura2/free acid (1.5 μM) with automated gentle stirring. Reuptake of Ca2+ into stores was achieved by addition of ATP (1 mM), creatine phosphate (20 mM), and creatine kinase (20 units/ml). At the end of each experiment, the free Ca2+ concentration was calibrated by addition of Ca2+/ionomycin (1 mM/2 μM) and subsequently by addition of EGTA/Tris (4/40 mM). The free Ca2+ concentration was calculated according to the formulas given by Thomas and Delaville, 1991.

**HPLC Analysis of the Metabolism of Exogenous cADPr**—Permeabilized T cells (3 × 106 cells in 750 μl of intracellular buffer) were incubated with cADPr (final concentration, 7 μM) for 0, 1, and 3 min. cADPr and its metabolites were extracted from permeabilized cells in a fashion similar to that described recently (Guse et al., 1995). Briefly, to an aliquot of the cell suspension (250 μl) 0.75 ml of ice-cold HClO4 (3 M) was added, and the resulting suspension was vortex mixed and freeze dried twice in liquid N2. After addition of 5 ml H2O and ultrasonication for 10 s, precipitated protein was removed at 15,000 × g (10 min, 4 °C). The supernatant was adjusted to pH 8.0 by addition of KOH. KCIO4 precipitate was pelleted at 15,000 × g (10 min, 4 °C). The supernatant was lyophilized and stored at −70 °C. The samples were reconstituted in HPLC buffer A (see below) and filtered through disposable 0.45-μm filters with low dead volume directly before placing them into the HPLC autosampler. cADPr was analyzed on an automated Kontron Instruments (Neufahrn, Germany) HPLC system equipped with a strong anion-exchange HPLC column (MonoQ HR 5/5, Pharmacia Biotech Inc.) using a gradient from buffer A (1 mM Tris-HCl, pH 8.0) to 150 mM trifluoroacetic acid at a flow rate of 0.75 ml/min. The gradient was (in percent buffer B): 0 min, 0%; 5 min, 0%; 7.5 min, 15%; 8.5 min, 40%; 13 min, 50%; 15 min, 70%; 17.5 min, 80%; and 22.5 min, 100%. The wavelength of the detector was adjusted to 270 nm. Retention times for standard compounds were: adenosine, 1.82 min; NAD+ 2.56 ± 0.05 min (n = 4); cADPr, 6.92 ± 0.05 min (n = 6); and ADP-ribose, 10.13 ± 0.01 min (n = 6).

