The Type 2 Ryanodine Receptor of Neurosecretory PC12 Cells Is Activated by Cyclic ADP-ribose

ROLE OF THE NITRIC OXIDE/cGMP PATHWAY*

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Emilio Clementi§, Maria Riccio§, Clara Sciorati§, Giuseppe Nistico, and Jacopo Meldolesi§

From the 1Department of Pharmacology, Faculty of Pharmacy, University of Reggio Calabria, 88021 Catanzaro, Italy, the 1Department of Biology, Mondino Neurobiology Centre, University of Roma “Tor Vergata,” 00133 Roma, Italy, and the §Consiglio Nazionale delle Ricerche, Molecular and Cellular Pharmacology Centre and the Department of Pharmacology, B. Ceccarelli Centre, University of Milano, Dipartimento di biotecnologie, San Raffaele Scientific Institute, 20132 Milano, Italy

Of two neurosecretory PC12 cell clones that respond to NO donors and 8-bromo-cGMP with similar increases in cADP-ribose and that possess molecularly similar Ca\(^{2+}\) stores, only one (clone 16A) expresses the type 2 ryanodine receptor, whereas the other (clone 27) is devoid of ryanodine receptors. In PC12-16A cells, activation of the NO/cGMP pathway induced slow [Ca\(^{2+}\)]\(_i\) responses, sustained by release from Ca\(^{2+}\) stores. In contrast, PC12-27 cells were insensitive to NO donors. Likewise, in PC12-16A cells preincubated with NO donors, Ca\(^{2+}\) stores were partially depleted, as revealed by a test with thapsigargin, whereas those in clone 27 were unchanged. The NO-induced Ca\(^{2+}\) release was increased synergistically by caffeine, and the corresponding store depletion was magnified by ryanodine. The specificity for the NO/cGMP pathway was confirmed by the effects of two blockers of cGMP-dependent protein kinase I, while the role of cADP-ribose was demonstrated by the effects of its antagonist, 8-amino-cADP-ribose, administered to permeabilized cells. These results demonstrate in neurosecretory cells a ryanodine receptor activation pathway similar to that known in sea urchin oocytes. The signaling events described here could be of great physiological importance, especially in the nervous system.

Ryanodine receptors are a family of intracellular Ca\(^{2+}\) channels coded by different genes, recognized to play important roles in the homeostasis of the cation. For quite some time, the expression of these channels was believed to be strictly muscle-specific, with types 1 and 2 sustaining excitation-contraction coupling in skeletal and cardiac fibers, respectively (1). Recently, however, the two types (as well as type 3, initially known in the ovary) of RyR receptors, sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCa) and endoplasmic reticulum Ca\(^{2+}\)-binding proteins, are similarly expressed in the two clones (19–21). By the systematic, comparative investigation of these clones, [Ca\(^{2+}\)]\(_i\) events of small dimension were unambiguously revealed by both direct and indirect experimental approaches. The analysis of these processes led, on the one hand, to the dissection of the signaling pathway going from NO generation to cGMP, cADP-ribose, and RyR activation and, on the other hand, to the identification of the physiological role of this system in PC12 cells, revealing possible implications for neuronal cell functions.

The abbreviations used are: RyR, ryanodine receptor; IP\(_3\), inositol 1,4,5-trisphosphate; SERCA, sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase; SNAP, 5-nitroso-N-acetylpenicillamine; (R\(_1\))-8-Br-cGMP-5-S, (R\(_3\))-8-bromoguanosine 3’5’-monophosphorothioate; SNP, sodium nitroprusside; Ab, antibody; KRM, Krebs-Ringer Heps; MOPS, 3-(N-morpholino)propanesulfonic acid.
EXPERIMENTAL PROCEDURES

