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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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 cloned from a cultured epithelial line) have been shown to be expressed also in a variety of nonmuscle cells, including neurons and neurosecretory cells (2, 3).

From the functional point of view, the various ryanodine receptors exhibit considerable differences. The primary activation mechanisms vary: direct coupling to surface Ca^{2+} channels for type 1 and Ca^{2+}-induced Ca^{2+} release for type 2 (1). Other events are also known to contribute to channel opening, including the binding of ATP and calmodulin, and phosphorylation of the channels (or of adjacent proteins) by cAMP-, cGMP- and Ca^{2+}/calmodulin-dependent protein kinases (1, 4). Moreover, studies carried out initially in sea urchin oocytes have revealed a role for a putative second messenger, the NAD^+ derivative cADP-ribose (5). This molecule is synthesized by a family of cytosolic enzymes, the ADP-ribosyl cyclases, the activities of which are controlled by NO working via the activation of guanylyl cyclase (6, 7). Except for oocytes, however, cADP-ribose physiology is still controversial. A trigger role for Ca^{2+} release has been reported in a few types of rat neurons and glandular cells (8–10). In contrast, in other systems, the effect of cADP-ribose, when present, was shown to consist of the positive modulation of otherwise stimulated Ca^{2+} release responses (11–15). As recently pointed out (4), these conflicting results may be explained, at least in part, by the critical dependence of RyR^1 opening on the concentration of ions (Mg^{2+} and Ca^{2+}) and nucleotides (ATP, ADP, and NAD^+). Even the specificity of cADP-ribose action (whether it is active only on type 2 or on both types 1 and 2 and possibly also on type 3) is still debated (4, 16–18).

Two isolated clones of PC12 pheochromocytoma cells, a widely employed nerve cell model, possess favorable properties for investigating the role of cADP-ribose in Ca^{2+} homeostasis. Of these clones, one is in fact completely devoid of RyR, and the other expresses a single identified type (type 2). In contrast, other components of the intracellular Ca^{2+} stores, i.e. inositol 1,4,5-trisphosphate (IP_3) receptors, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases (SERCAs), 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.

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[1] The abbreviations used are: RyR, ryanodine receptor; IP_3, inositol 1,4,5-trisphosphate; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; SNAP, S-nitroso-N-acetylpenicillamine; (R_p)-8-Br-cGMP-S, (R_p)-8-bromoguanosine 3′:5′-monophosphorothioate; SNP, sodium nitroprusside; Ab, antibody; KRH, Krebs-Ringer Hepes; MOPS, 3-(N-morpholino)propanesulfonic acid.

EXPERIMENTAL PROCEDURES

Materials—Culture sera and media were purchased from GIBCO (Basel, Switzerland). cADP-ribose was from Alexis Corp. (Läufelfingen, Switzerland). Fura-2, fluo-3, KT5823, *S*-nitroso-*N*-acetylpenicillamine (SNAP), thapsigargin, ryanodine, and ionomycin from Calbiochem (Baden Soden, Germany). (R_p)-8-Br-cGMP-S was from Biolog (Bremen, Germany). 8-Amino-cADP-ribose was from Molecular Probes (Leiden, The Netherlands). ^{45}Ca was purchased from Amersham International (Buckinghamshire, United Kingdom). Caffeine, sodium nitroprusside (SNP), 8-Br-cGMP, and the remaining chemicals were from Sigma (Milano, 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-IP$_3$ receptor, a rabbit polyclonal Ab, by Villa *et al.* (24). The polyclonal rabbit antisera specific for RyR types 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 week 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 KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 2 mM CaCl$_2$, 6 mM glucose, and 25 mM Hepes/NaOH (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 prepared 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 blotted bands were decorated with ^{125}I-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.

