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L. D. Partridge and C. F. Valenzuela

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A. J. Irving and G. L. Collingridge

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Activation of Ca^{2+} -Dependent Currents in Dorsal Root Ganglion Neurons by Metabotropic Glutamate Receptors and Cyclic ADP-Ribose Precursors

JANE H. CRAWFORD,¹ JOHN F. WOOTTON,² GUY R. SEABROOK,¹ AND RODERICK H. SCOTT³

¹Merck Sharp and Dohme, Neuroscience Research Centre, Harlow, Essex CM20 2QR, United Kingdom; ²Department of Physiology, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853-601; and ³Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, United Kingdom

Crawford, Jane H., John F. Wootton, Guy R. Seabrook, and Roderick H. Scott. Activation of Ca^{2+} -dependent currents in dorsal root ganglion neurons by metabotropic glutamate receptors and cyclic ADP-ribose precursors. *J. Neurophysiol.* 77: 2573–2584, 1997. Cultured dorsal root ganglion neurons were voltage clamped at -90 mV to study the effects of intracellular application of nicotinamide adenine dinucleotide (βNAD^+), intracellular flash photolysis of caged 3',5'-cyclic guanosine monophosphate (cGMP), and metabotropic glutamate receptor activation. The activation of metabotropic glutamate receptors evoked inward Ca^{2+} -dependent currents in most cells. This was mimicked both by intracellular flash photolysis of the caged axial isomer of cGMP [P-1-(2-nitrophenyl)ethyl cGMP] and intracellular application of βNAD^+ . Whole cell Ca^{2+} -activated inward currents were used as a physiological index of raised intracellular Ca^{2+} levels. Extracellular application of $10 \mu\text{M}$ glutamate evoked the activation of Ca^{2+} -dependent inward currents, thus reflecting a rise in intracellular Ca^{2+} levels. Similar inward currents were also activated after isolation of metabotropic glutamate receptor activation by application of $10 \mu\text{M}$ glutamate in the presence of $20 \mu\text{M}$ 6-cyano-7-nitroquinoline-2,3-dione and $20 \mu\text{M}$ dizocilpine maleate (MK 801), or by extracellular application of $10 \mu\text{M}$ *trans*-(1*S*,3*R*)-1-amino-1,3-cyclopentanedicarboxylic acid. Intracellular photorelease of cGMP, from its caged axial isomer, in the presence of βNAD^+ was also able to evoke similar Ca^{2+} -dependent inward currents. Intracellular application of βNAD^+ alone produced a concentration-dependent effect on inward current activity. Responses to both metabotropic glutamate receptor activation and cGMP were suppressed by intracellular ryanodine, chelation of intracellular Ca^{2+} by bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid, and depletion of intracellular Ca^{2+} stores, but were insensitive to the removal of extracellular Ca^{2+} . Therefore both cGMP, possibly via a mechanism that involves βNAD^+ and/or cyclic ADP-ribose, and glutamate can mobilize intracellular Ca^{2+} from ryanodine-sensitive stores in sensory neurons.

INTRODUCTION

Mobilization of intracellular calcium (Ca^{2+}) stores is now generally recognized to be controlled by two similar, although distinct, mechanisms. Inositol 1,4,5 trisphosphate (IP_3) is a well-characterized second messenger that releases Ca^{2+} from intracellular pools by activating the IP_3 receptor (for review see Berridge 1993). The second mechanism involves activation of ryanodine receptors (RyRs), of which there are at least three known isoforms (RyR1, RyR2, and RyR3) (Takeshima et al. 1989). These RyRs are thought to

be Ca^{2+} release channels that gate intracellular Ca^{2+} stores, and are defined as such by their sensitivity to the plant alkaloid ryanodine. Since their initial characterization in skeletal and cardiac muscle, RyRs are now also thought to play a role in neuronal excitability by regulating intracellular Ca^{2+} levels (Hua et al. 1994). Brain RyRs are sensitive to caffeine and Ca^{2+} (Bezprozvanny et al. 1991; McPherson et al. 1991) and are probably sites of Ca^{2+} -induced Ca^{2+} release in neurons (Lipscombe et al. 1988; Marrion and Adams 1992). Ryanodine itself has a dual action whereby, depending on the experimental conditions, it can either stimulate or inhibit Ca^{2+} release (Meissner 1986).