**Measurement of [Mg2+]i**—Jurkat T cells were centrifuged (Hereaus Varifuge 3.0R, 6 min, 1600 rpm, 18°C), and the cell pellet was resuspended in fresh, warm RPMI 1640 medium containing 10% newborn calf serum at a density of 107/ml. The cells were then incubated for 5 min at 37 °C. Subsequently, mag-Fura2/AM was added (final concentration, 4 μM), and the tube was protected from light and left for 20 min at 37 °C. The cell suspension was then diluted 5-fold and incubated for another 20 min at 37 °C. Finally, the cells were washed and centrifuged twice (Hereaus Varifuge 3.0R, 2 min, 3900 rpm, 18°C), and the cell pellet was resuspended in a buffer containing 140 mM NaCl, 3 mM KCl, 1 mM MgSO4, 1 mM CaCl2, 20 mM HEPES, 1 mM NaH2PO4, and 5.5 mM glucose, pH 7.4 (termed “extracellular buffer”) at a density of 2 × 106 cells/ml. [Mg2+]i of the cell suspension was transferred into a cuvette containing another 750 μl of the extracellular buffer. The cuvette was placed into a Hitachi F-2000 spectrofluorometer, and [Mg2+]i was measured at room temperature at alternating excitation wavelengths of 330 ± 5 and 370 ± 5 nm and at the corresponding alternating emission wavelengths of 491 ± 5 and 511 ± 5 nm. Ratios of the fluorescence intensities of the wavelength pairs were automatically calculated by the Hitachi software. Calculation was carried out at the end of each single measurement by addition of MgCl2 (35 mM final concentration) and the Mg2+-ionophore 4-bromo-A23187 (2.7 μM final concentration) to achieve the maximal ratio ([Rmax]). The minimal ratio ([Rmin]) was then determined by addition of Tris-EDTA (50 and 50 mM final concentrations). [Mg2+]i was calculated according to the formula [Mg2+]i = Kd [Q] / ([R] - [Rmin][Rmax] - [R]), where Kd = 1.5 mM, Q = minimal fluorescence divided by maxi-
Fig. 1. Effect of varying $[P_i]$ on cADPr- and Ins(1,4,5)P$_3$-induced Ca$^{2+}$ release from permeabilized Jurkat T lymphocytes. Jurkat T lymphocytes were permeabilized and [Ca$^{2+}$] was measured as detailed under "Experimental Procedures." The $[P_i]$ in the intracellular buffer was changed from the standard composition ($[P_i] = 5$ mM) to the values indicated. To maintain a similar osmolarity, the concentration of KCl was changed inversely. After charging of the Ca$^{2+}$ pools by addition of ATP (1 mM) and an ATP-regenerating system, cADPr (7 μM), Ins(1,4,5)P$_3$ (4 μM), and ionomycin (IM, 4 μM) were added where indicated. A–C, single representative Ca$^{2+}$ tracings at the $P_i$ concentrations indicated. D, data are presented as mean ± S.D. ($n = 4–8$). Shaded bars in D, physiological range of $[P_i]$ as determined in intact unstimulated T cells and T cells stimulated by OKT3 (10 μg/ml) for 30 min.

Maximum fluorescence ($F_{\text{max}}$/$F_{\text{min}}$) at the second wavelength (excitation, 370 nm; emission, 511 nm), and $R$ = ratio of fluorescence intensities of the wavelength pairs.

Determination of $[P_i]$ in Jurkat T Cells—Neutralized perchloric acid extracts of Jurkat cells were obtained as described in an earlier publication (Guse and Emmrich, 1991). Aliquots from 1 or 2 x 10$^6$ cells were used for colorimetric $P_i$ determination in 0.1 M HCl-washed, flat-bottom, 96-well plates. The sample or $P_i$ standard was added into the well in a
Regulation of cADPr-induced Ca^{2+} Release

**Table II**

| Composition | cADPr | Ins(1,4,5)P_3 |
|-------------|-------|--------------|
|             | Jurkat | HBP:ALL | Jurkat | HBP:ALL |
| % of total Ca^{2+} pool content | | | | |
| P_i, 0.5 mM | 10 ± 3 | 20 ± 6 | 59 ± 8 | 56 ± 5 |
| P_i, 5 mM | 26 ± 2 | 30 ± 3 | 51 ± 3 | 32 ± 13 |
| P_i, 10 mM | 40 ± 6 | 23 ± 3 | 42 ± 5 | 45 ± 1 |
| Mg^{2+}, 0 mM | 9 ± 3 | 23 ± 6 | 50 ± 6 | 52 ± 6 |
| Mg^{2+}, 1.07 mM | 26 ± 2 | 36 ± 5 | 51 ± 3 | 46 ± 8 |
| Mg^{2+}, 8.58 mM | 0 | 20 ± 4 | 63 | 47 ± 7 |

Effect of Varying [P_i] and [Mg^{2+}]_free on cADPr- and Ins(1,4,5)P_3-induced Ca^{2+} Release

The magnitude of cADPr-induced Ca^{2+} release was dependent on [P_i], showing only small release activity below 2 mM (about 10% of the total Ca^{2+} pool content; Fig. 1A), whereas at [P_i] of 4–10 mM considerably higher Ca^{2+} release was observed (about 25–38% of the total Ca^{2+} pool content; Fig. 1). A slightly negative relationship was observed for Ins(1,4,5)P_3-induced Ca^{2+} release, showing highest release activity at low [P_i] and reduced release activity above 4 mM [P_i] (Fig. 1). In HBP:ALL cells a comparable dependence of cADPr- and Ins(1,4,5)P_3-induced Ca^{2+} release was observed (Table II). However, high [P_i] (10 mM) did not result in a further enhancement of cADPr-induced Ca^{2+} release in these cells (Table II).