Purification and Measurement of cADP-ribose Concentration—PC12 cells, resuspended in KRH medium, were treated or not with SNP (300 μM) or with SNP plus KT5823 (10 μM) for 15 min at 37 °C. Cells were then centrifuged and rapidly frozen in liquid nitrogen. cADP-ribose was purified by an adaptation of the method of Walseth *et al.* (27). Essentially, 3 M perchloric acid was added to frozen cell pellets, which were allowed to thaw at the melting temperature of perchlorate (−10 °C). After rapid mixing, ice-cold water was added to dilute the acid to a final concentration of 0.5 M, and the extracts were sonicated for 30 s in a Branson 3200 apparatus. An aliquot of each suspension was saved for protein determination. Samples were then centrifuged at 15,000 × g for 10 min, and the supernatants were neutralized by the addition of 1 M KHCO$_3$. After centrifugation at 15,000 × g for 10 min, the supernatants were evaporated to dryness on a Savant SpeedVac concentrator and kept at −80 °C until use. The dried samples were reconstituted with 1 ml of 10 mM triethylammonium bicarbonate. cADP-ribose was purified on a Hi Trap Q anion-exchange column (Pharmacia-LKB, Uppsala) using a triethylammonium bicarbonate gradient (27) at a flow rate of 1 ml/min. Under these conditions, the cADP-ribose standard eluted at 6.8 min; ADP, ATP, and IP$_3$ 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. To determine cADP-ribose levels, a Ca^{2+} release bioassay using sea

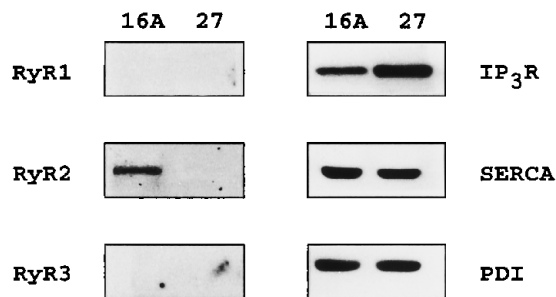FIG. 1. **Characterization of endoplasmic reticulum markers in PC12 cell clones 16A and 27.** 300 μg of protein from microsomal fractions were separated on 3–8% SDS-polyacrylamide gels and then transferred to nitrocellulose sheets. Western blots, decorated with Abs specific for ryanodine receptors (types 1–3), the IP$_3$ receptor (*IP$_3$R*), protein-disulfide isomerase (*PDI*), or SERCAs, were revealed by ^{125}I-protein A.

urchin egg homogenates was set up as described (27), in which Ca^{2+} released by each sample was compared with that released by known amounts of cADP-ribose standards (28). The results shown are means ± S.D. of three experiments.

[Ca^{2+}]$_i$ Measurements in Intact and Permeabilized Cells—PC12 cell suspensions were loaded for 30 min at 37 °C in KRH 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 KRH medium containing excess (3 mM) EGTA (Ca^{2+}-free medium; estimated [Ca^{2+}]$_o$ < 10^{-8} 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 (R_p)-8-Br-cGMP-S) for 10 min at 37 °C, a condition known to induce maximal effects in PC12 cells (29–31). In the experiments in which ryanodine was employed, the 10-min preincubation was performed in Ca^{2+}-containing KRH medium, after which the cells were centrifuged, washed three times in the same medium, and finally resuspended in EGTA-containing medium for the fluorometric analysis. The experiments were initiated by adding the Ca^{2+}-mobilizing agents (thapsigargin or caffeine) to the cell suspensions. [Ca^{2+}]$_i$ values were determined as described (19).

For the experiments measuring [Ca^{2+}] 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 MgCl$_2$, 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.

^{45}Ca Measurements—PC12 cells were grown as described above, except that during the last 72 h, their incubation medium was supplemented with ^{45}Ca (4 μCi/ml). Labeled cells were extensively washed and resuspended in plain KRH medium. An aliquot of the suspension was immediately centrifuged, and the ensuing pellet was used for the measurement of total cell ^{45}Ca content. The rest was incubated at 37 °C in EGTA-containing KRH medium. At different times, aliquots of 1 × 10^6 cells were centrifuged, and the ^{45}Ca recovered in the medium was assayed in a Beckman β-counter (for further details, see Ref. 20). Results shown are means ± S.D. of four separate experiments.

