cADP-ribose potentiates cytosolic Ca^{2+} elevation and Ca^{2+} entry via L-type voltage-activated Ca^{2+} channels in NG108-15 neuronal cells

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The effects of cADP-ribose (cADPR), a metabolite of $\beta\text{-NAD}^+$, on the elevation of cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and Ca^{2+} influx through voltage-activated Ca^{2+} channels (VACCs) were studied in NG108-15 neuroblastoma $\times$ glioma hybrid cells. NG108-15 cells were pre-loaded with fura-2 and whole-cell patch-clamped. Application of cADPR through patch pipettes did not by itself trigger any $[\text{Ca}^{2+}]_i$ rise at the resting membrane potential. A rise in $[\text{Ca}^{2+}]_i$ was evoked upon sustained membrane depolarization, and was significantly larger in cADPR-infused cells than in non-infused cells. This potentiation in the $[\text{Ca}^{2+}]_i$ elevation was reproduced by infusion of $\beta\text{-NAD}^+$, and was blocked by 8-bromo-cADPR and antagonized by external application of ryanodine or by pretreatment of cells with FK506. Nicotinamide inhibited $\beta\text{-NAD}^+$ -induced, but not cADPR-elicited, potentiation. $[\text{Ca}^{2+}]_i$ increases or Ca^{2+} influx,

measured by Mn^{2+} quenching, elicited by the same protocol of depolarization was blocked completely by nifedipine but not by ω -conotoxin. Ca^{2+} influx in cADPR- or $\beta\text{-NAD}^+$ -infused cells was steeper and greater than that in control cells, and was inhibited partly by ryanodine. In contrast, ryanodine accelerated Ca^{2+} influx in non-infused cells. These results show that cADPR amplifies both depolarization-induced $[\text{Ca}^{2+}]_i$ increase and Ca^{2+} influx through L-type VACCs. These results suggest that cADPR functions on ryanodine receptors as a direct agonist and also interacts with L-type VACCs as an indirect agonist, i.e. via a retrograde signal.

Key words: Ca^{2+} influx, FK506, $\beta\text{-NAD}^+$, ryanodine receptor, Ca^{2+} signalling.

INTRODUCTION

An elevation of the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) results either from Ca^{2+} entry through voltage-gated and receptor-operated Ca^{2+} channels, or from Ca^{2+} release from intracellular Ca^{2+} stores [1]. Ca^{2+} mobilization occurs by activation of inositol trisphosphate-sensitive and/or ryanodine-sensitive Ca^{2+} pools [2]. Increased $[\text{Ca}^{2+}]_i$ induces Ca^{2+} release from ryanodine receptor (RyR) Ca^{2+} -release channels, a mechanism called Ca^{2+} -induced Ca^{2+} release (CICR). cADP-ribose (cADPR) [3] has been suggested to be a second messenger or endogenous modulator for CICR [4], acting on specific subtypes of RyRs. The cardiac and brain isoforms of type-2 RyRs (RyR-2) and brain isoforms of type-3 RyRs (RyR-3) [5,6] are activated by cADPR [7,8], but the skeletal-muscle isoforms of type-1 RyRs (RyR-1) are not [8,9]. Whereas RyR-1 is coupled to L-type voltage-activated Ca^{2+} channels (VACCs) through protein–protein interactions [10], such direct coupling has not been shown in RyR-2 and RyR-3. In neurons, instead, a link from N-type VACCs to RyRs (an orthograde influence) [11] and a functional coupling from RyRs to L-type VACCs (a retrograde signal) [12] have been reported. The molecular mechanism and responsible second messengers for such interactions are not clear. Furthermore, in the latter study L-type VACCs were not well characterized.

cADPR is reported to potentiate the depolarization-induced $[\text{Ca}^{2+}]_i$ increase in bullfrog sympathetic neurons [13] and NG108-15 neuroblastoma $\times$ glioma hybrid cells [14]. These results led us to consider the possibility that cADPR could be a messenger

between RyRs and VACCs in neurons. Thus, the questions addressed here are whether or not VACCs are potentiated after cADPR-dependent activation of RyRs and, if so, which type (L or N) of VACC is involved. To pursue these issues, we used differentiated NG108-15 cells which possess low- and high-threshold VACCs [15] and RyRs that are sensitive to caffeine [14,16]. In this preparation, we have found that $\beta\text{-NAD}^+$ and cADPR function as modulators of the M-type K^+ channel [17] and have characterized ADP-ribosyl cyclase activity, which converts cADPR from $\beta\text{-NAD}^+$ [18]. In this context, NG108-15 cells provide several advantages for analysing how $[\text{Ca}^{2+}]_i$ and Ca^{2+} influx are modulated by cADPR. Here, we show that cADPR and $\beta\text{-NAD}^+$ amplify the nifedipine-sensitive depolarization-induced $[\text{Ca}^{2+}]_i$ rises and Ca^{2+} influx measured as fura-2 fluorescence intensity increase or its quenching by Mn^{2+} . Furthermore, we show that such facilitating effects of cADPR are sensitive to an L-type Ca^{2+} -channel inhibitor under the voltage-clamped condition. Finally, we demonstrate that these potentiations by $\beta\text{-NAD}^+$ -related compounds are antagonized by ryanodine, nicotinamide and the immunosuppressant FK506 [19], providing evidence for a novel bidirectional signal flow between L-type VACCs and RyRs.

