# Cyclic ADP-ribose and inositol 1,4,5-trisphosphate mobilizes $Ca^{2+}$ from distinct intracellular pools in permeabilized lacrimal acinar cells

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**Abstract In permeabilized lacrimal acinar cells, cyclic ADP-ribose (cADP-ribose) and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) release $Ca^{2+}$ in a dose dependent manner from distinct thapsigargin-sensitive $Ca^{2+}$ pools. Ryanodine specifically blocks the $Ca^{2+}$ response to cADP-ribose, whereas heparin strongly reduces the response to $Ins(1,4,5)P_3$ application. GTP causes a rapid $Ca^{2+}$ release by a ryanodine- and heparin-insensitive mechanism and potentiates $Ins(1,4,5)P_3$ but not cADP-ribose evoked $Ca^{2+}$ release. It is estimated that cADP-ribose can release 16 μmol $Ca^{2+}$/l cells, whereas $Ins(1,4,5)P_3$ can mobilize 55 μmol $Ca^{2+}$/l cells. The results suggest that cADP-ribose and $Ins(1,4,5)P_3$ release $Ca^{2+}$ from distinct internal stores and that a third $Ca^{2+}$ pool exists which can selectively interact with the $Ins(1,4,5)P_3$-sensitive $Ca^{2+}$ store by a GTP-mediated process.**

*Key words:* Cyclic ADP-ribose; Inositol trisphosphate; Ryanodine; Calcium; Lacrimal; Acinar cell

## 1. Introduction

Cyclic ADP-ribose (cADP-ribose) is a newly discovered $Ca^{2+}$ mobilizing nucleotide of $NAD^+$ that releases $Ca^{2+}$ through a mechanism distinct from that of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) [1–4]. cADP-ribose has been shown to be an endogenous regulator of the non-skeletal muscle type ryanodine receptor [5]. Recently, it was found that α-adrenergic receptor stimulation of lacrimal acinar cells induces $Ca^{2+}$ release without inositol phosphate synthesis [6,7], and it was suggested that cADP-ribose is involved in this $Ca^{2+}$ release process [6]. In exocrine acinar cells there is evidence for the presence of ryanodine- and $Ca^{2+}$-sensitive $Ca^{2+}$ release channels [8,9], that are thought to mediate the $Ca^{2+}$ response to cADP-ribose [6,10]. In lacrimal acinar cells, acetylcholine (ACh) promotes a rapid production of $Ins(1,4,5)P_3$ and a maximum level was attained within a few seconds after receptor activation [11–13]. Using permeabilized lacrimal acinar cells, we have investigated the heterogeneity of intracellular $Ca^{2+}$ stores and their sensitivity to the $Ca^{2+}$ mobilizing messengers cADP-ribose and $Ins(1,4,5)P_3$.

## 2. Materials and methods

### 2.1. Chemicals

cADP-ribose was obtained from Amersham (UK). $Ins(1,4,5)P_3$, thapsigargin, and ryanodine were from Alomone Labs (Israel). All other chemicals were obtained from Sigma (MO, USA).

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### 2.2. Isolation and preparation of permeabilized cells

Rat lacrimal acinar cells were isolated and permeabilized, as described previously [6].

### 2.3. Measurements of the free $Ca^{2+}$ concentration

Measurements of $[Ca^{2+}]$ were performed at 37°C in suspensions of permeabilized lacrimal acinar cells in a Perkin-Elmer LS5B fluorescence spectrophotometer as described priviously [6]. The permeabilized cells were resuspended in an intracellular-like solution containing 20 mM NaCl, 100 mM KCl, 1 mM $MgSO_4$, 20 mM HEPES, 1 mM ATP, 10 U/ml creatine phosphokinase, 10 mM phosphocreatine, 10 μg/ml oligomycin, and 1 μM fura-2 pentapotassium salt (pH 7.2). All data are given as mean values ± S.D. and $n$ is the number of experiments. Statistical significance was judged by Student's $t$-test for unpaired observations.

