Ca\textsuperscript{2+} Entry Induced by Cyclic ADP-ribose in Intact T-Lymphocytes*

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Cyclic ADP-ribose (cADPR) is a potent Ca\textsuperscript{2+}-mobilizing natural compound (Lee, H. C., Walseth, T. F., Bratt, G. T., Hayes, R. N., and Clapper, D. L. (1989) J. Biol. Chem. 264, 1608–1615) which has been shown to release Ca\textsuperscript{2+} from an intracellular store of permeabilized T-lymphocytes (Guse, A. H., Silva, C. P., Emmrich, F., Ashamu, G., Potter, B. V. L., and Mayr, G. W. (1995) J. Immunol. 155, 3353–3359). Microinjection of cADPR into intact single T lymphocytes dose dependently induced repetitive but irregular Ca\textsuperscript{2+} spikes which were almost completely dependent on the presence of extracellular Ca\textsuperscript{2+}. The Ca\textsuperscript{2+} spikes induced by cADPR could be blocked either by co-injection of cADPr with the specific antagonist 8-NH\textsubscript{2}-cADPr, by omission of Ca\textsuperscript{2+} from the medium, or by superfusion of the cells with Zn\textsuperscript{2+} or SK-F 96365. Ratiometric digital Ca\textsuperscript{2+} imaging revealed that single Ca\textsuperscript{2+} spikes were initiated at several sites (“hot spots”) close to the plasma membrane. These hot spots then rapidly formed a circular zone of high Ca\textsuperscript{2+} concentration below the plasma membrane which subsequently propagated like a closing optical diaphragm into the center of the cell. Taken together these data indicate a role for cADPr in Ca\textsuperscript{2+} entry in T-lymphocytes.

Intracellular Ca\textsuperscript{2+} signaling is one of the major events transducing extracellular signals into many different types of living cells. In Jurkat T-lymphocytes it is well accepted that d-myoinositol 1,4,5-trisphosphate (IP\textsubscript{3})\textsuperscript{3+} releases Ca\textsuperscript{2+} from an intracellular store located in the endoplasmic reticulum via its specific receptor (1–3). In addition, in T-lymphocytes a sustained long-lasting Ca\textsuperscript{2+} entry can be observed in response to stimulation of the T cell receptor-CD3 complex (4–6). This Ca\textsuperscript{2+} entry is necessary for clonal expansion of T cells and therefore essential for a functional immune response. One of the basic mechanisms underlying Ca\textsuperscript{2+} entry in electrically non-excitable cells appears to be the “capacitative” mechanism (reviewed in Refs. 7 and 8). The central idea of the capacitative mecha-

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The abbreviations used are: IP\textsubscript{3}, d-myoinositol 1,4,5-trisphosphate; cADPR, cyclic ADP-ribose; [Ca\textsuperscript{2+}], free intracellular Ca\textsuperscript{2+} concentration.

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T-lymphocytes was analyzed with a digital ratiometric imaging station (PhotoMed GmbH/Photon Technology, Wedel, Germany). The coated coverslips with cells were mounted on the stage of an inverted Axiovert 100 fluorescence microscope (Zeiss, Oberkochen, Germany). The excitation light source beam was split using an optical chopper, then passed through either a 340- or 380-nm optical filter, and guided into the microscope via fiber optics. The fluorescence intensity was filtered at 510 nm and then monitored using a CCD camera at a resolution of 525 × 487 pixels (type C2400–77, Hamamatsu, Garching, Germany). The data sampling rate usually was 1 ratio/5 s, in some experiments 1 ratio/s. Re-analysis of Ca$^{2+}$ image data was carried out using the so-called region-of-interest function of the ImageMaster software (PhotoMed GmbH/Photon Technology, Wedel, Germany). Region of images were set either to cover the whole cell or subregions of the cell. The numerical median ratios and the corresponding free Ca$^{2+}$ concentrations were calculated by the software using external calibration.