EXPERIMENTAL

Cell culture

NG108-15 neuroblastoma $\times$ glioma hybrid cells were cultured at 37 °C in 90% air/10% CO_2 in Dulbecco's modified Eagle's

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; RyR, ryanodine receptor; RyR-1, -2, -3, type-1, -2 and -3 RyR, respectively; CICR, Ca^{2+} -induced Ca^{2+} release; ADPR, ADP-ribose; cADPR, cADP-ribose; VACC, voltage-activated Ca^{2+} channel; fura-2 AM, fura-2 acetoxymethyl ester; 8-Br-cADPR, 8-bromo-cADPR; $[\text{Ca}^{2+}]_o$, extracellular Ca^{2+} concentration; PKA, cAMP-dependent protein kinase.

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Figure 1 Effects of cADPR on membrane-depolarization-evoked $[Ca^{2+}]_i$ increases in patch-clamped NG108-15 cells

(A), (B) and (E) Traces show $[Ca^{2+}]_i$ changes in fura-2-loaded cells. Cells were touched with pipettes filled without (A), or containing 10 μM (B) or 1 nM–100 μM cADPR (E) at about 1 min before the beginning of each trace. Then membranes were sealed and ruptured at times indicated by the arrows. Membrane potentials were voltage-clamped at time zero to -15 , 0 or $+15$ mV from -45 mV and held for 26 min throughout the recordings. (C) and (D) Plots of $[Ca^{2+}]_i$ levels at the peak (C) or at 10 min (D) after starting the voltage clamp as a function of holding membrane potentials in cells infused with (●) or without (○; Con) 10 μM cADPR. $[Ca^{2+}]_i$ level is represented by percentage of $[Ca^{2+}]_i$ at -45 mV. (F) Concentration-response curves for the action of cADPR at 0 mV, showing the concentration-dependent increase in $[Ca^{2+}]_i$ level both at the peak and at 10 min. Cells were infused without (○) or with (●) the indicated concentrations of cADPR. Each symbol is the mean $\pm$ S.E.M. in 4–8 experiments. *, Significantly different from the cADPR-free control value at each membrane potential ($P < 0.05$).

medium supplemented with 5% fetal calf serum, 100 μM hypoxanthine, 0.1 μM aminopterin and 16 μM thymidine, as described previously [17]. Cells were differentiated in Dulbecco's modified Eagle's medium supplemented with hypoxanthine, thymidine, 1% fetal calf serum and 0.25 mM dibutyl-cAMP for 7 days [15].

Measurement of $[Ca^{2+}]_i$ and Mn^{2+} quenching

Microspectrofluorometric determination of $[Ca^{2+}]_i$ using fura-2 was performed in differentiated NG108-15 cells cultured on polylysine-coated glass coverslips. The cells were loaded with fura-2 by 5 μM fura-2 acetoxymethyl ester (fura-2 AM). Fluorescence was measured at 36 $^{\circ}C$ at a determined site through a pin hole (10–20 μm) with alternating excitation wavelengths of 340 and 380 nm [20], using a Ca^{2+} microspectrofluorometric system (OSP-3 model, Olympus, Tokyo, Japan). For measurement of Mn^{2+} quenching, an excitation wavelength of 360 nm and an emission wavelength of 510 nm were used, according to the method described previously [21].

Patch voltage clamp and drug application

Patch voltage clamp on fura-2-loaded NG108-15 cells was performed in the whole-cell configuration, as described previously [15,21]. NG108-15 cells were superfused at 36 $^{\circ}C$ with a bath solution of the following composition: 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM glucose and 20 mM Hepes, pH 7.3, buffered with NaOH. Patch electrodes contained solution of the following composition: 150 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2ATP , 0.1 mM fura-2 and 10 mM Hepes, pH 7.2, buffered with KOH. The electrode resistance was 8–16 M Ω . About 90 s after touching the cells, the electrode tip was sealed to the cell body by suction (seal resistance > 1 G Ω), and the membrane in the patch electrode was disrupted by administering negative potentials. After successfully establishing a whole-cell recording, the cell was voltage-clamped with a single-electrode voltage-clamp amplifier (Axoclamp 2A, Axon Instruments, Foster City, CA, U.S.A.) in switching mode operation at about 1–3 kHz. $[Ca^{2+}]_i$ rises and Mn^{2+} influx were usually measured during depolarizations maintained for 1.6–25 min, as described previously [21]. cADPR (1 nM–100 μM), β -NAD $^{+}$ (100 μM), ADP-ribose (ADPR;

10 μM), 8-bromo-cADPR (8-Br-cADPR; 10 μM) and nicotinamide (5 mM) dissolved in the intracellular recording medium were applied by diffusion into the cytoplasm from the patch pipette. FK506 at concentrations of 1 nM–1 μM was dissolved in 0.01 % ethanol.

Sources of compounds

cADPR was purchased from Yamasa Shoyu (Choshi, Japan). Ryanodine, ADPR, 8-Br-cADPR, nicotinamide and ω -conotoxin were obtained from Sigma (St. Louis, MO, U.S.A.). Fura-2 and fura-2 AM were purchased from Dojindo Laboratories (Kumamoto, Japan) and β -NAD⁺ from Nacalai Tesque (Kyoto, Japan). FK506 was a kind gift of both Professor H. Okamoto (Tohoku University, Sendai, Japan) and Fujisawa Pharmaceutical Co. All other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan).