Materials—Culture sera and media were purchased from Gibco (Basel, Switzerland). cADP-ribose was from Alexion Corp. (Läufelfingen, Switzerland). Fura-2, fluo-3, KT5823, S-nitroso-N-acetylpenicillamine (SNAP), thapsigargin, ryanodine, and ionomycin from Calbiochem (Baden Soden, Germany). (R)-8-Br-cGMP-S was from Biolog (Bremen, Germany). 8-Amino-cADP-ribose was from Molecular Probes (Leiden, The Netherlands). cADP-ribose was purified using a triethylammonium bicarbonate gradient at a flow rate of 1 ml/min. Under these conditions, the cADP-ribose standard eluted at 6.8 min; ADP, ATP, and IP3 standards eluted at 14.2, 22.5, and 33 min, respectively. The eluate from PC12 samples was collected in 1-mL fractions. Fractions 6–11 were pooled, dried, and stored at −20 °C until use.

Purification and Measurement of cADP-ribose Concentration—PC12 cell suspensions were loaded for 30 min at 37 °C in KRHM medium with fura-2/AM (5 μM) and kept at 37 °C until use. Aliquots (4 × 10^6 cells in 1.5 ml) were suspended in KRHM containing excess (3 mM) EGTA (Ca^{2+}-free medium; estimated [Ca^{2+}]_i < 10^-6 M) and transferred to a thermostatted cuvette (37 °C) maintained under continuous stirring in a Perkin-Elmer LS-5B fluorometer. Samples were then preincubated with or without the drugs interfering with the NO pathway (SNP, SNAP, 8-Br-cGMP, KT5823, and (SNAP), thapsigargin, ryanodine, and ionomycin from Calbiochem (Baltimore, Md.). Caffeine, sodium nitroprusside (SNP), 8-Br-cGMP, and the remaining chemicals were from Sigma (Milan, Italy). The antibodies used have been described elsewhere: anti-protein-disulfide isomerase, a rabbit polyclonal antibody (Ab), by Villa et al. (22); anti-SERCA, a mouse monoclonal Ab, by Colyer et al. (23); and anti-IP₃ receptor, a rabbit polyclonal Ab, by Villa et al. (24). The polyclonal rabbit antisera specific for RyR proteins 1–3 (described in Ref. 3) were the kind gift of V. Sorrentino (Dipartimento di biotecnologie, Milano, Italy).

PC12 Cell Clone Selection and Culture—Of the previously isolated panel (19, 21), two PC12 cell clones were used (clones 16A and 27), the former sensitive and the latter insensitive to the RyR-active drugs, i.e. ryanodine and caffeine (19). PC12 cells were routinely grown as described (19) and used before the tenth passage of thawing. The day of the experiment, cell monolayers were detached from Petri dishes by a gentle flow of Krebs-Ringer Hepes (KRH) medium containing 125 mM NaCl, 5 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 6 mM glucose, and 25 mM HepesNaOH (pH 7.4). After three washes by centrifugation, cells were then resuspended in the medium necessary for the various experimental procedures. Viability in the presence or absence of the drugs employed was ±95% as assessed by the trypan blue exclusion test.

Microsome Preparation, SDS-Polyacrylamide Gel Electrophoresis, and Western Blotting—All operations were performed at 4 °C. Washed cell pellets were homogenized by 40 strokes of a Teflon/glass homogenizer in 0.32 m sucrose buffer containing 0.1 mM phenylmethylsulfonyl fluoride. Total microsomal fractions were separated as described (25), and protein content was assayed by the bicinchoninic acid procedure (Pierce). After the addition of SDS and β-mercaptoethanol, the samples were boiled, and 300 μg of protein/lane was loaded into the slots of 3–8% gradient SDS-polyacrylamide gels, which were run as described elsewhere (24). High efficiency transfer of proteins onto nitrocellulose membranes was carried out at 200 mA for 18 h in buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.3). After transfer, both the gels and the blots were routinely stained with Ponceau red. For Western blotting, the nitrocellulose sheets were processed at room temperature, first for 1 h with phosphate-buffered saline + 3% bovine serum albumin and then for 2 h with appropriate concentrations of the specific Abs in the same buffer. After washing five times for 5 min with 150 mM NaCl, 50 mM Tris-HCl, 0.05% Tween 20, and 5% powdered milk (pH 7.4), the membranes were decorated with 125I-protein A. The blots were washed five times for 10 min with the above buffer, dried, and finally autoradiographed at −80 °C for variable periods of time. Microdensitometry of the relevant bands of immunoblots was carried out using a Molecular Dynamics Imagequant apparatus (26). Results shown are representative of two to four experiments.