## 3. Results and discussion

The function of cADP-ribose and $Ins(1,4,5)P_3$ was studied in saponin-permeabilized acinar cells isolated from rat lacrimal glands. Following $Ca^{2+}$ accumulation in the cells and baseline stabilization, $[Ca^{2+}]$ amounted to 194 ± 64 nM ($n$ = 58), and stimulation with cADP-ribose (5 μM) evoked a rapid $Ca^{2+}$ release with the peak value lasting <5 s. This was followed by a pronounced re-uptake of $Ca^{2+}$ into the organelles (Fig. 1A). The $Ca^{2+}$ response following $Ins(1,4,5)P_3$ stimulation was rapid but more protracted and was also followed by a $Ca^{2+}$ re-uptake (Fig. 1A). cADP-ribose and $Ins(1,4,5)P_3$ released $Ca^{2+}$ in a dose dependent manner (Fig. 1B), with maximal concentrations of $Ins(1,4,5)P_3$ attained at 5 μM, causing a rise in $[Ca^{2+}]$ of 280 ± 35 nM ($n$ = 6), and also at 5 μM for cADP-ribose, causing an increase in $[Ca^{2+}]$ of 86 ± 18 nM ($n$ = 3). Half maximal effect for cADP-ribose was seen at ≈3 μM and at ≈2 μM for $Ins(1,4,5)P_3$ (Fig. 1B).

In order to obtain a quantitative measure of the amount of $Ca^{2+}$ released following stimulation, a pulse of 750 nM $CaCl_2$ (1.88 nmol $Ca^{2+}$) was added. This increased $[Ca^{2+}]$ by 234 ± 31 nM ($n$ = 3) and was followed by a fast uptake of the ion (Fig. 1C). Subsequent addition of 5 μM $Ins(1,4,5)P_3$ induced an increase in $[Ca^{2+}]$ amounting to 293 ± 26 nM (Fig. 1C), which is of the same magnitude as the maximal $Ca^{2+}$ release found in Fig. 1B (280 ± 35 nM). In these experiments the cell suspensions had a cytocrit of 1.7% (in 2.5 ml), and from the data it can be calculated that 55 μmol $Ca^{2+}$/l cells is released in response to maximal $Ins(1,4,5)P_3$ stimulation. Maximal cADP-ribose stimulation on average elevated $[Ca^{2+}]$ by 86 ± 18 nM ($n$ = 3), and thus released 16 μmol $Ca^{2+}$/l cells.

From these data it is possible to calculate the ratio of bound $Ca^{2+}$ to free $Ca^{2+}$ in the cytoplasm in intact cells. The maximal rise in $[Ca^{2+}]_i$ obtained in intact cells following ACh stimulation, resulting in production of $Ins(1,4,5)P_3$, amounted on average to 645 nM [6]. Assuming that maximal ACh stimulation causes release of $Ca^{2+}$ from internal pools to the same extent


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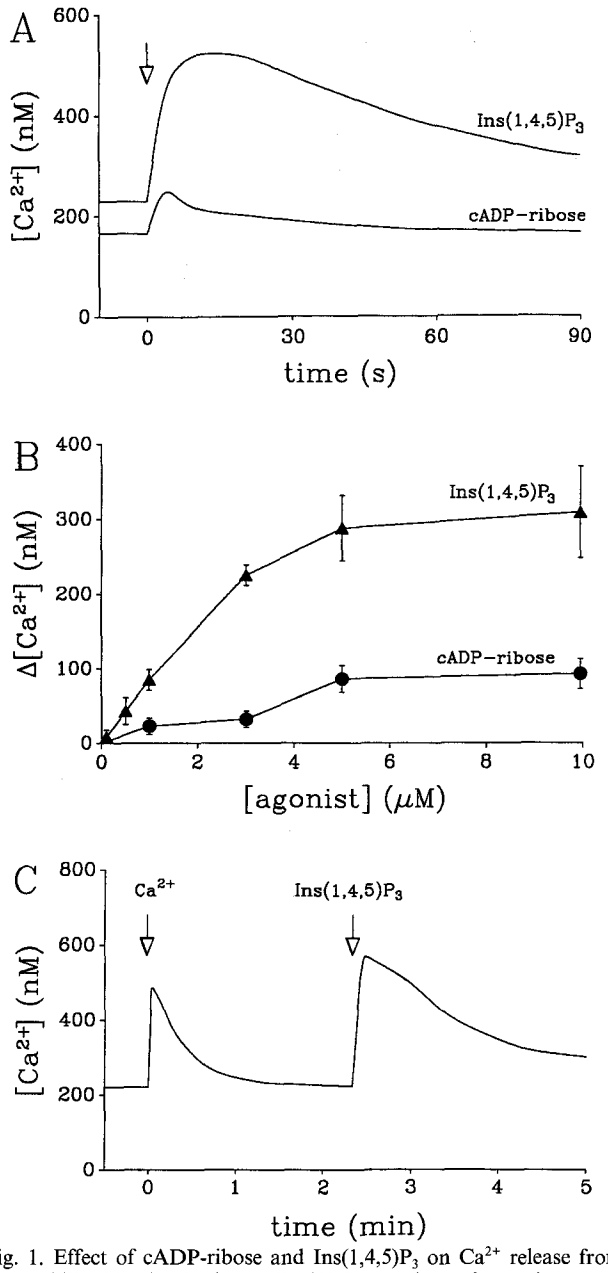