RESULTS

Effect of cADPR on $[\text{Ca}^{2+}]_i$ in NG108-15 cells at resting membrane potentials

NG108-15 cells loaded with fura-2 AM were sealed with patch pipettes in the presence of the extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) of 2 mM, and membrane potential and $[\text{Ca}^{2+}]_i$ were measured. The average resting membrane potential was 44 ± 2 mV (mean $\pm$ S.E.M., $n = 14$) and the average $[\text{Ca}^{2+}]_i$ values were 75 ± 3 and 75 ± 3 nM before and after membrane rupture (arrows on Figure 1A) respectively. The values in cells injected with 10 μM cADPR through patch pipettes were 45 ± 1 mV, and 72 ± 2 and 74 ± 2 nM ($n = 14$), respectively (arrows, Figure 1B). Therefore, our results show that, upon infusion, cADPR evokes no apparent changes in membrane potential and $[\text{Ca}^{2+}]_i$, which agrees well with the report in bullfrog sympathetic neurons [13]. Next, we examined the role of cADPR in $[\text{Ca}^{2+}]_i$ elevation at constant depolarization.

Effect of cADPR on $[\text{Ca}^{2+}]_i$ increases induced by depolarization steps under voltage-clamp conditions

$[\text{Ca}^{2+}]_i$ increases resulting from Ca^{2+} influx through VACCs were examined in voltage-clamped NG108-15 cells. Figure 1(A) shows examples of $[\text{Ca}^{2+}]_i$ increases evoked by three different depolarization steps at -15 , 0 and $+15$ mV from -45 mV in NG108-15 cells without cADPR infusion. $[\text{Ca}^{2+}]_i$ increased within 10 s after the voltage step and reached its maximal level, followed by a gradual decrease during the recording period of 5–20 min. The $[\text{Ca}^{2+}]_i$ level at the peak and at 10 min after the clamping is plotted as a function of the command potential in Figures 1(C) and 1(D). The maximal $[\text{Ca}^{2+}]_i$ increase was obtained at a step depolarization to 0 mV with an average value at the peak of 276 ± 37 % of the pre-injection level (72 ± 2 nM, $n = 5$). The $[\text{Ca}^{2+}]_i$ increase at 10 min was 132 ± 13 %.

The same protocol was applied to cADPR-injected NG108-15 cells, in which membrane depolarizations induced significantly higher and long-lasting $[\text{Ca}^{2+}]_i$ increases (Figure 1B and see also Figure 2A). The average $[\text{Ca}^{2+}]_i$ level at 0 mV was 423 ± 39 % (at the initial peak) and 195 ± 17 % (after 10 min) of the pre-injection level (73 ± 2 nM, $n = 5$; Figures 1C and D). This cADPR-elicited potentiation of depolarization-induced $[\text{Ca}^{2+}]_i$ increases occurred in a concentration-dependent manner (Figures 1E and 1F). The doses of cADPR causing 50 % potentiation (EC_{50}) were around 400 and 340 nM at the initial peak and after 10 min, respectively.

Figure 2 Effect of cADPR and β -NAD⁺ on $[\text{Ca}^{2+}]_i$ at various holding membrane potentials in NG108-15 cells

Time courses of the holding membrane potentials are shown at the top in each panel. The traces in the middle show the fluorescence-ratio signals for $[\text{Ca}^{2+}]_i$. The lower-most bars indicate the duration of whole-cell mode, with intracellular injection of 10 μM cADPR through the patch pipette (A), with injection of 100 μM β -NAD⁺ (B) or without any treatment (C).

The cADPR-elicited potentiation of depolarization-induced $[\text{Ca}^{2+}]_i$ increases was suppressed by extracellular application of ryanodine at 10 μM (results not shown) and even 100 μM (Figure 3A). The level of $[\text{Ca}^{2+}]_i$ at 0 mV in cADPR-injected NG108-15 cells was 318 ± 42 % of the value at -40 mV (77 ± 4 nM, $n = 4$) in the absence of ryanodine (Figures 2A and 3A). Ryanodine (100 μM) decreased this to 164 ± 17 % ($n = 4$), which was indistinguishable from the control level (149 ± 12 %, $n = 5$). Thus ryanodine appears to block only the additional facilitation by cADPR of the depolarization-induced (VACC-dependent) response, rather than the response itself. Also, application of ryanodine (10 μM) to control cells could not abolish the basal response (144 ± 4 %, $n = 6$; results not shown). These results, together with those shown in bullfrog sympathetic neurons [13], suggest that RyRs are not usually activated by Ca^{2+} influx through VACCs.