For the experiments measuring [Ca^{2+}]_i in permeabilized, cell-free systems, aliquots of 6 × 10^6 cells were washed twice with an intracellular-like solution supplemented with an ATP-regenerating system (containing 100 mM KCl, 20 mM NaCl, 3.5 mM MgCl2, 1 mM ATP, 10 mM phosphocreatine, 3 units/ml phosphocreatine kinase, and 20 mM MOPS (pH 7.2)). After resuspension in 0.7 ml of the same medium, the cells were transferred to the thermostatted cuvette, supplemented with 4 μM fluo-3, and subsequently permeabilized with 60 μg of digitonin. This treatment yielded >95% cells permeable to trypan blue. Results are shown as traces, representative of results obtained in six experiments, and graphs, showing means ± S.D. of four to eight experiments.

RESULTS

Fig. 1 shows the characterization of important molecular components active in the rapidly exchanging Ca^{2+} stores of the two PC12 cell clones selected for this study, clones 16A and 27. When tested by Western blotting using three antisera, each specific for one of the three RyR types, clone 16A was found to express only type 2, whereas no signal at all was detected from clone 27. This observation is consistent with previous data demonstrating the lack of response of clone 27 cells to caffeine and ryanodine, whereas clone 16A was responsive to both these RyR-specific drugs (19). The difference in RyR expression between the clones con-
trasts with the similar levels of expression of the other Ca^{2+}
store components such as the SERCA Ca^{2+}-pumps, the IP_{3}
receptor, and endoplasmic reticulum luminal Ca^{2+}-binding
proteins, i.e. protein-disulfide isomerase (Fig. 1), calreticulin,
and BiP (21). Moreover, the two clones were similarly respon-
sive to IP_{3} generated via surface receptor activation (19), while
their resting cytosolic cADP-ribose levels differed by ~35%
(Table I), falling, however, in both cases within the range of
values previously reported in rat brain (27).

When incubated in the presence of the NO donor, SNP (300
µM), the cells of both clones showed similar, ~100% increases
in the cADP-ribose level, which were largely (~90%) prevented
if the treatment was carried out in the presence of the specific
cGMP-dependent protein kinase I blocker, KT5823 (10 µM)
(Table I) (30, 32). The present observations identify ADPribose
cyclase as a new target of NO/cGMP, a transduction
pathway known to be present and functional in PC12 cells
(29–31, 33).

In a first attempt to reveal any NO-initiated, cADP-ribose-
triggered stimulation of Ca^{2+} release, cells from the two PC12
dones were loaded with the specific Ca^{2+} dye, fura-2, and then
exposed to various concentrations of two NO donors, SNP and
SNAP, while suspended in EGTA-containing, Ca^{2+}-free
medium. Under these conditions, no appreciable [Ca^{2+}]i increase
responses were detected (data not shown). Because of the high
Ca^{2+} buffering capacity of the cell cytosol (34) and the conti-
nuous efflux due to the plasmalemma Ca^{2+} pump and the Na^{+}/
Ca^{2+} exchanger, slow kinetics of [Ca^{2+}]i increase can escape
direct measurement with the fura-2 technique (25). When the
experiments were carried out in the presence of 100 µM La^{3+}
(a blocker of the pump), using a Na^{+}-free, lightly buffered sucrose
medium to block the exchanger (0.3 M sucrose, 10% gelatin, and
5 mM Hepes/Tris (pH 7.4)), Ca^{2+} release responses became
appreciable, but only in the clone 16A cells (Fig. 2, left trace).
As can be seen, simple incubation under the above conditions
failed to modify [Ca^{2+}]i. However, when either SNP (300 µM) or
SNAP (data not shown) was administered, a slow, yet signifi-
cant rise started, reaching levels ~30% above resting values
within 6–7 min. In contrast, in the RyR-defective clone 27 cells,
the NO donors failed to induce any effect on [Ca^{2+}]i, even when
administered in the La^{3+}-containing, Na^{+}-free medium (Fig. 2,
right trace).