Fig. 1. Effect of cADP-ribose and Ins(1,4,5)$P_3$ on $Ca^{2+}$ release from permeabilized lacrimal acinar cells. (A) Suspensions of saponin-permeabilized cells were stimulated with 5 $\mu$M cADP-ribose or 5 $\mu$M Ins(1,4,5)$P_3$ at the time indicated by the arrow. Each trace is a representative of 4 experiments. (B) Dose–response relationship for cADP-ribose and Ins(1,4,5)$P_3$. The changes in the free $Ca^{2+}$ concentration ($\Delta[Ca^{2+}]$) were calculated by substrating the unstimulated $[Ca^{2+}]$ from the peak $[Ca^{2+}]$ measured 2–4 s after stimulation with cADP-ribose and 6–8 s after Ins(1,4,5)$P_3$ stimulation. The values are averages of 3–6 experiments. (C) $CaCl_2$ (750 nM) and Ins(1,4,5)$P_3$ (5 $\mu$M) was added as indicated by the arrows. The trace is a representative of 3 experiments.

as the Ins(1,4,5)$P_3$ evoked $Ca^{2+}$ release in Fig. 1B, it is calculated that ≈85 times more $Ca^{2+}$ has to be released in order to cause the observed increase in $[Ca^{2+}]_i$ of 645 nM. This finding shows that the ratio of bound $Ca^{2+}$ to free $Ca^{2+}$ in the cytoplasm is 85, revealing a high cellular $Ca^{2+}$ buffer capacity in this cell type. This number, however, represents a maximum value, since maximal ACh stimulation could conceivably cause an Ins(1,4,5)$P_3$ release to the cell cytoplasm that is smaller than the 5 $\mu$M Ins(1,4,5)$P_3$ used to obtain a maximal response in permeabilized cells. However, a cellular $Ca^{2+}$ buffer capacity of this magnitude implies that $Ca^{2+}$ gradients, as a result of local $Ca^{2+}$ release processes, should be standing for a considerable period of time since both the low diffusion coefficient of $2.2 \cdot 10^{-6}$ $cm^2/s$ as well as the immobilization of diffusible $Ca^{2+}$ ions will restrict the dissipation of such gradients [14]. Such effects have been observed in the luminal area in parotid acinar cells [12].

In the following experiment we investigated to what extent the intracellular $Ca^{2+}$ pools are physically and functionally separated with respect to their responsiveness to the two types of cellular messengers. Ryanodine (100 $\mu$M) completely inhibited the cADP-ribose (5 $\mu$M) induced $Ca^{2+}$ release while subsequent stimulation with Ins(1,4,5)$P_3$ (5 $\mu$M) was unaffected (Fig. 2A). Heparin (200 $\mu$g/ml), a competitive inhibitor of the Ins(1,4,5)$P_3$ receptor [15], dramatically reduced the Ins(1,4,5)$P_3$ induced $Ca^{2+}$ release but had no effect on $Ca^{2+}$ release following subsequent addition of cADP-ribose (5 $\mu$M) (Fig. 2B). In heparin treated cells the $Ca^{2+}$ response to 10 $\mu$M Ins(1,4,5)$P_3$ was reduced to 65 ± 12 nM ($n$ = 4). Furthermore, heparin reduced the unstimulated $[Ca^{2+}]$ by 20–40 nM (Fig. 2B). This is in accordance with a previous study [16] revealing a significant $Ca^{2+}$ leak through the Ins(1,4,5)$P_3$ receptors.