RESULTS

Fig. 1 shows the characterization of important molecular components active in the rapidly exchanging Ca^{2+} stores of the two PC12 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-

PC12 cell suspensions were treated with the drugs indicated for 10 min at 37 °C. Cells were then pelleted and frozen. cADP-ribose was extracted and purified from cell lysates on a Hi Trap Q anion-exchange column and measured with a bioassay system using sea urchin oocyte homogenates. Results given are means ± S.D. of three experiments.

Treatment	cADP-ribose	
	16A	27
	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

trasts with the similar levels of expression of the other Ca^{2+} store components investigated: 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 responsive 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 ADP-ribosyl 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 clones 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 continuous 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 (26). 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 significant 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. Evidence was also obtained with cells bathed in the conventional EGTA-containing, Ca^{2+}-free medium using an indirect approach. 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
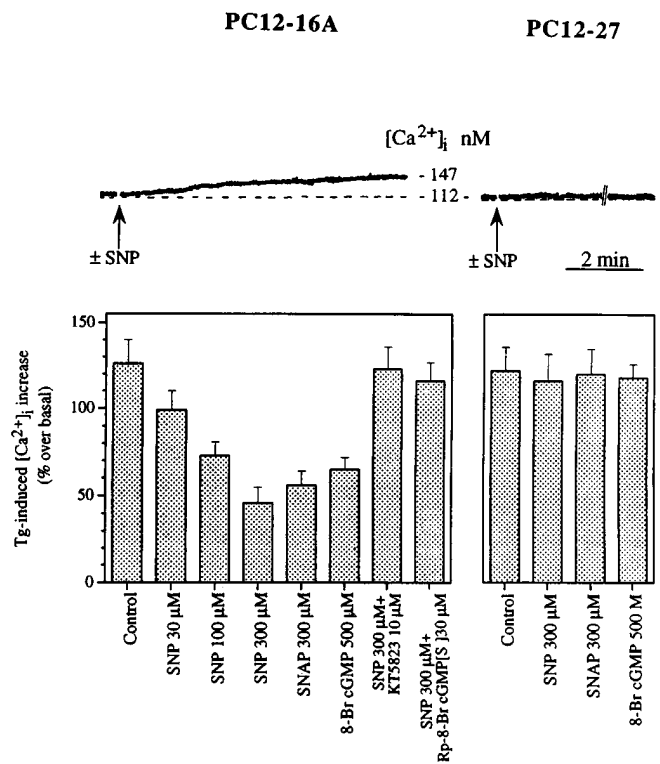
PC12-16A   **PC12-27**

FIG. 2. **Effects of SNP, SNAP, 8-Br-cGMP, KT5823, and (R_p)-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 (*Tg*; 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.