Effect of β -NAD⁺ on $[\text{Ca}^{2+}]_i$ increases produced via nifedipine-sensitive VACC activation

Intracellularly infused β -NAD⁺ enhanced the depolarization-induced $[\text{Ca}^{2+}]_i$ rise (Figure 2B). This effect of 100 μM β -NAD⁺ on the $[\text{Ca}^{2+}]_i$ increase with depolarizations between -30 and $+30$ mV is shown in Figure 3(B), as a function of membrane potential. Quantitatively, $[\text{Ca}^{2+}]_i$ evoked by depolarization to 0 mV in β -NAD⁺-infused cells was 219 ± 10 % ($n = 6$) of the value

Figure 3 Effects of ryanodine, 8-Br-cADPR, ω -conotoxin, nifedipine, nicotinamide and FK506 on $[Ca^{2+}]_i$ at various holding membrane potentials in cADPR- or β -NAD $^{+}$ -infused NG108-15 cells

The average $[Ca^{2+}]_i$ as a function of holding membrane potentials in NG108-15 cells, measured as in Figure 2. The $[Ca^{2+}]_i$ levels were determined 100 s after stepping to the specified potential, and are expressed as percentages of the values at -40 mV. (A) The $[Ca^{2+}]_i$ level is shown for cADPR-infused cells (●), cADPR-infused cells pretreated with ryanodine (○; Rya) or control cells (▲; Con). The values in cells treated with cADPR from -30 to $+30$ mV are significantly higher than those in control cells at $P < 0.05$. (B) $[Ca^{2+}]_i$ levels in cells infused without (▲), or with $100 \mu M$ β -NAD $^{+}$ alone (■), $100 \mu M$ β -NAD $^{+}$ / $10 \mu M$ 8-Br-cADPR (□; +8-Br) and $10 \mu M$ 8-Br-cADPR alone (△; 8-Br). The values in cells treated with β -NAD $^{+}$ from -30 to $+30$ mV are significantly higher than those in control cells at $P < 0.05$. (C) $100 \mu M$ β -NAD $^{+}$ alone (■), $100 \mu M$ β -NAD $^{+}$ -infused cells with an extracellular application of either $10 \mu M$ ω -conotoxin (□; + ω -Tx) or $10 \mu M$ nifedipine (hatched square; +Nifed), or control cells (▲). Values in cells treated with β -NAD $^{+}$ alone or β -NAD $^{+}$ plus ω -conotoxin from -30 to $+30$ mV are significantly higher, and values in cells with nifedipine from -20 to $+30$ mV are significantly lower than those in the control cells at $P < 0.05$. (D) $[Ca^{2+}]_i$ levels in cells infused with $10 \mu M$ cADPR alone (●), $10 \mu M$ cADPR + 5 mM nicotinamide (○; +Nico), $100 \mu M$ β -NAD $^{+}$ alone (■), $100 \mu M$ β -NAD $^{+}$ + 5 mM nicotinamide (□), and in control cells (▲). Note that addition of nicotinamide significantly reduced the β -NAD $^{+}$ -induced elevation in $[Ca^{2+}]_i$ at -10 to $+30$ mV at $P < 0.05$. (E) Cells infused with $100 \mu M$ β -NAD $^{+}$ pretreated with 1 (□), 10 (●), 100 nM (◇) and $1 \mu M$ (▲) FK506. The $[Ca^{2+}]_i$ levels recorded with (—; NAD) or without (·····; Con) β -NAD $^{+}$ infusion (from B) are superimposed. The values in cells pretreated with 100 nM and $1 \mu M$ FK506 at 0 mV are significantly different from those in cells with $100 \mu M$ β -NAD $^{+}$ alone at $P < 0.05$. Each point shows the mean $\pm$ S.E.M. from 3–7 cells.

at -40 mV, and was significantly higher than the control value ($144 \pm 3\%$, $n = 8$; $P < 0.05$).

To confirm that the β -NAD $^{+}$ -induced potentiation of $[Ca^{2+}]_i$ elevation is mediated through its conversion to cADPR, 8-Br-cADPR, a cADPR competitor [22], was used. $[Ca^{2+}]_i$ at 0 mV in cells injected with both $100 \mu M$ β -NAD $^{+}$ and $10 \mu M$ 8-Br-cADPR was $159 \pm 8\%$ ($n = 4$) of the value at -40 mV (Figure 3B). 8-Br-cADPR ($10 \mu M$) antagonized the $10 \mu M$ cADPR-induced response to a level of $155 \pm 11\%$ ($n = 6$), and had no influence on the basal response ($151 \pm 9\%$, $n = 6$). This observation shows that 8-Br-cADPR had no effect on the Ca^{2+} -mobilization elicited by openings of VACCs, suggesting that resting endogenous cADPR does not contribute to CICR. Furthermore, no potentiation was induced by $10 \mu M$ ADPR ($142 \pm 12\%$ of the value at -40 mV, $n = 5$), another metabolite of β -NAD $^{+}$ and/or a direct metabolite of cADPR [23].

Next, we used ω -conotoxin and nifedipine to determine which types of VACC are responsible for the depolarization-induced $[Ca^{2+}]_i$ rise that is sensitized by cADPR and β -NAD $^{+}$. Application of $10 \mu M$ ω -conotoxin, an N-type VACC antagonist, did not have an effect on β -NAD $^{+}$ -induced potentiation: $[Ca^{2+}]_i$ at 0 mV was $238 \pm 16\%$ ($n = 3$) of the value at -40 mV (Figure 3C). On the other hand, $10 \mu M$ nifedipine, an L-type VACC antagonist, completely abolished the depolarization-induced $[Ca^{2+}]_i$ rise as well as the β -NAD $^{+}$ -induced enhancement: $[Ca^{2+}]_i$ at 0 mV was $108 \pm 3\%$ ($n = 7$) of the value at -40 mV (Figure 3C). The differential sensitivity of the depolarization-induced $[Ca^{2+}]_i$ rise to ω -conotoxin and nifedipine was identical in cADPR-infused NG108-15 cells (results not shown). These results and the voltage-dependence suggest that Ca^{2+} influx through L-type VACCs is the main source of Ca^{2+} for both β -NAD $^{+}$ - and cADPR-induced amplification of $[Ca^{2+}]_i$ elevations.