Revelation of Ca^{2+} release by NO donors did not necessarily
require the use of the La^{3+}-containing, Na^{+}-free medium. Ev-
idence was also obtained with cells bathed in the conventional
EGTA-containing, Ca^{2+}-free medium using an indirect ap-
proach. The method is based on comparison of the [Ca^{2+}]i
responses elicited by thapsigargin, an irreversible SERCA
blocker that induces leakage of Ca^{2+} from the endoplasmic
reticulum (35), administered in parallel to cells pretreated or
not for 10 min with NO donors. If, during the preincubation,
the stores were depleted, at least in part, by activation of
ryanodine receptors, the subsequent thapsigargin treatment
was expected to yield diminished [Ca^{2+}]i responses, but only in the
RyR-expressing clone 16A. The results shown in the graphs of
Fig. 2 demonstrate that this is indeed the case. The effects of
the two NO donors on clone 16A were similarly dose-depend-
ent, and they were mimicked by incubation of the cells with
8-Br-cGMP (500 µM) (Fig. 2, left panel). However, when the NO
donors were administered together with cGMP-dependent pro-
tein kinase I blockers, either KT5823 (10 µM) or the structur-
ally unrelated compound (R_{2})-8-Br-cGMP-S (30 µM) (30, 36),
the inhibition of the thapsigargin responses was prevented
(Fig. 2, left panel). When the RyR-defective clone 27 cells were
incubated with SNP, SNAP, or 8-Br-cGMP administered at the
same concentrations as described above, no changes in the
subsequent thapsigargin-induced [Ca^{2+}]i responses were ob-
erved (Fig. 2, right panel). Taken together, these data suggest
that NO is able to induce partial depletion of intracellular Ca^{2+}
stores via an action ultimately occurring at the level of RyR2
and involving the activation of the cGMP/cGMP-dependent
protein kinase I signal transduction pathway.

Further evidence confirming the role of agents generated in
response to activation of the NO/cGMP-dependent protein ki-
ase I pathway in the control of RyR activity was obtained by
experiments with the plant alkaloid ryanodine (Fig. 3). When
administered at low concentration, this drug is known to in-
duce a persistent activation of ryanodine receptors that, how-
ever, is use-dependent, i.e. it occurs only when the channels
have been induced to open by another agent (1, 19). In this
series of experiments, fura-2-loaded PC12 cells, while sus-

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### Table I

| Treatment          | cADP-ribose, pmol/mg protein |
|--------------------|-----------------------------|
| None               | 2.82 ± 0.08, 2.05 ± 0.18    |
| SNP (300 µM)       | 5.76 ± 0.41, 4.10 ± 0.17    |
| SNP (300 µM) + KT5823 (10 µM) | 3.24 ± 0.33, 2.29 ± 0.36 |

