Cyclic ADP-ribose Enhances Coupling between Voltage-gated Ca\textsuperscript{2+} Entry and Intracellular Ca\textsuperscript{2+} Release*

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Ca\textsuperscript{2+} release from intracellular stores can be activated in neurons by influx of Ca\textsuperscript{2+} through voltage-gated Ca\textsuperscript{2+} channels. This process, called Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, relies on the properties of the ryanodine receptor and represents a mechanism by which Ca\textsuperscript{2+} influx during neuronal activity can be amplified to large intracellular Ca\textsuperscript{2+} signals. In a differentiated neuroblastoma cell line, we show that caffeine, a pharmacological activator of the ryanodine receptor, released Ca\textsuperscript{2+} from intracellular stores in a Ca\textsuperscript{2+}-dependent and ryanodine-sensitive manner. The pyridine nucleotide, cyclic ADP-ribose, thought to be an endogenous modulator of ryanodine receptors also amplified Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release in these neurons. Cyclic ADP-ribose enhanced the total cytoplasmic Ca\textsuperscript{2+} levels during controlled Ca\textsuperscript{2+} influx through voltage-gated channels, in a concentration-dependent and ryanodine-sensitive manner and also increased the sensitivity with which a small amount of Ca\textsuperscript{2+} influx could trigger additional release from the ryanodine-sensitive intracellular Ca\textsuperscript{2+} stores. Single cell imaging showed that following the Ca\textsuperscript{2+} influx, cyclic ADP-ribose enhanced the spatial spread of the Ca\textsuperscript{2+} signal from the edge of the cell into its center. These powerful actions suggest a role for cyclic ADP-ribose in the functional coupling of neuronal depolarization, Ca\textsuperscript{2+} entry, and global intracellular Ca\textsuperscript{2+} signaling.

Calcium-induced calcium release (or CICR),\textsuperscript{1} is a means of amplifying intracellular cytosolic Ca\textsuperscript{2+} signals. Ca\textsuperscript{2+} entering a cell, for example through voltage-sensitive channels, can trigger the release of further Ca\textsuperscript{2+} from the intracellular stores (1–3). The mechanism relies upon the distinct properties of the ryanodine receptor, in particular its ability to control Ca\textsuperscript{2+} release from the store and the sensitivity of its gating mechanism to Ca\textsuperscript{2+} (4, 5). A wide variety of neurons, both cultured and acutely isolated, possess ryanodine-sensitive intracellular Ca\textsuperscript{2+} stores (6–9) and also express ryanodine receptors (9–12). A number of recent studies have emphasized the importance of Ca\textsuperscript{2+} release from intracellular stores during physiological neuronal activity (13), during changes in synaptic efficacy that may be involved in learning processes (14–16), as part of the mechanism of neurotransmitter release (17), and during neuronal development (18). Caffeine (4) and cyclic ADP-ribose, the novel Ca\textsuperscript{2+}-mobilizing pyridine nucleotide originally discovered in the sea urchin egg (19), both release Ca\textsuperscript{2+} from intracellular stores via modulation of the ryanodine receptor (20, 21). In the last few years cyclic ADP-ribose has begun to emerge as a potential physiological regulator of ryanodine-sensitive Ca\textsuperscript{2+}-dependent processes in a number of intact mammalian systems. Cyclic ADP-ribose modulates excitation-contraction coupling in the heart (22), it alters excitability of pancreatic acinar cells (23) and dorsal root ganglion cells (24), and stimulates Ca\textsuperscript{2+} release from the intracellular stores of T-lymphocytes (25). Using a combination of electrophysiology and Ca\textsuperscript{2+} imaging we show here, in intact mammalian cultured neuroblastoma cells (26), that release from ryanodine-sensitive intracellular stores is coupled to Ca\textsuperscript{2+} influx via voltage-activated channels and potentiated by cyclic ADP-ribose applied through the recording electrode.

EXPERIMENTAL PROCEDURES

NG108-15 neurons, a mouse neuroblastoma \times rat glioma hybrid culture (obtained from the European Collection of Cell Cultures, Porton, UK), were cultured as described previously (26, 27).