Figure 4 Effect of ryanodine on depolarization-evoked $[\text{Ca}^{2+}]_i$ increases and Mn^{2+} influx in control or cADPR- and $\beta\text{-NAD}^+$ -infused NG108-15 cells

Experiments were carried out in the presence of 2 mM $[\text{Ca}^{2+}]_o$. Membranes were voltage-clamped to -15 mV from -40 mV, as shown at the top. Three pairs of traces were obtained, for cells infused with 10 μM cADPR (**A**), 100 μM $\beta\text{-NAD}^+$ (**B**) and control solution (**C**). Membranes were ruptured 100 s prior to the beginning of each trace. Ryanodine (Ry, 25 μl of 20 μM , final concentration 1 μM) was applied at the arrow. Middle traces show $[\text{Ca}^{2+}]_i$ changes in three cells. Lower traces show changes in fluorescence at an excitation wavelength of 360 nm to estimate Mn^{2+} influx in three other cells. MnCl_2 (Mn, 25 μl of 5 mM, final concentration 250 μM) was added at the arrows prior to application of ryanodine. The ordinates on the lower traces are in arbitrary units. Each trace shown is typical findings from 3–6 different experiments.

Table 1 Effects of ryanodine, nicotinamide and FK506 on depolarization-evoked $[\text{Ca}^{2+}]_i$ increases and Mn^{2+} influx in control or cADPR- and $\beta\text{-NAD}^+$ -infused NG108-15 cells

The Mn^{2+} influx was determined by net fluorescence changes over 1 min before (Pre) and after (Post) application of vehicle or ryanodine (+Rya, 1 μM). Experiments were carried out in cells with or without infusion of 10 μM cADPR or 100 μM $\beta\text{-NAD}^+$, as shown in the lower panels of Figures 4 and 5. $[\text{Ca}^{2+}]_i$ levels were determined at 1 min after application of 1 μM ryanodine (+Rya) or vehicle alone. Experiments were carried out as shown in middle panel of Figure 4. Values are expressed as a percentage of the pre-application level. The total amount of Mn^{2+} influx over 10 min: Mn^{2+} influx was determined by subtracting the fluorescence level at 10 min after clamping to 0 mV from the level at time zero in control cells (corresponding to middle panel of Figure 5), cells co-injected with 5 mM nicotinamide or cells pretreated with 100 nM FK506 for 24 h. Each value is the mean $\pm$ S.E.M. from 4–8 cells.

	Ratio of Mn^{2+} influx before and after application (Post/Pre; %)				$[\text{Ca}^{2+}]_i$ changes after 1 min (%) at 2 mM $[\text{Ca}^{2+}]_o$		Net Mn^{2+} influx over 10 min (%) at 100 μM $[\text{Ca}^{2+}]_o$		
	2 mM $[\text{Ca}^{2+}]_o$		100 μM $[\text{Ca}^{2+}]_o$		Vehicle	+ Rya	Control	Nicotinamide-treated	FK506-pretreated
Control	82 $\pm$ 2	176 $\pm$ 20*	69 $\pm$ 8	348 $\pm$ 103*	94 $\pm$ 3	115 $\pm$ 7	33 $\pm$ 9	Not determined	24 $\pm$ 4
cADPR	90 $\pm$ 3	58 $\pm$ 11*	95 $\pm$ 8	47 $\pm$ 2*	94 $\pm$ 2	80 $\pm$ 3*	63 $\pm$ 4†	63 $\pm$ 8†	39 $\pm$ 6
$\beta\text{-NAD}^+$	101 $\pm$ 14	66 $\pm$ 4*	96 $\pm$ 6	45 $\pm$ 7*	97 $\pm$ 2	79 $\pm$ 2*	61 $\pm$ 6†	30 $\pm$ 6	28 $\pm$ 3

*, Significantly different from the value in vehicle-treated cells at $P < 0.05$.

†, Significantly different from the value in control cells at $P < 0.05$.

Effects of nicotinamide on $\beta\text{-NAD}^+$ - and cADPR-induced potentiation of $[\text{Ca}^{2+}]_i$ increase

Since it has been shown that nicotinamide inhibits cADPR-mediated Ca^{2+} signalling in sea urchin eggs [24], the effect of nicotinamide was tested in our NG108-15 cells. Nicotinamide was introduced through patch pipettes. Whereas $[\text{Ca}^{2+}]_i$ at 0 mV in cells injected with both 100 μM $\beta\text{-NAD}^+$ and 5 mM nicotinamide [$153 \pm 7\%$ ($n = 5$) of the value at -40 mV] was lower than that with $\beta\text{-NAD}^+$ alone, $[\text{Ca}^{2+}]_i$ in cells injected with both 10 μM cADPR and nicotinamide was as high as those with cADPR alone ($313 \pm 31\%$, $n = 6$; Figure 3D). This inhibitory effect of nicotinamide on $\beta\text{-NAD}^+$ but not on cADPR is consistent with that in sea urchin eggs and suggests that the effect

of nicotinamide occurs through an inhibition of ADP-ribosyl cyclase activity.