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-dependent, 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 protein kinase I blockers, either KT5823 (10 μM) or the structurally unrelated compound (R_p)-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 observed (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 kinase 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 induce a persistent activation of ryanodine receptors that, however, 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-
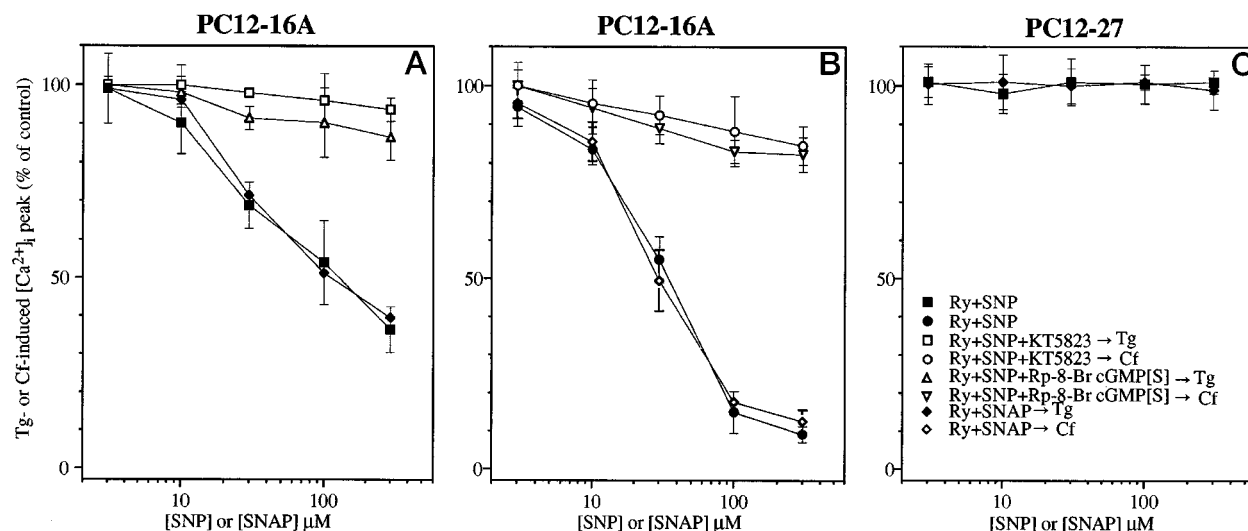
PC12-16A   PC12-16A   PC12-27

FIG. 3. **Effects of SNP, SNAP, 8-Br-cGMP, KT5823, and (R_p)-8-Br-cGMP-S on Ca^{2+} release responses in the presence of ryanodine.** *A* and *B* refer to PC12 cell clone 16A, and *C* refers to PC12 cell clone 27. Fura-2-loaded PC12 cells were pretreated for 10 min in Ca^{2+}-containing medium supplemented with various concentrations of SNP in the presence of 3 μM ryanodine (*Ry*), with or without KT5823 (10 μM) or (R_p)-8-Br-cGMP-S (30 μM). Cells were then washed, resuspended in Ca^{2+}-free medium, and challenged with thapsigargin (*Tg*; 100 nM) (*A* and *C*) or caffeine (*Cf*; 100 mM) (*B*). Results are means ± S.D. of the peak $[Ca^{2+}]_i$ increase responses observed in six experiments, expressed as percent of the response to thapsigargin or caffeine obtained in cells pretreated with medium alone.

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 μM). 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 (30 mM) 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, the subsequent thapsigargin or caffeine release tests revealed considerable and dose-dependent depletions of intracellular Ca^{2+} stores (Fig. 3, *A* and *B*) (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. 3*C*).

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 $[Ca^{2+}]_i$ responses. To obtain direct evidence, two different experimental approaches were employed. The first is based on the quantitative evaluation of the $[Ca^{2+}]_i$ responses triggered by different concentrations of caffeine in PC12-16A cells, preincubated or not with SNP (300 μM, 1 min) while bathed in Ca^{2+}-containing medium. As can be seen (Fig. 4), the $[Ca^{2+}]_i$ responses to the latter drug were markedly (up to 100%) increased by the pretreatment with the NO donor,