For Ca\textsuperscript{2+} measurements in intact cells, the cells were loaded with Fura-2 using the acetoxymethyl ester loading technique for a maximum loading period of 15 min. Before commencing experiments the cells were washed three times in the perfusion buffer containing 130 mM NaCl, 10 mM HEPES, 5 mM KCl, 5 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 25 mM glucose, and the stage of the upright microscope (Zeiss, Oberkochen, Germany). To depolarize the cells, 30 mM KCl (and an equimolar reduction in Na\textsuperscript{+} ions) was switched into the perfusion flow (rate varied between 1 and 1.5 ml/min). Changes in Ca\textsuperscript{2+} were measured every 4 s in these experiments using ratio metric determinations of image intensity following excitation with 340- and 380-nm wavelength light supplied by a TILL Photonics monochromator (Planegg, Germany) controlled by a digitizing (Leicester, UK) “Ionvision” software (as described previously (28)). An in vitro calibration using the Ionvision software was used to average Ca\textsuperscript{2+} changes, over the whole cell, in individual cells from the pseudocolor images, as described previously. The same experimental setup and analysis was used to determine Ca\textsuperscript{2+} changes during the electrophysiological experiments, except that an image was captured every 2 s. Variations in intracellular Ca\textsuperscript{2+} in different regions of the cell were also measured with the same calibrations and software using small rectangular regions of interest. Electrophysiological recordings were made in the whole cell patch clamp mode using an Axopatch 200A (Axon Instruments, Foster City, CA) with pipettes of resistance 2–6 M\textohm. Seal resistances prior to breakthrough were always greater than 1 G\ohm. Cells settled and filled with Fura-K” for approximately 10 min and then broke through. Current and voltage were digitized using an ITC-16 A/D converter (Intruchtech Corp., Great Neck, NY) and the experiments controlled using the Axodata program (Axon Instruments) through the same interface. The intracellular solution contained 135 mM CaCl\textsubscript{2}, 10 mM HEPES, 1 mM Mg-ATP, 100 \textmu M Fura-2-penta- pottassium salt, and extracellular solution contained 130 mM NaCl, 10 mM HEPES, 5 mM KCl, 5 mM tetraethylammonium chloride, 5 mM CaCl\textsubscript{2}, 5 mM 4-aminoypyridine, 1 mM MgCl\textsubscript{2}, 25 mM glucose, and 1 mM tetrodo- toxin. Both intra- and extracellular solutions were designed to block K\textsuperscript{+} and Na\textsuperscript{+} channels, so that during cellular depolarization the area beneath the inward current, measured with Axograph (Axon Instruments), an indication of the charge entering the cell, represented the entry of Ca\textsuperscript{2+} and not other cations. Ca\textsuperscript{2+} (or charge) entry was controlled by changing the duration of the +60- or +80-mV voltage step evoked from a holding potential of –70 or –90 mV. Voltage steps were applied in a random order, every 20–30 s to avoid excessive run-down of the Ca\textsuperscript{2+} current with linear on-line leak subtraction. For each voltage step the peak Ca\textsuperscript{2+} change was measured and divided by the

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FIG. 1. A, Intracellular Ca$^{2+}$ levels were increased by 50 mM caffeine, indicated by the filled bar. B, depolarization of the cells with 30 mM external K$^+$, as shown by the open bar, lead to an increase in Ca$^{2+}$ levels through the opening of N- and L-type voltage-sensitive Ca$^{2+}$ channels (30). Subsequent application of 50 mM caffeine (filled bar) leads to a marked increase in Ca$^{2+}$ levels, as shown for all cells in C. In the absence of extracellular Ca$^{2+}$, as shown by the dotted line, depolarizations did not lead to significant rises in intracellular Ca$^{2+}$, nor did it enhance caffeine-induced Ca$^{2+}$ release, rather Ca$^{2+}$ changes were not significantly different to the results when caffeine was applied alone, 172 ± 20 nM Ca$^{2+}$ (n = 5) p = 0.24, t-test. These responses were reduced by extracellular application of 10 μM ryanodine, mean caffeine-induced Ca$^{2+}$ rises were 658 ± 113 reduced to 91 ± 25 nM Ca$^{2+}$ (n = 8, p = 0.004, paired t test). D, concentration response curve showing the sharp increase in the predopolarization caffeine-induced intracellular Ca$^{2+}$ rises in response to raised caffeine concentrations, values are means ± S.E. of the mean for at least three separate experiments and between five and nine cells.