When thapsigargin (0.5 $\mu$M) was added to permeabilized lacrimal acinar cells, a slow increase in $[Ca^{2+}]$ was observed, reaching a maximum value after 10 min (Fig. 3A). Subsequent stimulation with 5 $\mu$M cADP-ribose and 5 $\mu$M Ins(1,4,5)$P_3$ did not cause further $Ca^{2+}$ release (Fig. 3A). However, if 5 $\mu$M

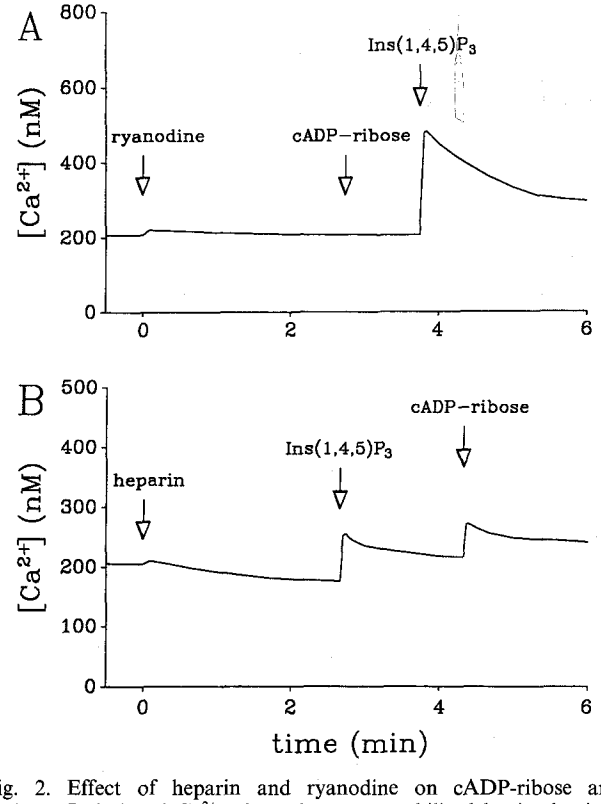


Fig. 2. Effect of heparin and ryanodine on cADP-ribose and Ins(1,4,5)$P_3$ induced $Ca^{2+}$ release from permeabilized lacrimal acinar cells. Ryanodine (100 $\mu$M; A) or heparin (200 $\mu$g/ml; B) was added 3 min before stimulation with 5 $\mu$M of cADP-ribose and Ins(1,4,5)$P_3$ as indicated. Each trace is a representative of 3 experiments.