Microinjection—Microinjections were done with an Eppendorf system (transjector type 5246, micromanipulator type 5171; Eppendorf-Netheler-Hinz, Hamburg, Germany) using Femtotips II as pipettes. The compounds to be microinjected were diluted to their final concentrations in intracellular buffer (20 mM Hepes, 110 mM KCl, 2 mM MgCl$_2$, 5 mM KH$_2$PO$_4$, 10 mM NaCl, pH 7.2) and filtered (0.2 μm) directly before filling into the Femtotips. Injections were made using the semiautomatic mode of the Eppendorf system at a pipette angle of 45° and the following instrumental settings: injection pressure 80 hPa, compensatory pressure 60 hPa, injection time 0.5 to 1 s, and velocity of the pipette 700 μm/s. Under such conditions the injection volume was 1–1.5% of the cell volume as measured by microinjection of a fluorescent compound (Fura2-free acid) and subsequent determination of its quantity in a spectrofluorimeter at 360 nm (excitation) and 500 nm (emission).

**RESULTS**

Ca$^{2+}$ Signaling Induced by Microinjection of cADPr—In response to microinjection of cADPr, the free intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) increased in an oscillatory manner; either two to several irregular Ca$^{2+}$ spikes or a combination of spikes and sustained elevated Ca$^{2+}$ levels were observed (Fig. 1, B, C, and D). The effect of cADPr was dose-dependent showing no increases in [Ca$^{2+}$] when intracellular buffer was injected, and increasing responses when cADPr in the pipette was elevated stepwise to 100 μM (Fig. 1). During every injection about 1–1.5% of the cell volume was injected. We assumed a fast dilution of cADPr in the cell being comparable to the diffusion reported for IP$_3$ in cytosolic extracts of *Xenopus laevis* (23). Considering the delay between injection and onset of the signal (Fig. 1, B, C, and D), a dilution factor of at least 50 must be assumed when estimating the effective intracellular concentration. Thus, at 1 μM cADPr in the pipette, which was the threshold concentration (Fig. 1, B and F), the intracellular concentration of cADPr should be about 20 nM. Ca$^{2+}$ spikes with amplitudes up to 1.5 μM [Ca$^{2+}$], were observed at a pipette concentration of 10 μM cADPr, amounting to an effective concentration of about 200 nM cADPr in the cell (Fig. 1, C and G). At 100 μM cADPr in the pipette (about 2 μM cADPr in the cell) usually 1 or 2 fast and large spikes were observed followed by a decline to an elevated level which was maintained for a relatively long period of time (Fig. 1, D and H). A pipette...
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**Fig. 2.** Specific antagonists inhibit both cADPr- and IP$_3$-induced Ca$^{2+}$ signaling. [Ca$^{2+}$], was measured in Fura2-loaded Jurkat T-lymphocytes using the digital ratiometric Ca$^{2+}$ imaging system. The cells were then microinjected with ligands as described under “Exper-
imental Procedures.” Data are presented as typical tracings from 1 individual cell. During reanalysis of the image data, regions of interest were set to cover the whole cell; data obtained from these regions of interest are plotted against time. Shown are (n = number of experi-
ments): A, microinjection of cADPr (10 μM) in the presence of extracellular Ca$^{2+}$ (n = 30); B, co-injection of cADPr (10 μM) with a 10-fold excess of the antagonist 8-NH$_2$-cADPr (n = 5); C, microinjection of IP$_3$ (4 μM) in the presence of 1 mM extracellular Ca$^{2+}$ (n = 15); D, co-
injection of IP$_3$ (4 μM) with a 10-fold excess of the partial antagonist d-myo-inositol 1,4,6-trisphosphorothioate in the presence of 1 mM extracellular Ca$^{2+}$ (n = 7). Arrows mark the time point of microinjection.

**Concentration of 100 μM cADPr as compared with 10 μM re-
sulted in a considerably faster onset of the Ca$^{2+}$ signal (com-
pare Fig. 1, G and H1); however, later after microinjection, e.g. after 600 s, high Ca$^{2+}$ spikes were still observed at 10 μM, but not at 100 μM cADPr in the pipette (Fig. 1, C and D). At a pipette concentration of 1 mM cADPr (data not shown), the Ca$^{2+}$ signals were very similar to the ones obtained at 100 μM indicating saturation of the dose-response relationship.**

Ca$^{2+}$ Signaling Induced by cADPr and IP$_3$ Can Be Blocked Specifically—The effect of cADPr was specific, because it could be blocked by co-injection of a 10-fold excess of the specific antagonist 8-NH$_2$-cADPr (Refs. 24 and 25; Fig. 2, A and B). For comparison, IP$_3$ was also microinjected. The resulting Ca$^{2+}$ spike pattern was somewhat more regular and usually not as long-lasting as compared with cADPr (Fig. 2C). Specificity was demonstrated by inhibition of the effect by co-injection with a 10-fold excess of the partial antagonist d-myo-inositol 1,4,6-
phosphorothioate (Fig. 2D).