The area beneath the current trace (pA × ms or pC) to express the unit Ca$^{2+}$ transient (29), Ca$^{2+}$/pC (pA × s) charge entering the cell. Three voltage steps were used to calculate a mean value of the unit Ca$^{2+}$ transient at each step duration. All experiments were conducted at room temperature. All values are compared with a Student's t test, and values are means ± standard error of the mean. All materials were obtained from Sigma (Poole, UK) except Fura-2, which was from Molecular Probes.

RESULTS AND DISCUSSION

Initial studies showed that these differentiated neuroblastoma cells responded to high concentrations of caffeine, 20–50 mM, with small increases in intracellular Ca$^{2+}$ (Fig. 1A). The responses persisted in the absence of extracellular Ca$^{2+}$, indicating that these cells possess an intracellular, caffeine-sen- sitive Ca$^{2+}$ store. If the NG108–15 cells were first depolarized with 30 mM K$^+$, we observed a rise in intracellular Ca$^{2+}$ as observed previously (30), consistent with influx of Ca$^{2+}$ through N and L-type voltage-sensitive Ca$^{2+}$ channels present on the plasma membrane (open bar, Fig. 1B). Application of caffeine, immediately after the depolarization, then gave a fast and large intracellular Ca$^{2+}$ rise (Fig. 1, B and C). The responses resembled those seen in bullfrog and rat sympathetic neurons and also in rodent central neurons (6, 7, 31). The caffeine responses were blocked by ryanodine (5–10 μM), an antagonist of the ryanodine receptor at these concentrations (see legend to Fig. 1). This result shows that Ca$^{2+}$ entry by prior depolarization sensitized the ryanodine receptors on the intracellular stores to subsequent activation by caffeine and represented a form of CICR. A concentration response curve (Fig. 1D) shows a steep response to caffeine in the presence of the prior depolarization, strongly indicating the operation of an amplification process.