Effects of FK506 on $\beta\text{-NAD}^+$ - and cADPR-induced potentiation of $[\text{Ca}^{2+}]_i$ increase

The $\beta\text{-NAD}^+$ -elicited potentiation in depolarization-induced $[\text{Ca}^{2+}]_i$ increases was suppressed in NG108-15 cells preincubated for 24 h with FK506, a known competitor of cADPR at RyRs [25,26]. The $\beta\text{-NAD}^+$ -elicited potentiation in depolarization-induced $[\text{Ca}^{2+}]_i$ increases was suppressed. As shown in Figure 3(E), the levels of $[\text{Ca}^{2+}]_i$ at 0 mV in the presence of 1, 10, 100 and 1000 nM FK506 decreased dose-dependently to 218 ± 14 , 198 ± 14 , 151 ± 22 and $142 \pm 16\%$ of the value at -40 mV

Figure 5 Effect of ryanodine on depolarization-evoked Mn^{2+} influx in control or cADPR- and $\beta\text{-NAD}^+$ -infused NG108-15 cells

Experiments were carried out in the reduced extracellular Ca^{2+} concentration of $100\ \mu\text{M}$ and in $250\ \mu\text{M}$ Mn^{2+} . Upper traces of each set of records show holding membrane potential. Two sets of traces show changes in Mn^{2+} quenching upon depolarization in cells infused with $10\ \mu\text{M}$ cADPR (**A**), $100\ \mu\text{M}$ $\beta\text{-NAD}^+$ (**B**) or control solution (**C**). The arrow indicates application of $25\ \mu\text{M}$ of $20\ \mu\text{M}$ ryanodine (Ry, final concentration $1\ \mu\text{M}$). Each trace shown is typical findings from 4–8 experiments. The ordinates represent arbitrary units.

($72 \pm 1\ \text{nM}$; $n = 3$ –6), respectively. The concentration of FK506 that produced 50% inhibition of $[\text{Ca}^{2+}]_i$ increase was $44\ \text{nM}$. FK506 ($100\ \text{nM}$) also inhibited the cADPR-induced potentiation: $[\text{Ca}^{2+}]_i$ levels at $0\ \text{mV}$ in $10\ \mu\text{M}$ cADPR-infused cells and control cells were $143 \pm 11\%$ ($n = 4$) and $146 \pm 9\%$ ($n = 4$) of the values at $-40\ \text{mV}$, respectively. These results show that FK506 pretreatment antagonizes only the fraction enhanced by cADPR and $\beta\text{-NAD}^+$, leaving VACC activity intact.

Effect of ryanodine on depolarization-induced Mn^{2+} influx and $[\text{Ca}^{2+}]_i$ increase

The cellular fluorescence elicited at $360\ \text{nm}$ decreased when $200\ \mu\text{M}$ MnCl_2 was added to the medium 60–90 s after clamping to $-15\ \text{mV}$ from $-40\ \text{mV}$ with patch pipettes containing $10\ \mu\text{M}$ cADPR (Figure 4A), $100\ \mu\text{M}$ $\beta\text{-NAD}^+$ (Figure 4B) or control solution (Figure 4C). The decrease was steeper and larger in cells perfused with cADPR and $\beta\text{-NAD}^+$ than in control cells. Addition of $1\ \mu\text{M}$ ryanodine slowed down the Mn^{2+} quenching in cells infused with cADPR and $\beta\text{-NAD}^+$, but accelerated it in control cells. These changes are summarized quantitatively in Table 1. Figure 4 also shows the effect of ryanodine application on $[\text{Ca}^{2+}]_i$ in the middle of a long depolarization. Ryanodine decreased $[\text{Ca}^{2+}]_i$ to an extent from increased levels in cADPR- and $\beta\text{-NAD}^+$ -infused cells (Figures 4A and 4B), but increased it in non-infused cells (see Table 1). The result suggests that ryanodine functions as the inhibitor for cADPR-activated RyRs and as the activator for unstimulated RyRs.

As shown in upper panels of Figure 5, Ca^{2+} influx was examined in reduced $[\text{Ca}^{2+}]_o$ at $100\ \mu\text{M}$ (in the presence of

$250\ \mu\text{M}$ MnCl_2), under which conditions it is expected that competition with extra- and intracellular Ca^{2+} on Mn^{2+} influx is minimized. Under such conditions, the cADPR- and $\beta\text{-NAD}^+$ -elicited potentiation of depolarization-induced Mn^{2+} influx at $10\ \text{min}$ is summarized in Table 1. The EC_{50} of cADPR was $210\ \text{nM}$, which was calculated from a concentration-response curve (results not shown). The opposite effects of ryanodine on Mn^{2+} quenching (an inhibition in cADPR- and $\beta\text{-NAD}^+$ -infused cells and an increase in control cells) were not only reproduced but also intensified in the reduced $[\text{Ca}^{2+}]_o$ condition (lower panels of Figure 5 and Table 1).