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**Fig. 2. Effects of SNP, SNAP, 8-Br-cGMP, KT5823, and (R_{2})-8-Br-cGMP-S on [Ca^{2+}]i responses.** Traces illustrate results in PC12-16A and PC12-27 fura-2-loaded cells suspended in La^{3+}-containing, Na^{+}-free medium. Dashed traces refer to untreated controls; continuous traces indicate cells challenged with SNP (300 µM), added where indicated. Bar graphs illustrate the results in fura-2-loaded cells, suspended in EGTA-containing, Ca^{2+}-free medium, pretreated or not for 10 min with the various drugs at the indicated concentrations and then challenged with thapsigargin (30 nM). Traces are representative of six consistent experiments. Graphs show means ± S.D. of eight experiments, expressed as the percent increase in [Ca^{2+}]i over basal resting values, measured at the peak of the response.
pended in Ca\(^{2+}\)-containing (instead of Ca\(^{2+}\)-free) medium, were pretreated with various combinations of NO donors (SNP and SNAP), 8-Br-cGMP, and cGMP-dependent protein kinase I blockers, with or without ryanodine (3 \(\mu M\)). At the end of the preincubation, the cells were rapidly washed and transferred to EGTA-containing, Ca\(^{2+}\)-free medium, after which thapsigargin (100 nm) or caffeine (100 nm) was administered. Under these conditions, preincubations of PC12-16A cells with ryanodine, KT5823, or (\(R_p\))-8-Br-cGMP-S alone were without appreciable effect on the subsequent thapsigargin- and caffeine-induced responses (data not shown). Likewise, preincubations with SNP, SNAP, or 8-Br-cGMP alone remained ineffective, suggesting that the release revealed when experiments were entirely carried out in Ca\(^{2+}\)-free medium (as in Fig. 2) had been compensated by Ca\(^{2+}\) reuptake during incubation and washing. In contrast, when PC12-16A cells were pretreated with SNP, SNAP, or 8-Br-cGMP together with ryanodine, KT5823, or (\(R_p\))-8-Br-cGMP-S alone were without appreciable effect on the subsequent thapsigargin- and caffeine-induced responses (data not shown). These effects of the combinations of NO donors and 8-Br-cGMP with ryanodine were almost completely prevented when cGMP-dependent protein kinase I inhibitors were administered during preincubation. When, on the other hand, similar experiments were performed with the RyR-defective clone 27, none of the above preincubation treatments had any appreciable effect on the subsequent thapsigargin-induced responses, irrespective of the presence of ryanodine (Fig. 3C).

Most of the evidence so far presented in favor of the role of the NO/cGMP-dependent protein kinase I pathway in the control of intracellular Ca\(^{2+}\) release was obtained indirectly, by measuring the effects of drugs interfering with the NO/cGMP signaling on the thapsigargin- and caffeine-induced [Ca\(^{2+}\)] responses. To obtain direct evidence, two different experimental approaches were employed. The first is based on the quantitative evaluation of the [Ca\(^{2+}\)] responses triggered by different concentrations of caffeine in PC12-16A cells, preincubated or not with SNP (300 \(\mu M\), 1 min) while bathed in Ca\(^{2+}\)-containing medium. As can be seen (Fig. 4), the [Ca\(^{2+}\)] responses to the latter drug were markedly (up to 100%) increased by the pretreatment with the NO donor, revealing a synergistic interaction between two mechanisms of RyR activation: Ca\(^{2+}\)-induced Ca\(^{2+}\) release, whose threshold is known to be lowered by caffeine (1), and the mechanism initiated by NO/cGMP.