Having established that these neurons possessed a ryanodine-sensitive CICR capability, we next sought to establish whether cadPDR, a positive modulator of the ryanodine receptor, could potentiate CICR in these cells. Since cADPR is not membrane-permeable, we applied it to the cells via a whole cell patch pipette and simultaneously used voltage clamp to control the membrane potential of the cell. This allowed us to control Ca$^{2+}$ influx through voltage-sensitive Ca$^{2+}$ channels. Previous studies have successfully used a method called the unit Ca$^{2+}$ transient to relate the change in intracellular Ca$^{2+}$ levels directly to the amount of charge entering the cell (29, 32, 33). The amount of charge entering the cell was estimated from the area beneath the current trace that represented Ca$^{2+}$ entry, since K$^+$ and Na$^+$ channels were blocked. By dividing the peak Ca$^{2+}$ rise simultaneously recorded during the voltage step, by the area beneath the current trace, we calculated a unit Ca$^{2+}$ transient expressed as nanomolar Ca$^{2+}$ release per picocoulomb of charge entry. As shown in Fig. 2A, increasing the length of the voltage step from 100 to 1000 ms allowed the Ca$^{2+}$ channels to stay open for longer, thus allowing more Ca$^{2+}$ to enter the neuron. The cell in Fig. 2 was recorded using a pipette containing 10 μM cADPR, and it can be seen that relative to a rather modest increase in the area beneath the Ca$^{2+}$ current traces, the longer voltage steps evoked a large increase in the intracellular, simultaneously measured Ca$^{2+}$ level (Fig. 2B). This was consistent with extra Ca$^{2+}$ being released from the intracellular stores triggered by the initial Ca$^{2+}$ entry and had the effect of increasing the absolute value of the unit Ca$^{2+}$ transient, above that seen in a control cell (Fig. 2C). Note also that the red Ca$^{2+}$ trace (Fig. 2B) showed an additional later Ca$^{2+}$ release following the initial peak, a common occurrence in cells treated with cADPR. The effects of cADPR present in the patch pipette are illustrated by the images of two cells with similar charge entry during a depolarizing pulse (Fig. 2D). Particularly notable is the larger Ca$^{2+}$ rise in the cADPR-treated cell compared with control. Also apparent, after application of the depolarizing pulse, was the rise in Ca$^{2+}$ at the edge of the cell before Ca$^{2+}$ rose in the center of the cell, and a striking difference was the relatively larger elevation in Ca$^{2+}$ at the center of the cell compared with control cells (n = 9). This suggested that cADPR increased the likelihood with which elevated Ca$^{2+}$ levels close to the plasma membrane propagated to the center of the cell to give a global Ca$^{2+}$ signal over the whole of the cell (34). When Ca$^{2+}$ levels over the whole cell were measured and compared for all cells, as the duration of the voltage step was increased, the increase in the unit transient was greatest in the...
cells treated with cADPR (Fig. 3A). Controls also demonstrated a rise in the unit transient (33) as more Ca$^{2+}$ entered the cell, and it is tempting to suggest that this was due to low levels of endogenous cADPR found in a number of brain preparations (35). In response to the short 100-ms duration voltage step, the unit Ca$^{2+}$ transient was approximately 1.4 when charge (or Ca$^{2+}$) entry was 23.4 $\pm$ 3.6 pC, rising to a value of around 2.5 (see Fig. 3A), indicating additional release of Ca$^{2+}$ from the internal stores, as the amount of charge (or Ca$^{2+}$) entry was increased to 101.6 $\pm$ 14.5 pC as the length of the voltage step increased to 1000 ms. Addition of 10 $\mu$M cADPR to the patch pipette increased the unit Ca$^{2+}$ transient in two ways. First it increased the value of the unit Ca$^{2+}$ transient, compared with control, even following a short, 100-ms duration voltage step (Fig. 3, filled circles compared with open squares, $p = 0.007$, t test). A direct comparison shows that for a similar charge (or Ca$^{2+}$) entry to the controls, 30.7 $\pm$ 11.6 pC ($p = 0.24$, t test), during the shortest 100-ms duration voltage step, the presence of cADPR in the pipette gave rise to a unit Ca$^{2+}$ transient of approximately 2.8, consistent with release of Ca$^{2+}$ from intracellular stores. Since a similar charge entry in controls failed to elicit Ca$^{2+}$ release from the intracellular stores, but 100 pC could, our result indicates that cADPR reduced, by approximately 3-fold, the amount of Ca$^{2+}$ entry required to trigger additional Ca$^{2+}$ release from the internal stores. Second, as the length of the voltage step was increased and more Ca$^{2+}$ entered the cell, the presence of cADPR resulted in a relatively larger increase in the unit Ca$^{2+}$ transient, compared with the controls (even though the absolute values of charge entry were similar between control and cADPR-treated cells, $p = 0.34$, t test). This suggested that cADPR allowed the extra Ca$^{2+}$ entry evoked by depolarizations longer than 100 ms, to trigger even further release of Ca$^{2+}$ from the intracellular stores. We noted that the values of the unit transient were similar to those observed in isolated dorsal root ganglion cells (33, 36), but approximately 10 $\times$ larger than those observed in bullfrog neurons (29). This difference could relate to the much larger amplitude of the Ca$^{2+}$ currents in the bullfrog neurons and may reflect a greater degree of Ca$^{2+}$ buffering of the larger Ca$^{2+}$ entry compared with the smaller currents evoked in these mammalian neurons.

Inositol triphosphate also enhances release of Ca$^{2+}$ from intracellular stores in a Ca$^{2+}$-dependent manner, so called inositol triphosphate-induced Ca$^{2+}$ release (ICR) (2, 34). However we did not observe any effect on the unit Ca$^{2+}$ transient when 50 $\mu$M InsP$_3$ was included in the patch pipette, even though a number of studies suggest that these neuroblastoma cells express InsP$_3$-sensitive intracellular Ca$^{2+}$ stores (37–39) (mean value in the presence of InsP$_3$ following a 1-s pulse was
Ca$^{2+}$ transient to a level 3-fold over control, indicating that cADPR action reached a maximum.

Our results show that this neuronal cell line possessed ryanodine- and caffeine-sensitive intracellular Ca$^{2+}$ stores. Cyclic ADP-ribose, in the presence of Ca$^{2+}$ influx, increased the amount of Ca$^{2+}$ released from the intracellular store in these cells and also increased the sensitivity with which Ca$^{2+}$ entry triggered additional Ca$^{2+}$ release leading to a global intracellular Ca$^{2+}$ rise. These actions of cADPR suggest a requirement for powerful regulatory mechanisms to control the production of cytosolic levels of cADPR from its ubiquitous precursor, β-NAD$^+$ (40). As Ca$^{2+}$ release from internal stores becomes more widely recognized as part of neuronal Ca$^{2+}$ homeostasis, an increased understanding of the factors controlling endogenous levels of cADPR in neurons is now required.

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