High [P_i] did not enhance the sensitivity of the Ca^{2+} release system to cADPr. Dose-response curves for cADPr recorded at different [P_i] did not result in a leftward shift of the curves at high [P_i]. Instead, remarkable differences in the amplitude of the Ca^{2+} release were observed (Fig. 2A).

When increasing [P_i], the absolute Ca^{2+} release by cADPr paralleled the increase of the total Ca^{2+} pool content, whereas for Ins(1,4,5)P_3 a saturation was observed at 5 mM [P_i] (Fig. 2B). In other words, high [P_i] selectively increased the pool size of the cADPr-sensitive Ca^{2+} pool. The underlying mechanism is demonstrated in Fig. 2C. The rate of Ca^{2+} resequestration after addition of cADPr was higher compared with Ins(1,4,5)P_3 and, most importantly, increased at higher [P_i]. In contrast, the rate of Ca^{2+} resequestration after addition of Ins(1,4,5)P_3 was generally lower compared with cADPr and did not increase significantly at higher [P_i] (Figs. 1, A–C, and 2C). It should be noted that the absolute Ca^{2+} concentrations observed after addition of cADPr at high versus low [P_i] (e.g. 348 nM [Ca^{2+}] at 10 mM P_i versus 180 nM [Ca^{2+}] at 0.5 mM P_i; see Fig. 1, A and C) only had a minor influence on Ca^{2+} reuptake by the cADPr-sensitive stores (data not shown).

To determine whether the observed effects were specific for P_i, either oxalate or AMP were used in substitution for P_i. Fig. 2B shows that using 0.5 mM P_i plus 4.5 or 9.5 mM oxalate or AMP resulted in reduced uptake of Ca^{2+} into the Ca^{2+} stores. When oxalate was used in substitution for P_i, the magnitudes of cADPr- and Ins(1,4,5)P_3-induced Ca^{2+} release were lower than with 5 mM P_i and, in the case of Ins(1,4,5)P_3, even lower than with 0.5 mM P_i (Fig. 2B). When AMP was used, neither cADPr nor Ins(1,4,5)P_3 could release any Ca^{2+} from the permeabilized cell suspension (Fig. 2B). Under such conditions, Ca^{2+} could only be released by the ionophore ionomycin, indicating that either the second messenger-sensitive Ca^{2+} pools were not charged or, less likely, high concentrations of both oxalate and AMP somehow inhibited second messenger-mediated Ca^{2+} release. Likewise, Ca^{2+} reuptake was lower or not detectable when oxalate or AMP was used in substitution for P_i (Fig. 2C).

Low and very high [Mg^{2+}]_free also resulted in reduced cADPr-induced Ca^{2+} release (Fig. 3), whereas Ins(1,4,5)P_3-induced Ca^{2+} release was not significantly influenced by different [Mg^{2+}]_free (Fig. 3). Similar results for [Mg^{2+}]_free were obtained using HPB:ALL cells (Table II), although as for [P_i], the effects of low and high [Mg^{2+}]_free were not as pronounced as in Jurkat cells (Table II).