Cyclic adenosine 5'-phosphoribose (cADPribose) has recently been proposed as an endogenous modulator of Ca^{2+} -induced calcium release via modulation of RyRs. The Ca^{2+} -mobilizing properties of cADPribose were initially discovered in sea urchin eggs (Lee et al. 1989) and have subsequently been reported in a variety of cell types including mammalian dorsal root ganglion (DRG) neurons and bullfrog sympathetic neurons (Currie et al. 1992; Hua et al. 1994). cADPribose is a metabolite of nicotinamide adenine dinucleotide (βNAD^+) and its formation is catalyzed by the enzyme ADP-ribosyl cyclase (Rusinko and Lee 1989). Recently 3',5'-cyclic guanosine monophosphate (cGMP)-dependent protein phosphorylation has been shown to upregulate ADP-ribosyl cyclase activity, leading to an increased production of cADPribose (Galione et al. 1993). In sensory neurons, intracellular application of exogenous cADPribose liberates caffeine-sensitive Ca^{2+} stores, as measured by the subsequent activation of Ca^{2+} -dependent Cl^- and cation currents (Currie et al. 1992).

The excitatory neurotransmitter L-glutamate has also been shown to activate Ca^{2+} -dependent currents in cultured DRG neurons (Crawford et al. 1995). Glutamate can activate several classes of receptors that include ionotropic and metabotropic glutamate receptor (mGluR) subtypes. To date, eight mGluR subtypes have been cloned (mGluR1–mGluR8) along with splice variants of mGluR1, mGluR4, and mGluR5. These receptor subtypes are classified according to their structure and pharmacology. In particular, group I receptor subtypes (mGluR1 and mGluR5) couple to phosphoinositide turnover and subsequent mobilization of Ca^{2+} from IP_3 -sensitive intracellular stores (for review see Pin and Duvoisin 1995). To determine which receptor subtype

was responsible for the glutamate-induced activation of Ca^{2+} -dependent currents in these cells, we examined the effects of selective ionotropic antagonists and metabotropic agonists. Because direct intracellular application of exogenous cADPribose has also been shown to regulate Ca^{2+} release in DRG neurons, we consequently investigated the novel possibility that mGluRs induced Ca^{2+} release from ryanodine-sensitive stores by a mechanism involving cADPribose formation. With this objective in mind, the effects of mGluR activation were contrasted with the effects of the possible formation of endogenous cADPribose by flash release of cGMP in the presence of its precursor βNAD^+ .

METHODS

Cell culture

Primary cultures of DRG neurons were prepared from 2-day-old Wistar rats. The DRG neurons were enzymatically (trypsin and collagenase) and mechanically (trituration) dissociated and then plated on laminin-polyornithine-coated coverslips. The neurons were maintained in, and fed every 7 days, with Ham's F14 nutrient mixture (Imperial Laboratories, Andover, UK) supplemented with 10% horse serum (GIBCO, Grand Island, NY), penicillin/streptomycin (Flow, McLean, VA), and nerve growth factor (10 mg/ml; Sigma, St. Louis, MO). The cultured neurons were kept at 37°C in humidified air with 5% CO_2 . Electrophysiological recordings were made from DRG neurons maintained in culture for 1–14 days.

Electrophysiology

The whole cell configuration of the patch-clamp technique (Hamill et al. 1981) was used to record from DRG neurons at room temperature (23°C). Low-resistance borosilicate patch pipettes (3–8 M Ω) were used and the cells were voltage clamped with an Axoclamp-2A amplifier operated at a sampling rate of 20–25 kHz in the discontinuous single-electrode voltage-clamp mode. For some experiments, Axopatch-1D was used for continuous single-electrode voltage clamp. Both amplifiers were triggered, and the step duration was controlled, by a Digitimer D4030 pulse generator. Sodium currents were blocked with tetrodotoxin, and potassium currents with extracellular tetraethylammonium and intracellular Cs^+ , leaving intact the voltage-activated Ca^{2+} currents, Ca^{2+} -activated chloride currents, and Ca^{2+} -activated nonselective cation currents (Currie et al. 1992). The extracellular recording medium contained (in mM) 130 choline chloride, 3.0 KCl, 0.6 MgCl_2 , 1.0 NaHCO_3 , 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 4.0 glucose, 25 tetraethylammonium, 0.0025 tetrodotoxin, and 2.0 CaCl_2 , pH adjusted to 7.4 with NaOH, osmolarity adjusted to 320 mOsm with sucrose. " Ca^{2+} -free" extracellular recording medium had the same ionic constituents except for the lack of CaCl_2 and the addition of 1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA). In some experiments, a modified Ca^{2+} -free solution was used in which the CaCl_2 was replaced with BaCl_2 (no EGTA was added in this case). The patch pipette solution contained (in mM) 140 CsCl, 2.0 MgCl_2 , 1.1 EGTA, 0.1 CaCl_2 , 2.0 ATP, and 10 HEPES, pH was adjusted to 7.2 with tris(hydroxymethyl)aminomethane, osmolarity adjusted to 310 mosM with sucrose.