Ca$^{2+}$ Entry Induced by cADPr—Since in most of the experiments, microinjection of cADPr did not result in a single Ca$^{2+}$ spike, but in longer lasting trains of spikes (Figs. 1, 2A, and 3A), the involvement of Ca$^{2+}$ entry in these sustained signals was investigated. Such cADPr-induced Ca$^{2+}$ signals were nearly completely abolished when the cells were superfused with extracellular buffer containing no Ca$^{2+}$ (Fig. 3B). Moreover, when cells superfused with 1 mM Ca$^{2+}$ and microinjected with 10 μM cADPr were challenged with Zn$^{2+}$ (1 mM) after the first Ca$^{2+}$ spike, further spikes were completely inhibited (Fig. 3C). The effect of Zn$^{2+}$ could be washed out (data not shown), indicating that Zn$^{2+}$ acted by blocking Ca$^{2+}$ entry at the plasma membrane level. Further evidence for Ca$^{2+}$ entry in response to microinjected cADPr was obtained by blocking the Ca$^{2+}$ signals with the drug SK-F 96365 (Ref. 26; Fig. 3D). The IP$_3$-induced Ca$^{2+}$ spikes also were largely dependent on the presence of external Ca$^{2+}$ (data not shown).

Spatial Development of Ca$^{2+}$ Spikes Induced by cADPr—The spatial development of a single Ca$^{2+}$ spike in response to microinjection of cADPr is characterized by (i) a rapid increase of [Ca$^{2+}$], to a slightly elevated level within 5 to 10 s throughout the cell (Fig. 4, upper panel, image 1–3), (ii) a slowly generated wave of high [Ca$^{2+}$], initiating from distinct sites (“hot spots”) which then formed a circular zone close to the plasma membrane (Fig. 4, upper panel, images 3 and 4) and propagating like a closing optical diaphragm into the center of the cell (Fig.
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DISCUSSION

The mechanism of Ca²⁺ entry in T-lymphocytes as well as in other electrically non-excitable cells is not well understood. The trigger to switch on the capacitative Ca²⁺ entry has been reported to be likely the depletion of the IP₃-sensitive intracellular Ca²⁺ pool in T cells (4–6). The nature of the subsequent signal to stimulate Ca²⁺ entry itself is discussed to be either the soluble calcium-influx factor CIF, protein-protein interaction between the IP₃-receptor and the Ca²⁺ entry channel(s), or a G-protein-mediated process (reviewed in Refs. 7 and 8). We confirmed the potential role of IP₃ in this process by showing that microinjection of this second messenger induced a train of Ca²⁺ signals which depended on extracellular Ca²⁺ (Fig. 2C).

As a novel finding we now add cADPr as a compound activating Ca²⁺ entry in response to microinjection. During our experiments in nominally Ca²⁺-free medium only very small Ca²⁺ signals were observed regardless whether cADPr or IP₃ was microinjected. However, these Ca²⁺ signals were more pronounced at the site of microinjection, as compared with the signals averaged from the whole cell. Our data indicate that both ligands induced Ca²⁺ release from their distinct target Ca²⁺ stores, as has been clearly demonstrated for IP₃ and cADPr in permeabilized cells (16) and in vesicular membrane subfractions separated by density gradient centrifugation (17).

The relatively small and local Ca²⁺ release induced by both cADPr and IP₃ was microinjected. However, these Ca²⁺ signals were more pronounced at the site of microinjection, as compared with the signals averaged from the whole cell. Our data indicate that both ligands induced Ca²⁺ release from their distinct target Ca²⁺ stores, as has been clearly demonstrated for IP₃ and cADPr in permeabilized cells (16) and in vesicular membrane subfractions separated by density gradient centrifugation (17).