HPLC Analysis of cADPr Metabolism in Permeabilized T Cells—The effects of varying [P_i] and [Mg^{2+}]_free on cADPr-induced Ca^{2+} release could be explained by: (i) a direct effect of the different ionic conditions on the putative cADPr-responsive Ca^{2+} release system, including the Ca^{2+} pool size; (ii) modulation of the proportion of free cADPr, e.g. cADPr that is not bound unspecifically to cellular proteins; or (iii) the differences...
incatabolism of cADPr by endogenous cADPr hydrolases under different ionic conditions. To investigate the latter, we incubated permeabilized cells under such different ionic conditions with cADPr and analyzed the metabolism of this exogenously added cADPr by HPLC. The incubation conditions were identical to the experiments carried out to study Ca²⁺ release fluorometrically, except that no Fura2/free acid, ATP, creatine phosphate, and creatine kinase were added. Indeed, increasing [Pi] resulted in a reduced catabolism of cADPr compared with low [Pi]. However, since the effects of cADPr on Ca²⁺ release are very rapid (see Fig. 2), and since even at low [Pi] after 1 min about 85% of the initial cADPr (about 5.95 mM) was still present, it is not very likely that the effects of [Pi] on cADPr-induced Ca²⁺ release were due to the enhanced catabolism at low [Pi].

Measurement of intracellular concentrations of Mg²⁺ and Pi—At least two mechanisms for the regulation of cADPr-induced Ca²⁺ release are possible: (i) the concentration of cADPr is regulated by stimulation of receptors in the plasma membrane, as is known for Ins(1,4,5)P₃, e.g. by the TCR/CD3 complex; or (ii) receptor-dependent changes of other compounds may regulate cADPr-induced Ca²⁺ release. Since we observed such dependencies on both [Pi] and [Mg²⁺], we measured the intracellular concentrations of these ions under resting and activated conditions.

As demonstrated for low [Pi], low [Mg²⁺]free also resulted in a somewhat enhanced but statistically nonsignificant catabolism of cADPr. Significant differences between normal (1.07 mM) and high (8.58 mM) [Mg²⁺]free were not observed.
Mg²⁺, mag-Fura2. In resting, mag-Fura2-loaded Jurkat T cells, [Mg²⁺]ᵢ amounted to 0.941 ± 0.348 mM (mean ± S.D., n = 5) as indicated by a shaded bar in Fig. 3D. Stimulation of the TCRζCD3 complex using the anti-CD3 monoclonal antibody OKT3 did not result in a significant change of [Mg²⁺]ᵢ (data not shown). Interestingly, other unphysiological activation procedures, such as addition of thapsigargin or cytosolic alkalinization using 4-aminopyridine (Guse et al., 1994), resulted in a more pronounced elevation of [Mg²⁺]ᵢ (data not shown).

The intracellular [Pi] was determined by a colorimetric assay for Pi in neutralized perchloric acid-quenched cell samples. As indicated in Fig. 4, there was a rapid increase in [Pi] in cells stimulated by OKT3, which remained elevated for at least 30 min. The [Pi] in quiescent cells was about 2.8 mM, whereas on addition of OKT3, values up to about 4.5 mM were observed.

**DISCUSSION**

In this report we demonstrate that: (i) the Ca²⁺ pool content of permeabilized T lymphocytes was significantly altered by changing the standard composition of the intracellular buffer used; (ii) [Pi] and [Mg²⁺] both can modulate cADPr-induced Ca²⁺ release in permeabilized T lymphocyte cell lines; (iii) the stimulatory effect of increasing [Pi] is likely due to an increased loading of the cADPr-sensitive intracellular Ca²⁺ pool; and (iv) [Pi], increased on stimulation of TCR-CD3, whereas [Mg²⁺] did not.