Addition of nicotinamide through the patch pipette abolished the $\beta\text{-NAD}^+$ -induced potentiation of Mn^{2+} influx, whereas it had no effect on the cADPR-induced one (Table 1). Pretreatment with FK506 ($100\ \text{nM}$) for 24 h of cADPR- or $\beta\text{-NAD}^+$ -infused cells similarly suppressed Mn^{2+} influx to the control level (Table 1). Since the depolarization-induced and ryanodine-sensitive Mn^{2+} influx shown in Figures 4 and 5 was not observed at the resting membrane potential (results not shown), Mn^{2+} influx mostly reflects Ca^{2+} entry through VACCs, rather than the capacitative Ca^{2+} -entry pathway.

Effects of membrane potential and nifedipine on Mn^{2+} influx in $\beta\text{-NAD}^+$ -infused cells

Ca^{2+} influx through VACCs was characterized by examining membrane-potential-dependence and the effect of nifedipine on fluorescence intensity in NG108-15 cells perfused with $250\ \mu\text{M}$ Mn^{2+} and $100\ \mu\text{M}$ Ca^{2+} , as in Figure 5. The Mn^{2+} quenching was

Figure 6 Effects of membrane potential on Mn^{2+} quenching measured in NG108-15 cells infused with $\beta\text{-NAD}^+$

(A) Two NG108-15 cells were infused with $100 \mu\text{M}$ $\beta\text{-NAD}^+$ at the arrow. The cells were perfused at first in the absence of $[\text{Ca}^{2+}]_o$, and then with bath solution containing $250 \mu\text{M}$ MnCl_2 and $100 \mu\text{M}$ CaCl_2 , as indicated. Membrane potential was increased in 10-mV steps from -40 to $+40$ mV every 100 s. Traces represent the cellular fluorescence at a wavelength of 360 nm obtained from cells in the presence (+Nif) and absence (–Nif) of $10 \mu\text{M}$ extracellular nifedipine. Note that the trace without nifedipine shows maximal Mn^{2+} influx at holding potentials between -10 and 0 mV, but no such increase in the +Nif cell. (B) Depolarization-induced Mn^{2+} quenching shown in (A) was plotted against the membrane potential in the presence (Δ , +Nif) and absence ($\bullet$, –Nif) of $10 \mu\text{M}$ nifedipine. The changes in fluorescence intensity were determined over 100 s of holding at the potentials shown.

apparent at membrane potentials between -20 and $+30$ mV, with a peak at 0 mV in the absence of extracellular nifedipine, although such changes disappeared in the presence of $10 \mu\text{M}$ nifedipine (Figure 6). The profile of the Mn^{2+} quench as a function of membrane potential is plotted in Figure 6(B), which is quite similar to that of the voltage-dependent $[\text{Ca}^{2+}]_i$ increase, as shown in Figure 3. The voltage-profile and nifedipine sensitivity of Mn^{2+} quenching resemble those of electrophysiologically characterized L-type VACCs in NG108-15 cells [27], indicating that this Ca^{2+} influx is mediated through L-type VACCs, but not through the so-called capacitative Ca^{2+} -entry pathway.

DISCUSSION

The present results show that cADPR applied through a patch pipette does not by itself trigger the $[\text{Ca}^{2+}]_i$ rise under the clamp conditions used in this study. This observation suggests that cADPR does not act as a 'primary ligand' to open either RyRs

or VACCs at the resting membrane potential, as suggested by Sitsapesan et al. [28]. Our results also show that both cADPR and $\beta\text{-NAD}^+$ amplify the increase in $[\text{Ca}^{2+}]_i$ induced by membrane depolarization (Figures 1–3). In NG108-15 cells, the increased levels of both cADPR and Ca^{2+} , which are produced by intracellular injection and by activation of VACCs, induces potentiation in $[\text{Ca}^{2+}]_i$, suggesting that cADPR augments the depolarization-induced $[\text{Ca}^{2+}]_i$ elevation. This potentiation is due to the activation of L-type VACCs, judging from the following results. (i) The $[\text{Ca}^{2+}]_i$ profile for the potential range between -30 and $+30$ mV with a maximal $[\text{Ca}^{2+}]_i$ rise at 0 mV resembles the potential profile of currents due to activation of L-type VACCs in NG108-15 cells [27]. (ii) Prolonged depolarization (up to 30 min) did not occlude the channels. (iii) Potentiation of $[\text{Ca}^{2+}]_i$ was blocked by nifedipine, but not by ω -conotoxin, as are L-type channels. The present study, however, does not exclude the possible link between N-type VACCs and RyRs, which has been shown previously in superior cervical ganglia [11].

In our experiment, the involvement of L-type VACCs in cADPR-induced enhancement of $[\text{Ca}^{2+}]_i$ was proved directly by measuring Mn^{2+} influx (shown in Figures 4–6). This kind of confirmation had not been reported before. cADPR potentiated depolarization-induced Ca^{2+} influx, which was also blocked by nifedipine and whose membrane-potential-dependence resembles that of L-type VACCs.

The cADPR-induced enhancement in the depolarization-evoked $[\text{Ca}^{2+}]_i$ increase was blocked by ryanodine application or FK506 pretreatment (Figure 3), suggesting that cADPR acts on RyRs but not on VACCs. Our findings indicate an orthograde signal pathway for the cADPR-dependent enhancement of the depolarization-induced $[\text{Ca}^{2+}]_i$ increase, as follows. Without cADPR, Ca^{2+} entry through L-type VACCs by itself is insufficient to activate Ca^{2+} channels of RyRs (i.e. CICR), as shown in Scheme 1(A). cADPR, if present, together with increased Ca^{2+} , may enhance CICR, as shown in Scheme 1(B). This action of cADPR for $[\text{Ca}^{2+}]_i$ increase by VACC activation was already suggested in previous studies [13,14]. The present results, however, are new, in that L-type VACCs could be involved in this process in neurons.