PC12-16A
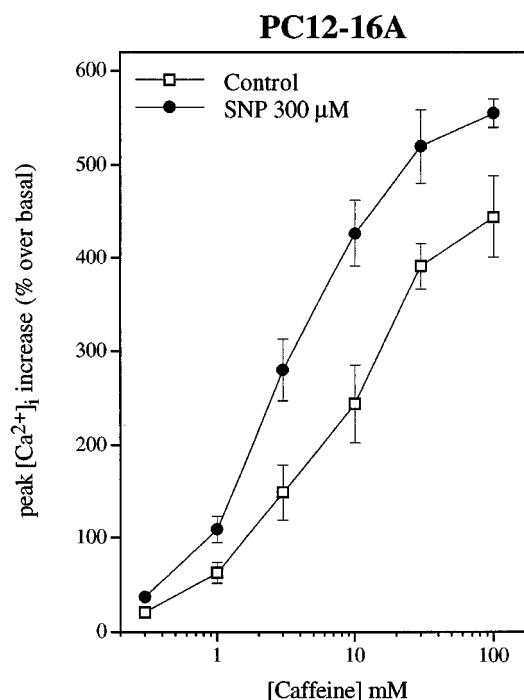

FIG. 4. **Effects of pretreatment with SNP on caffeine-induced $[Ca^{2+}]_i$ responses.** Fura-2-loaded cells suspended in Ca^{2+}-containing KRH medium were pretreated or not for 1 min with SNP (300 μM) and then challenged with the indicated concentrations of caffeine. The graph shows means ± S.D. of five experiments, expressed as the percent increase in $[Ca^{2+}]_i$ peak responses measured over basal resting $[Ca^{2+}]_i$ values. Basal $[Ca^{2+}]_i$ values were, on the average, 107 ± 12 nM and did not change upon SNP addition.

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
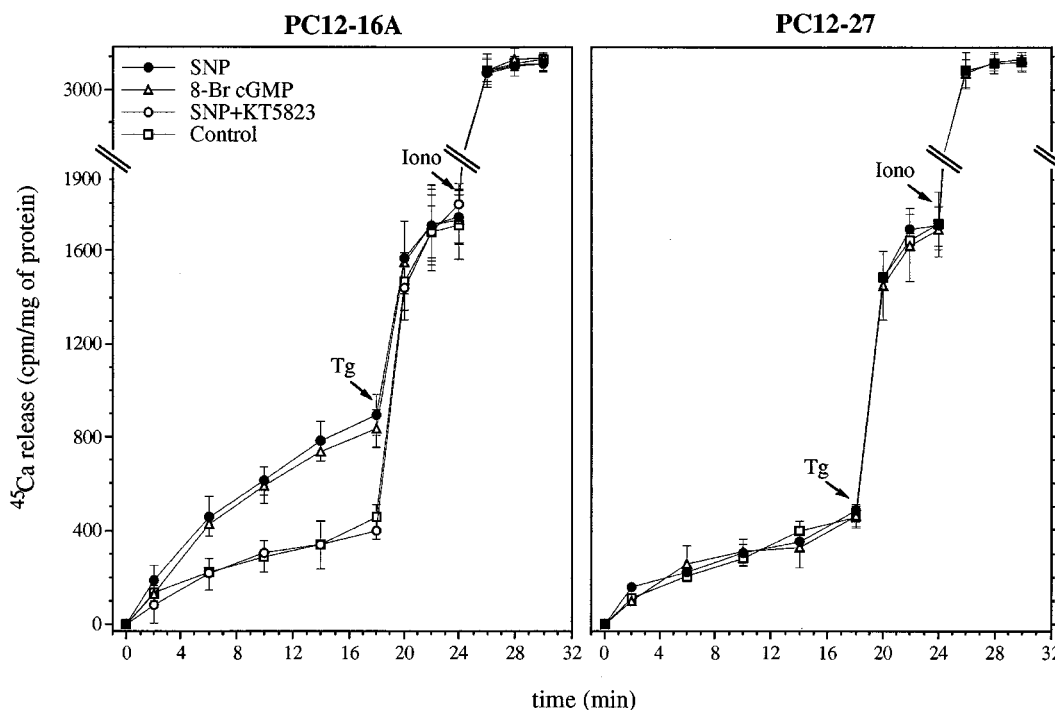
PC12-16A   **PC12-27**

FIG. 5. **Effects of SNP, 8-Br-cGMP, and KT5823 on ^{45}Ca release from the thapsigargin- and ionomycin-sensitive intracellular Ca^{2+} pools.** PC12 cells were loaded to equilibrium with ^{45}Ca and then incubated at 37 °C in Ca^{2+}-free KRH medium alone (control) or supplemented with SNP (300 μM), SNP plus KT5823 (10 μM), or 8-Br-cGMP (500 μM). Results illustrated are means ± S.D. of four experiments after subtraction of the basal ^{45}Ca leak estimated before challenging the cells by sequential addition of thapsigargin (*Tg*; 100 nM) followed by ionomycin (*Iono*; 1 μM), administered where indicated.