The dependence of cADPr-induced Ca²⁺ release on [Pi] has not been investigated so far. Not only did [Pi] influence cADPr-induced Ca²⁺ release, it also had a pronounced effect on Ca²⁺ pool loading in permeabilized T cell lines. Therefore, four dif-
Most importantly, when analyzing the [P], in intact Jurkat T cells, a TCR-CD3-mediated increase was observed. In unstimulated T cells [P], was about 2.8 mM, in other words, in a concentration range in which the Ca²⁺ release by cADPr was still small in permeabilized cells (Fig. 1D). Therefore, the TCR-CD3-mediated increase of [P] to about 4.5 mM, at which cADPr released considerably more Ca²⁺ in permeabilized cells (Fig. 1D), may be a switch to turn on cADPr-induced Ca²⁺ release inside the intact T cell. A prerequisite for such a model would be a constantly high intracellular concentration of cADPr. Although an exact quantification of endogenous cADPr is very difficult due to its chemical lability in extraction media (e.g. 3 M HClO₃) and the fact that various endogenous compounds show very similar behavior in anion-exchange as well as ion pair reversed phase HPLC, such a high endogenous concentration has been measured in Jurkat cells using two different HPLC systems, amounting to about 16 μM (Guse et al., 1995), assuming the intracellular volume to be 198 μl/10⁶ cells (Guse et al., 1993). However, it is not clear: (i) which proportion of this total amount of cADPr is freely available in the cytosol, in other words, how much cADPr is bound unspecifically to proteins or structures other than the cADPr-dependent Ca²⁺ release system; and (ii) whether the observed increase of [P] could have significantly increased the amount of free cADPr.

A modulatory effect of [Mg²⁺] on cADPr-induced Ca²⁺ release has already been described for sea urchin egg homogenates (Graeff et al., 1995). Similar to our results, Graeff et al. (1995) described an inhibitory effect of high [Mg²⁺] (>6 mM) on cADPr-induced Ca²⁺ release, but not on Ins(1,4,5)P³-induced Ca²⁺ release. In sea urchin egg homogenates 1 mM Mg²⁺ was the optimal concentration for cADPr-induced Ca²⁺ release (Graeff et al., 1995); this result and the fact that higher [Mg²⁺] acted in an inhibitory way was confirmed in our permeabilized Jurkat T cells. Since [Mg²⁺] at >5 mM is a known blocker of the RyR (Meissner, 1994) our data from T lymphocytes add another piece of evidence that RyR is one of the central players in cADPr-induced Ca²⁺ release. However, in contrast to [P], which increased in response to stimulation of TCR-CD3, no changes in [Mg²⁺], were observed, indicating that Mg²⁺-dependent modulation of cADPr-induced Ca²⁺ release does not play a major role, if any, in vivo.

In conclusion, our results indicate that cADPr-induced Ca²⁺ release may be regulated by [P] in Jurkat T lymphocytes in vivo, since: (i) the filling state of the cADPr-sensitive Ca²⁺ store and, thereby, cADPr-induced Ca²⁺ release in permeabilized T cells was modulated by [P]; and (ii) [P] increased in intact Jurkat T cells stimulated via the TCR-CD3 complex. Such a model would also explain our recent finding of high endogenous concentrations of cADPr, which remained unchanged during stimulation of the TCR-CD3 complex (Guse et al., 1995).

Acknowledgments—We are grateful to Karin Müller and Charlotte Armah for excellent technical assistance.

REFERENCES
Ashamu, G. A., Galione, A., and Potter, B. V. L. (1995) J. Chem. Soc. Chem. Commun. 1599–1600. Corren (1995) J. Chem. Soc. Chem. Commun. 1929. Berridge, M. J. (1993) J. Biol. Chem. 268, 17917–17922. Chini, E. N., Beers, K. W., Chini, C. C. S., and Dousa, T. P. (1995) Am. J. Physiol. 269, C1042–C1047. Dargie, P. J., Agre, M. C., and Lee, H. C. (1990) Cell Regul. 1, 279–290. Fabiato, A. (1991) in Cellular Calcium, A Practical Approach (McCormack, J. G., and Cobbold, P. H., eds) pp. 159–176, Oxford University Press, New York. Galione, A., and White, A. (1994) Trends Cell Biol. 4, 431–436. Galione, A., Lee, H. C., and Busa, W. A. (1991) Science 253, 1143–1146.