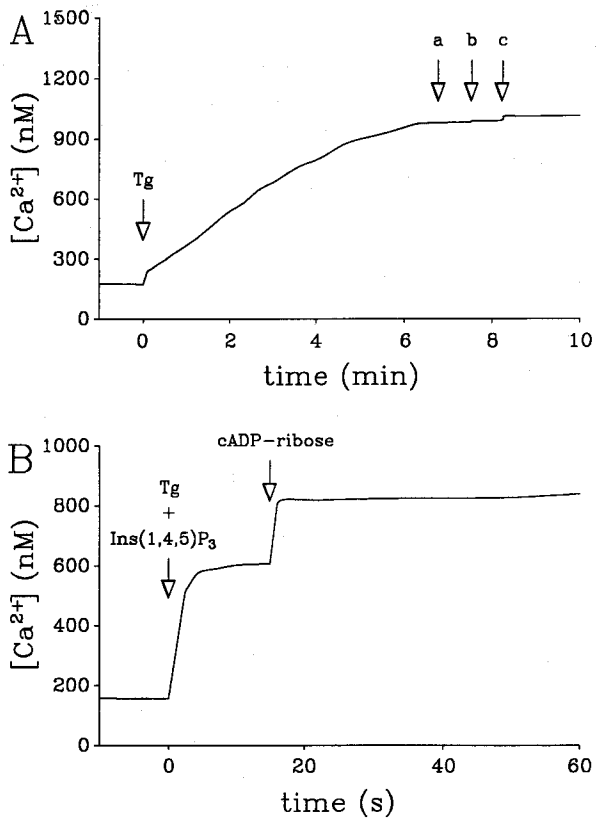


Fig. 3. Effect of thapsigargin on cADP-ribose and Ins(1,4,5)$P_3$ evoked $Ca^{2+}$ release. (A) Permeabilized lacrimal acinar cells were treated with 0.5 $\mu$M thapsigargin (Tg) for 8 min before stimulation with: a, 5 $\mu$M cADP-ribose; b, 5 $\mu$M Ins(1,4,5)$P_3$; c, 100 $\mu$M GTP. (B) A cocktail of 5 $\mu$M Ins(1,4,5)$P_3$ and 0.5 $\mu$M thapsigargin (Tg) was added followed by 5 $\mu$M cADP-ribose, as indicated by the arrows. Each trace is a representative of 3 experiments.

Ins(1,4,5)$P_3$ and 0.5 $\mu$M thapsigargin were added together, a rapid and strong $Ca^{2+}$ release was observed (378 ± 69 nM, $n = 3$) which was not down-regulated due to inhibition of the $Ca^{2+}$-ATPases. Subsequent addition of 5 $\mu$M Ins(1,4,5)$P_3$ did not cause further $Ca^{2+}$ release, indicating that the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores were emptied already after the first stimulation (data not shown). However, if 5 $\mu$M cADP-ribose was added shortly after stimulation with Ins(1,4,5)$P_3$ and thapsigargin, a second $Ca^{2+}$ release was observed, revealing that cADP-ribose can mobilize $Ca^{2+}$ from a pool not sensitive to Ins(1,4,5)$P_3$ (Fig. 3B). Surprisingly, the $Ca^{2+}$ release evoked by cADP-ribose following Ins(1,4,5)$P_3$ and thapsigargin stimulation amounted to 167 ± 24 nM ($n = 3$), which is larger than that observed in control experiments (cf. Fig. 1B). This could be partially due to the lack of $Ca^{2+}$ sequestration but also that the ryanodine receptors might be more sensitive to cADP-ribose at the elevated [$Ca^{2+}$] at which stimulation took place (Fig. 3B).

The ability of GTP to induce membrane fusion, and thus establishment of continuity between distinct Ins(1,4,5)$P_3$-sensitive and -insensitive $Ca^{2+}$ pools [17], was used to investigate the possibility that an Ins(1,4,5)$P_3$ and cADP-ribose insensitive $Ca^{2+}$ pool exists in lacrimal acinar cells. Addition of 100 $\mu$M GTP caused a rapid $Ca^{2+}$ release from a thapsigargin-sensitive $Ca^{2+}$ pool (Fig. 3A) through a heparin- and ryanodine-insensitive mechanism (data not shown). The effect was specific for GTP, since the non-hydrolysable GTP analogue, GTP$\gamma$S, had no effect on its own and inhibited GTP induced $Ca^{2+}$ release. Following maximal GTP stimulation, [$Ca^{2+}$] increased by 143 ± 31 nM ($n = 6$). Subsequent stimulation with 5 $\mu$M of cADP-ribose or Ins(1,4,5)$P_3$ 6–8 s after maximal GTP stimulation caused a second $Ca^{2+}$ release (Fig. 4). The cADP-ribose evoked $Ca^{2+}$ release amounted to 79 ± 20 nM ($n = 3$) which is not different from control responses in the absence of GTP (86 ± 18 nM). In contrast, the Ins(1,4,5)$P_3$ induced $Ca^{2+}$ release was larger than control responses: 366 ± 8 nM vs. 280 ± 35 nM ($P \leqq 0.001$; $n = 6$). The results are also consistent with the existence of a third thapsigargin-sensitive $Ca^{2+}$ pool based on its responsiveness to GTP. Furthermore, a physical interaction between this $Ca^{2+}$ pool and the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool was mediated by GTP. The latter observation supports previous findings in different cell types that GTP mediates the transfer of $Ca^{2+}$ from an Ins(1,4,5)$P_3$-insensitive to an Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool, thereby enlarging the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool and resulting in an enhanced $Ca^{2+}$ release following Ins(1,4,5)$P_3$ stimulation [17–20]. The GTP evoked $Ca^{2+}$ release is probably an artifact due to the fact that the cells were permeabilized. Thus, if GTP causes membrane fusion between intact and non-intact membrane structures, the net effect would be release of $Ca^{2+}$ into the medium. The requirements for GTP hydrolysis and inhibition by non-hydrolysable analogues appear to exclude the involvement of conventional G proteins in this translocation process, since GTP hydrolysis would not be required for G protein activity.