The relatively small and local Ca²⁺ release induced by both cADPr and IP₃ was also explained by the dilution of cADPr and IP₃ within the cell after microinjection (pipette concentrations 10 and 4 μM; assumed intracellular effective concentrations about 200 and 80 nM). In the presence of extracellular Ca²⁺, the small Ca²⁺ release then was followed for both ligands, IP₃ and cADPr, by a secondary Ca²⁺ entry of much higher magnitude. This interpretation is in agreement with recent reports show-

FIG. 4. Ca²⁺ wave induced by microinjection of cADPr. A single Fura2-loaded Jurkat T cell was microinjected with cADPr (10 μM) in the presence of 1 mM extracellular Ca²⁺ resulting in a train of Ca²⁺ spikes out of which the first spike is shown in detail. During reanalysis of the image data, three different regions of interest (ROI) (A-C) have been located inside the cell to display [Ca²⁺]i between the plasma membrane (ROI A) and the nucleus (ROI C). Pseudocolor ratiometric images taken at individual time points as indicated by the numbered arrows are displayed in an upper panel (showing the development of the Ca²⁺ signal) and in a lower panel (showing the decrease of the Ca²⁺ signal). Note that the Ca²⁺ signal started in a circular manner (image 2) propagating like a closing optical diaphragm to a very high intracellular concentration except a region close to the plasma membrane (image 6). The sampling rate of each ratio image was 5 s.

4, upper panel, images 4–6). At the top of the spike very high [Ca²⁺], were observed in the central part of the cell where the nucleus is located (Fig. 4, upper panel, image 6). At a slightly slower velocity, Ca²⁺ was removed from the central part of the cell reaching a level that was slightly elevated (Fig. 4, lower panel, images 7–9). This level in the central part of the cell was then maintained for some 100 s, while Ca²⁺ oscillations were observed during this period of time in the subplasmalemmal cytoplasm (Fig. 4, lower panel, images 10 and 11). Finally, the cytoplasm also nearly reached basal Ca²⁺ levels (Fig. 4, lower panel, image 12), before the next Ca²⁺ spike appeared (data not shown). Such cADPr-induced Ca²⁺ waves propagating at high [Ca²⁺] throughout the whole cell including the large nucleus of T-lymphocytes were not seen in all cases; in a number of cells Ca²⁺ signals of high amplitude developed mainly in the cytoplasm reaching at the top of the spike a status comparable to image 4 in Fig. 4. Then [Ca²⁺], in this circular zone returned to basal concentrations. However, irrespective of whether cADPr might have been injected into the cytoplasm or in the nucleus, Ca²⁺ waves were always initiated in the subplasmalemmal space pointing toward a crucial role of Ca²⁺ influx in both cases.
ing fast abrogation of Ca\(^{2+}\) spikes induced by extracellular stimuli by omission of extracellular Ca\(^{2+}\) in individual T cells (27–29). Also, the type of Ca\(^{2+}\) wave (Fig. 4) argues for Ca\(^{2+}\) influx since the wave started in a circular manner close to the plasma membrane all around the cell and propagated like a closing optical diaphragm into the center of the cell.

The mechanism by which cADPr stimulated Ca\(^{2+}\) entry is not yet clear. However, at least three models are possible: first, microinjected cADPr released Ca\(^{2+}\) from its target Ca\(^{2+}\) store, namely membrane vesicles which are located close to surface receptor-capped structures (17). This primary event then led to activation of a Ca\(^{2+}\) entry mechanism similar to the capacitative mechanism. The fact that SK-F 96365 inhibited both cADPr-and IP\(_3\)-mediated Ca\(^{2+}\) entry2 may argue for this possibility. Second, cADPr may have opened directly Ca\(^{2+}\) channels in the plasma membrane. This possibility appears to be less likely, since no such action of cADPr has ever been described. Furthermore, this hypothetical cADPr-responsive Ca\(^{2+}\) channel then must have the same pharmacological properties, e.g. inhibition by 8-NH\(_2\)-cADPr, which also is not very likely. As a third possibility, cADPr may have activated IP\(_3\) receptors. However, all experiments carried out in permeabilized T cells suggest that cADPr acts completely independent of IP\(_3\) receptors (16).

In conclusion, we add as a novel observation that Ca\(^{2+}\) release by cADPr can induce Ca\(^{2+}\) entry by a mechanism obviously being analogous to the capacitative mechanism induced by microinjection of IP\(_3\). Thus, cADPr, an intracellular ligand which is modulated in its activity indirectly by the T cell receptor-CD3 complex (30), may represent a new intracellular tool to control Ca\(^{2+}\) entry in T cells. The recent discovery and molecular cloning of a whole family of human homologues of the Drosophila photoreceptor trp-channel points toward diversity in the structural basis for Ca\(^{2+}\) entry (31) and opens up the possibility of identifying the Ca\(^{2+}\) entry channel involved in cADPr-mediated Ca\(^{2+}\) signaling in the future.

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