C. P. da Silva and A. H. Guse, unpublished results.

FIG. 4. Determination of [P], in intact Jurkat T lymphocytes. [P], was determined in intact Jurkat T cells as explained under “Experimental Procedures.” The anti-CD3 monoclonal antibody OKT3 (10 μg/ml) was added at time point 0 min. Data are presented as mean ± S.D. (n = 3–4).
Regulation of cADPr-induced Ca\textsuperscript{2+} Release

Galione, A., White, A., Willmott, N., Turner, M., Potter, B. V. L., and Watson, S. P. (1993) *Nature* **365**, 456–459

Gerasimenko, O. V., Gerasimenko, J. V., Tepikin, A. V., and Petersen, O. H. (1995) *Cell* **80**, 439–444

Graeff, R. M., Podein, R. J., Aarhus, R., and Lee, H. C. (1995) *Biochem. Biophys. Res. Commun.* **206**, 786–791

Guse, A. H., and Emmrich, F. (1991) *J. Biol. Chem.* **266**, 24498–24502

Guse, A. H., Roth, E., and Emmrich, F. (1992) *Biochem. J.* **286**, 489–495

Guse, A. H., Greiner, E., Emmrich, F., and Brand, K. (1993) *J. Biol. Chem.* **268**, 7129–7133

Guse, A. H., Roth, E., and Emmrich, F. (1994) *Biochem. J.* **301**, 83–88

Guse, A. H., da Silva, C. P., Emmrich, F., Ashamu, G. A., Potter, B. V. L., and Mayr, G. W. (1995) *J. Immunol.* **155**, 3353–3359

Hakamata, Y., Nishimura, S., Nakai, J., Nakashima, Y., Ria, T., and Imoto, K. (1994) *FEBS Lett.* **352**, 206–210

Kuenen, J. F., and Makhlof, G. M. (1995) *J. Biol. Chem.* **270**, 25488–25494

Lee, H. C. (1993) *J. Biol. Chem.* **268**, 293–299

Lee, H. C. (1994) *Cell. Signalling* **6**, 591–600

Lee, H. C., and Aarhus, R. (1993) *Cell Regul.* **2**, 203–209

Lee, H. C., Aarhus, R., Graeff, R., Gurnack, M. E., and Walseth, T. F. (1994) *Nature* **370**, 307–309

Meissner, G. (1994) *Annu. Rev. Physiol.* **56**, 485–508

Takahashi, K., Kukimoto, I., Tokita, K.-I., Inaguma, K., Inoue, S.-I., Kontani, K., Hoshino, S.-I., Nishina, H., Kanaho, Y., and Katada, T. (1995) *FEBS Lett.* **371**, 204–208

Thomas, A. P., and Delaville, F. (1991) in *Cellular Calcium, A Practical Approach* (McCormack, J. G., and Cobbold, P. H., eds) pp. 1–54, Oxford University Press, New York

Walseth, T. F., and Lee, H. C. (1993) *Biochim. Biophys. Acta* **1178**, 235–242

Walseth, T., Aarhus, R., Zeleznikar, R. J., Jr., and Lee, H. C. (1991) *Biochim. Biophys. Acta* **1094**, 113–120

Walseth, T. F., Aarhus, R., Keer, J. A., and Lee, H. C. (1993) *J. Biol. Chem.* **268**, 26686–26691
Regulation of cADP-ribose-induced Ca\(^{2+}\) Release by Mg\(^{2+}\) and Inorganic Phosphate
Andreas H. Guse, Cristina P. da Silva, Karin Weber, Gloria A. Ashamu, Barry V. L. Potter
and Georg W. Mayr

*J. Biol. Chem.* 1996, 271:23946-23953.
doi: 10.1074/jbc.271.39.23946

Access the most updated version of this article at [http://www.jbc.org/content/271/39/23946](http://www.jbc.org/content/271/39/23946)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 26 references, 10 of which can be accessed free at
[http://www.jbc.org/content/271/39/23946.full.html#ref-list-1](http://www.jbc.org/content/271/39/23946.full.html#ref-list-1)