In the second approach, attention was moved from the cytosol, where slow Ca\(^{2+}\) release responses can be hidden by buffering (34), to the extracellular space, which is the ultimate
destination of most of the released cation, as indicated, for example, by the results obtained in the La$^{3+}$-containing, Na$^{+}$-free medium reported in Fig. 2. To obtain quantitative data, cells were loaded at equilibrium (72 h) with $^{45}$Ca, and the release of radioactivity to the extracellular medium was measured (Fig. 5). Incubation of clone 16A cells in EGTA-containing, Ca$^{2+}$-free medium with SNP (300 $\mu$M), SNAP (300 $\mu$M), or 8-Br-cGMP (500 $\mu$M) induced sustained increases of $^{45}$Ca release to the medium, distinctly greater than the release from control, untreated cells. Such release was greatly enhanced when NO donors were administered together with ryanodine (data not shown). Co-incubation with either KT5823 (10 $\mu$M) or (R)-8-Br-cGMP-S (30 $\mu$M) completely abolished the effects of NO donors (Fig. 5, left panel) (data not shown). Based on these results and the data in Ref. 20, the rate of the NO-induced release of Ca$^{2+}$ from the cells can be calculated to be $-0.12$ nmol/mg of protein/min. That this release originates from the rapidly exchanging stores is shown by the nonadditive nature of the $^{45}$Ca release elicited by the subsequent administration of thapsigargin. In contrast, under these conditions, no changes were observed in the effect induced by the ensuing administration of ionomycin, a Ca$^{2+}$/H$^+$ exchanger ionophore that releases Ca$^{2+}$ from all stores except those with an acidic luminal environment (20). When similar experiments were performed in the RyR-defective clone 27, neither the basal $^{45}$Ca release nor that induced by thapsigargin and ionomycin was changed by pretreatment with NO donors (Fig. 5, right panel). Based on these results, we conclude that the Ca$^{2+}$ release responses of the RyR-defective cells from those observed in untreated controls (Fig. 6B).

The permeabilized cell approach was also employed to reveal the concentration dependence and the magnitude of the cADP-ribose-induced Ca$^{2+}$ release responses. Fig. 7 shows the results obtained by measuring directly the [Ca$^{2+}$] changes observed after application of cADP-ribose and the results of the thapsigargin test investigated in parallel. As can be seen in the graph, the thapsigargin-induced responses in PC12-16A cells were eliminated in a dose-dependent manner by pretreatment with cADP-ribose in the 0.1–30 $\mu$M range. cADP-ribose-induced Ca$^{2+}$ release into the medium, monitored using fluo-3, was clearly visible at 0.3 $\mu$M cADP-ribose and above (Fig. 7, graph and continuous trace). No such responses were observed when the tests with PC12-16A cells were carried out in the presence of 100 $\mu$M 8-amino-cADP-ribose, a specific antagonist of cADP-ribose (37), the effect of the NO donors was no longer seen (Fig. 6A). Similar experiments were performed also with clone 27 cells. As with the assays in intact cells, also after permeabilization, the treatment with NO donors did not modify significantly the responses of the RyR-defective cells from those observed in untreated controls (Fig. 6B).

**DISCUSSION**

Since its discovery (38), cADP-ribose has been extensively investigated as an activator of RyR, but with conflicting results. In particular, conclusive evidence concerning both the Ca$^{2+}$ release activity and the intracellular generation pathway has been obtained in only sea urchin oocytes (5–7), where, however, ryanodine receptors are molecularly different from...
This latter event is shown here to induce moderate activation of cGMP-dependent protein kinase I (29–31, 33). The application of NO donors with generation of cGMP and other. Moreover, PC12 cells were already known to respond to the series of events initiated by NO and mediated by the pharmacological criteria, appropriate for the demonstration of range. The operational approach was based on widely accepted and other possible RyR regulators are in the physiological range. The results we obtained after stimulation of the NO/cGMP pathway revealed a modest but consistent activation of type 2 ryanodine receptors, detected by both direct and indirect approaches. Such an activation (i) took place when NO donors (or cGMP) were administered to otherwise resting cells; (ii) required the function of cGMP-dependent protein kinase I inasmuch as it was inhibited by specific blockers; and (iii) was entirely due to cADP-ribose generation since the antagonist, 8-amino-cADP-ribose, was able to completely block the effects of either the gaseous messenger or the cyclic nucleotide. The simplest, although experimentally not demonstrated, explanation of our data is that of a phosphorylation by cGMP-dependent protein kinase I up to increased cADP-ribose levels. The results we obtained after stimulation of the NO/cGMP pathway revealed a modest but consistent activation of type 2 ryanodine receptors, detected by both direct and indirect approaches. Such an activation (i) took place when NO donors (or cGMP) were administered to otherwise resting cells; (ii) required the function of cGMP-dependent protein kinase I inasmuch as it was inhibited by specific blockers; and (iii) was entirely due to cADP-ribose generation since the antagonist, 8-amino-cADP-ribose, was able to completely block the effects of either the gaseous messenger or the cyclic nucleotide. The simplest, although experimentally not demonstrated, explanation of our data is that of a phosphorylation by cGMP-dependent protein kinase I.