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 μM), SNAP (300 μM), or 8-Br-cGMP (500 μM) 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 μM) or (R_p)-8-Br-cGMP-S (30 μM) 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 SNP, SNAP, or 8-Br-cGMP preincubation (Fig. 5, *right panel*).

The final step of this investigation was the demonstration that cADP-ribose is the ultimate messenger responsible for the effect of the NO/cGMP-dependent protein kinase I signaling pathway on RyR2. To this end, experiments were carried out with cells permeabilized by digitonin in the presence of the specific Ca^{2+} dye, fluo-3. When, under these conditions, clone 16A cells were loaded with four pulses of 10 μM Ca^{2+} and then incubated for 10 min with SNP (300 μM (Fig. 6A) or SNAP (300

μM) (data not shown), a large reduction of the thapsigargin-sensitive pool was observed. When cells were simultaneously treated with 8-amino-cADP-ribose (100 μM), 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).

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 μM range. cADP-ribose-induced Ca^{2+} release into the medium, monitored using fluo-3, was clearly visible at 0.3 μM 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 μM 8-amino-cADP-ribose or when cells from the RyR-defective PC12-27 clone were used (*graph and dashed trace*). From these results, we conclude that the Ca^{2+} release responsiveness of PC12-16A cells to cADP-ribose is specific and depends on the expression of RyR2.

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
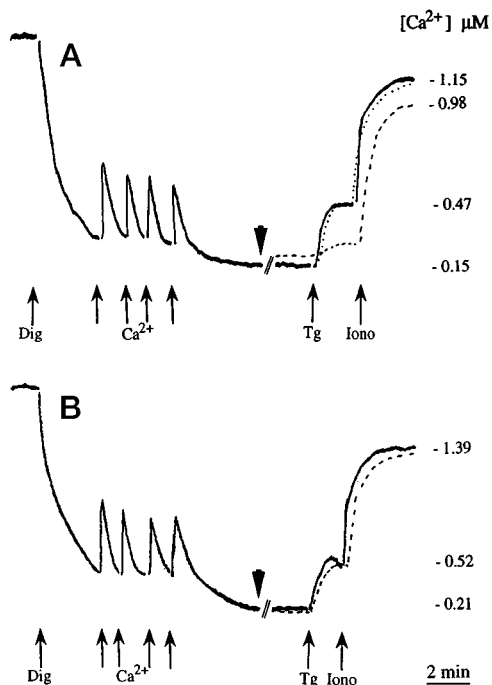
FIG. 6. **Effects of SNP and 8-amino-cADP-ribose on Ca²⁺ release from permeabilized PC12 cells.** *A*, clone 16A; *B*, clone 27. Cells were suspended in an intracellular-like solution containing the ATP-regenerating system and the Ca²⁺ dye, fluo-3, and then permeabilized with digitonin (*Dig*), loaded with four consecutive 10 μM (final concentration) Ca²⁺ pulses, and finally treated for 10 min (//) with medium alone (*continuous traces*), SNP (300 μM) (*dashed traces*), or SNP plus 8-amino-cADP-ribose (100 μM; *dotted trace*, present only in *A*), added where indicated by the *arrowheads*. Thapsigargin (*Tg*; 100 nM) and ionomycin (*Iono*; 1 μM) were added where indicated. [Ca²⁺] values are specified on the right. Traces shown are representative of results obtained in six consistent experiments.
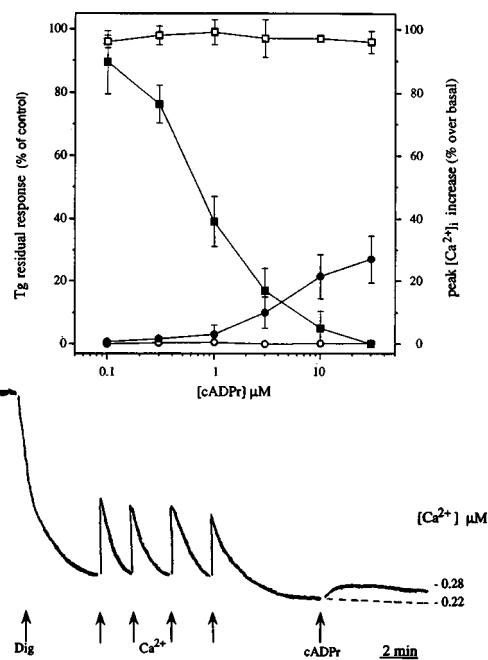