Our data suggest that the endoplasmic reticulum (ER) is

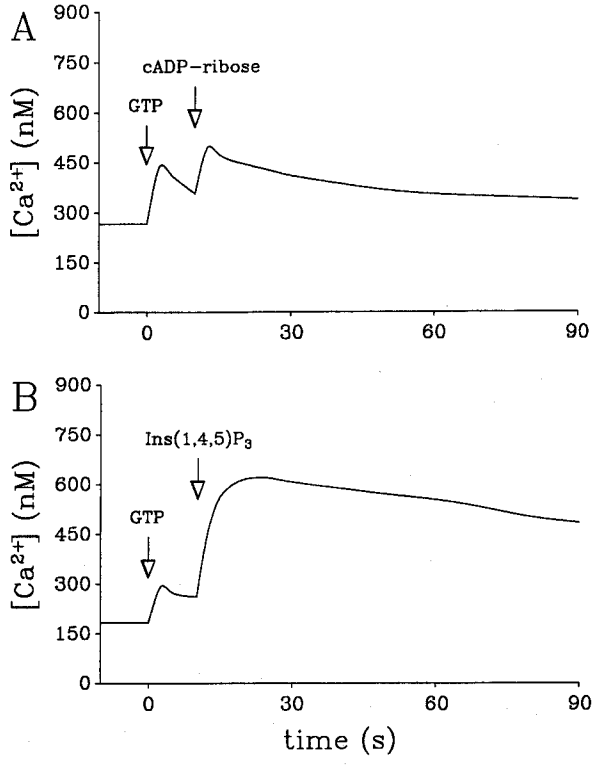


Fig. 4. Effect of GTP on cADP-ribose or Ins(1,4,5)$P_3$ induced $Ca^{2+}$ release. Permeabilized lacrimal acinar cells were stimulated with 100 $\mu$M GTP followed by (A) 5 $\mu$M cADP-ribose or (B) 5 $\mu$M Ins(1,4,5)$P_3$. The traces are representative of 3 experiments.

heterogeneous in terms of $Ca^{2+}$ release, implicating that this function is either concentrated in specialized ER subcompartments or separate organelles. In a previous study on lacrimal acinar cells it was found that the non-mitochondrial $Ca^{2+}$ pool was homogeneous in intact cells, whereas in permeabilized cells this pool behaved as if it had been partitioned into Ins(1,4,5)$P_3$-sensitive and -insensitive compartments [21]. However, we believe that our studies on permeabilized rat lacrimal acinar cells truly reflect the behavior of the intracellular $Ca^{2+}$ pools in intact cells for the following reasons: (i) if ER was fragmented randomly, one would expect to find an overlap of receptors for the different intracellular messengers on the different $Ca^{2+}$ pools; (ii) GTP specifically enlarged the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool; and (iii) in intact lacrimal acinar cells we have provided evidence for discrete agonist-sensitive $Ca^{2+}$ pools [6]. However, in intact lacrimal acinar cells the physiological heterogeneity may not necessarily be reflected in an anatomical heterogeneity, since subcompartments of ER could mediate this effect, and GTP may allow translocation of $Ca^{2+}$ between $Ca^{2+}$ pumping compartments and the Ins(1,4,5)$P_3$-sensitive compartments.

Lacrimal acinar cells have proven to be a useful model system for investigating intracellular signalling. The present and previous results [6] strongly indicate an intracellular messenger role for cADP-ribose in lacrimal acinar cells. This conclusion is further supported by recent data on pancreatic acinar cells that both cADP-ribose and Ins(1,4,5)$P_3$ sensitive $Ca^{2+}$ release channels are required for agonist evoked $Ca^{2+}$ spiking [10]. The effect of cADP-ribose could be to activate or sensitize the ryanodine receptors for $Ca^{2+}$, which is in marked similarity to the Ins(1,4,5)$P_3$ receptor [1,22]. Thus, the ryanodine receptor may, like the Ins(1,4,5)$P_3$ receptor, operate under second messenger and $Ca^{2+}$ dependent regulation.

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