There is another hypothesis that activation of RyRs by cADPR and Ca^{2+} can further augment Ca^{2+} influx through VACCs. Such a signal is termed retrograde signalling [10]. Chavis et al. have shown a similar signal in cerebellar granule cells [12], where RyRs activated by type-1 metabotropic glutamate receptors increase VACC activity. Since it has been reported that cerebellar granule cells possess RyR-2 and RyR-3 [5,6], the retrograde signal from RyR-2 or RyR-3 may target the L-type VACCs. Our results support and extend this novel idea that L-type VACCs and RyRs are functionally coupled, and we provide the first evidence for the involvement of cADPR in this coupling, as shown in Scheme 1. This coupling may not be the direct interaction, because ryanodine had no inhibitory effect on the control VACC activity [12].

For the retrograde signal in neurons, Chavis et al. [12] postulated that some intermediate factor physically links RyRs with the L-type VACCs (Scheme 1C). An alternative idea is that Ca^{2+} exhaustion in ryanodine-sensitive Ca^{2+} stores elicited by cADPR and CICR may trigger VACC activation, which in turn refills the stores by Ca^{2+} influx by a positive-feedback mechanism (Scheme 1D). The latter theory seems to be more plausible in NG108-15 cells, since cADPR-sensitive RyRs can be activated by CICR [29] and subsequently refilled via VACCs, as reported in pheochromocytoma and sympathetic neurons [2,30]. Although we have shown that RyR activation via cADPR/ Ca^{2+} triggers further Ca^{2+} entry, the evidence that links this further influx

Scheme 1 Models for reciprocal interactions between RyRs and L-type VACCs in NG108-15 cells

(A) Ca²⁺ entry through L-type VACCs [VACC (L)] induces [Ca²⁺]_i elevation. RyRs are not normally activated in the absence of cADPR. (B) Orthograde signalling from L-type VACCs to RyRs. Ca²⁺ enters through L-type VACCs, which induces [Ca²⁺]_i elevation. This Ca²⁺, together with cADPR, co-operatively activates RyRs to release more Ca²⁺ (CICR). This process is blocked by FK506. (C) and (D) Two models for retrograde signalling from RyRs to L-type VACCs. (C) Some intermediate factor (?) that bridges RyRs and L-type VACCs potentiates further Ca²⁺ entry through VACCs after activation of RyRs by both cADPR and increased cytosolic Ca²⁺. (D) Ca²⁺ entered through L-type VACCs, together with cADPR, causes activation of RyRs (i.e. CICR). This induces depletion of ryanodine-sensitive Ca²⁺ stores. This exhaustion works as a signal and leads to the further increase of L-type VACC activity to refill the Ca²⁺ store. In this Figure, the capacitative Ca²⁺-entry pathway for refilling the ryanodine-sensitive store is not considered.

through the L-type VACCs is not conclusive. Although the initial trigger of the pathway is the L-type VACCs, which activate the RyR in the presence of cADPR, the next step is not necessarily the L-type VACCs: the capacitative Ca²⁺-entry pathway could be involved [2]. This point needs further study.

Such bidirectional coupling by cADPR might function physiologically if some cell-surface-receptor stimulation is linked to both cADPR production and VACC activation. A candidate for this is the receptor-activated cAMP-dependent protein-kinase (PKA) cascade. It has been shown that activation of PKA by stimulation with acetylcholine increases ADP-ribosyl cyclase activity in chromaffin cells [31], facilitates CD38 expression in Jurkat T cells [32] and enhances L-type VACC activity targeted by the PKA-anchoring protein [33].

β-NAD⁺ efficiently potentiated the [Ca²⁺]_i increase and Mn²⁺ quenching due to depolarization (Figures 2–5). The effect of β-NAD⁺ was antagonized by 8-Br-cADPR, which eliminates the ability of cADPR to activate Ca²⁺ release [22], or by nicotinamide, which inhibits ADP-ribosyl cyclase activity [24]. Since ADPR has no facilitating effect, the action of β-NAD⁺ seems to be mediated by cADPR [23,24], after conversion of β-NAD⁺ to cADPR by ADP-ribosyl cyclase in NG108-15 cells. Recently, we have reported membrane-associated intrinsic ADP-ribosyl cyclase modulated by muscarinic receptor stimulation [18].

RyRs may be involved in synaptic Ca²⁺ signalling through VACCs in normal brain neurons, judging from the result that Ca²⁺ transients produced by VACCs are prolonged by caffeine, at both distal and proximal dendrites and soma of neurons [34].

If we assume that the mechanism found in NG108-15 neuronal cells exists in intact brain neurons, cADPR may be involved in synaptic Ca²⁺ signalling by enhancing CICR resulting from activation of L-type VACCs. Of course, in this case, it is necessary to determine which condition activates production of cADPR in neurons. In conclusion, our present results strongly support the idea that cADPR is an endogenous mediator for bidirectional coupling between RyRs and VACCs, and is capable of modulating neuronal signalling.

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Received 20 April 1999/7 July 1999; accepted 29 October 1999