FIG. 7. **Effects of cADP-ribose on Ca²⁺ release from permeabilized PC12 cells.** Experimental conditions were as described for Fig. 6. After permeabilization and Ca²⁺ loading, the cells were treated for 10 min with increasing concentrations of cADP-ribose (*cADPr*). Thapsigargin (*Tg*; 100 nM) was subsequently 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.

those of mammalian cells.[2] In the latter cells, positive results (16, 39) have been questioned because of a possible competition of cADP-ribose binding by ATP (13, 17, 18). Also, the biosynthetic pathway of cADP-ribose remains largely unknown in mammalian cells. Recently, increased cADP-ribose formation has been described in intestinal longitudinal muscle upon cholecystokinin administration and subsequent Ca²⁺ influx stimulation (12). In this case, again, the signaling steps leading to ADP-ribosyl cyclase activation were not investigated.

In this study, the two-PC12 clone system we selected offered considerable advantages. Expression of a single RyR type (type 2) by clone 16A cells excluded the involvement of the other types, with different pharmacological and functional characteristics. The parallel study of the RyR-defective clone 27 was instrumental in excluding the involvement of other, nonspecific mechanisms. This aspect of our study was strengthened by the fact that, apart from the RyR, other properties of the rapidly exchanging Ca²⁺ stores in the two clones were similar to each other. Moreover, PC12 cells were already known to respond to the application of NO donors with generation of cGMP and activation of cGMP-dependent protein kinase I (29–31, 33). This latter event is shown here to induce moderate (~2-fold), yet clearly appreciable increases in cADP-ribose, similar in the two clones. Finally, most of our experiments were carried out with intact cells, *i.e.* in an environment where ATP, cations, and other possible RyR regulators are in the physiological range. The operational approach was based on widely accepted pharmacological criteria, appropriate for the demonstration of the series of events initiated by NO and mediated by the

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[2] A. Galione, personal communication.

cGMP/cGMP-dependent protein kinase I pathway 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 of ADP-ribosyl cyclase(s), cADP-ribose hydrolase(s), or regulators of cADP-ribose metabolic pathways.

An important observation emerging clearly from these results is that the effect of cADP-ribose on RyR takes place not only in stimulated cells, but also independently from other treatments, *i.e.* it consists of a real activation rather than a positive modulation. Convincing evidence for this conclusion comes from the experiments with ryanodine, a drug known to convert its receptor to a long-lived, low conductance state following its activation by an independent mechanism (use dependence) (1, 19). The lack of any effect of ryanodine alone in PC12-16A cells and the appearance of effects during co-incubation with NO donors can only be explained by a process of RyR activation independently triggered by cADP-ribose.

Another interesting property of the cADP-ribose-induced Ca²⁺ release, *i.e.* its synergism with Ca²⁺-induced Ca²⁺ re-

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 $[Ca^{2+}]_i$ 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 modulate negatively the generation of IP_3, with ensuing blunting of the $[Ca^{2+}]_i$ 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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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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