Another interesting property of the cADP-ribose-induced Ca²⁺ release, i.e., its synergism with Ca²⁺-induced Ca²⁺ release from permeabilized PC12 cells. Experimental conditions were as described for Fig. 6. After permeabilization and Ca²⁺ loading, the cells were treated for 10 min with increasing concentrations of cADP-ribose (cADPR). Thapsigargin (Tg; 100 nM) was subsequently added. Black and white symbols in the graph refer to PC12-16A and PC12-27 cells, respectively. Squares indicate the response to thapsigargin, measured as percent of the response observed in cells incubated without cADP-ribose. Circles indicate the peak [Ca²⁺]i increase induced by cADP-ribose, measured as the percent increase over basal values. Traces show typical responses to 10 μM cADP-ribose, added where indicated, in PC12-16A (continuous trace) and PC12-27 (dashed trace) cells. Results illustrated in the graph are means ± S.D. of four experiments; traces are representative of six experiments. Dig, digitonin.

**Table 2** A, Galione, personal communication.
lease, already demonstrated in sea urchin oocytes (40), was revealed by the experiments with caffeine, a drug known to act by lowering the threshold of the latter process (1). When a NO donor was administered together with the xanthine, the overall effect was a doubling of the already considerable response to caffeine alone, much greater than the sum of the latter with the modest, NO-initiated response, which by itself was hardly appreciable by the fura-2 approach.

In spite of their modest size, the Ca^{2+} release responses initiated by NO could be of great physiological importance, especially in neurons. Most of these cells appear to express type 2 ryanodine receptors (2, 3) together with the Ca^{2+}-dependent, constitutive type I NO synthase and to use NO for regulatory functions of crucial importance (41). In these neurons, appropriate increases in [Ca^{2+}], are expected to activate ryanodine receptors by the two synergistically interactive mechanisms: not only Ca^{2+}-induced Ca^{2+} release, but also the NO/cGMP/cADP-ribose pathway. Because of its well known property of rapid diffusion, the gaseous messenger and the ensuing cGMP/cADP-ribose events could then facilitate the spread of RyR activity to adjacent areas of the same and even of surrounding cells, thus contributing significantly not only to the regulation, but also to the extension of the response. Moreover, in some neurons, IP_{3} and ryanodine/cADP-ribose-sensitive areas of the Ca^{2+} stores have been shown to be distinct (42). Therefore, in these cells, a response initiated in one area by receptor-triggered IP_{3} generation could then move to different areas when sustained by NO and cADP-ribose. It should also be emphasized that activation of cGMP-dependent protein kinase I is known to regulate negatively the generation of IP_{3}, with ensuing blunting of the [Ca^{2+}], responses mediated by that second messenger (30). Taken together, these dynamic processes might ultimately be of great importance in the subtle, microdomain-associated events that sustain, for example, neuronal plasticity.

Finally, our experiments with permeabilized PC12 cells have revealed that the Ca^{2+} release responses induced by direct application of cADP-ribose can be greater than those induced via the NO/cGMP pathway. These results suggest that a limiting step in cADP-ribose-induced intracellular Ca^{2+} release is cADP-ribose formation. In other cells, in particular in some neurons, it appears reasonable to expect the contribution of cADP-ribose to the Ca^{2+} release to be greater than shown here for PC12 cells. The extension of this study to well characterized neuron populations might therefore ultimately contribute to shedding light on a variety of aspects of cell physiology that so far have not been adequately investigated.

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