Drugs applied to the extracellular environment were dissolved in recording medium and applied by low-pressure ejection via a micropipette (tip diameter 10 μm) positioned within 100 μm of the cell being recorded. Drugs were supplied by the following companies: L-glutamate and caffeine, Sigma; 6-cyano-7-nitroquinoline-2,3-dione (CNQX) and *trans*-(1*S*,3*R*)-1-amino-1,3-cyclopentanedicarboxylic acid [(1*S*,3*R*)-ACPD], RBI, Natick,

MA; and (\pm)-1-aminocyclopentane-*cis*-1,3-dicarboxylic acid (*cis*-ACPD) and dizocilpine maleate (MK 801), Merck, Sharp & Dohme, Harlow, UK. In specific experiments, modified patch solutions were used in which either 10–100 μM βNAD^+ (Sigma), 10 mM bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA) (Calbiochem Novabiochemicals UK), or 10 μM ryanodine (Calbiochem Novabiochemicals UK and RBI) were included in the patch solution. After the membrane patch was broken and the whole cell configuration was achieved, the cultured DRG neuron was then left for 5 min before experiments were started. This period allowed appropriate equilibration between the intracellular environment and the patch solution. This was particularly important when "caged" photolabile compounds were used.

Flash photolysis

Both adenosine 3',5'-cyclic monophosphate (cAMP) and cGMP were photoreleased inside the DRG neurons by including the P-1-(2-nitrophenyl)ethyl caged axial isomers of cAMP (100 μM) or cGMP (200 μM) in the appropriate modified patch solution. A Hi-Tech Scientific XF-10 xenon flashlamp with a UG11 band-pass filter provided a 1-ms flash of near-ultraviolet light of high intensity. A 200 V lamp discharge (200 V flash) gave 100 mJ of near-ultraviolet light that photolyzed 10% of the axial isomer of caged cGMP. The estimate of the amount of cGMP photoreleased inside the cells was determined by flashing 12 μl samples of caged compound made up in Krebs solution (composition, in mM: 120.8 NaCl, 4.7 KCl, 1.2 KH_2PO_4 , 1.2 MgSO_4 , and 24.9 NaHCO_3) and analyzing the conversion from caged compound to uncaged product by high-performance liquid chromatography (Currie et al. 1995; Walker et al. 1988). An equivalent 200 V flash yielded ~42% cAMP from its caged axial isomer. This was estimated with the use of the same method. The caged compounds were synthesized and characterized by previously published methods (Wootton and Trentham 1989). The flashlamp was triggered by an external signal from a Digitimer DS-2 isolated stimulator under the control of the same Digitimer D4030 pulse generator used to trigger and set the duration of the voltage step commands generated by the Axoclamp/patch amplifier.

Analysis

Electrophysiological data were captured and stored on digital audio tape recorder (Biologic DTR 1200 recorder) and, in some experiments, on a Gould 2200S twin-channel pen recorder (Gould, Cleveland, OH). Analysis of data were performed off-line with the use of a Tandon personal computer and Cambridge Electronic Design (Cambridge, UK) patch and voltage-clamp analysis software (version 6.0). All voltage-gated current data were subjected to subtraction of scaled linear leakage and capacitance currents unless stated otherwise. Data are presented as means \pm SE and statistical significance was determined with the use of Student's paired or independent *t*-tests where appropriate.

RESULTS

Ca^{2+} -activated currents evoked by glutamate receptor activation

It has previously been shown that intracellular Ca^{2+} release from caffeine-sensitive stores can activate Ca^{2+} -dependent Cl^- and nonspecific cation currents in cultured DRG neurons (Currie et al. 1992). In the present study, DRG neurons were voltage clamped at a holding potential of -90 mV and loaded with CsCl-based patch pipette solution. In 80% (8 of 10) of neurons, extracellular application of 5 mM caffeine resulted in inward currents sometimes accompanied by underlying

transient events (Fig. 1*Ai*). These transient events are considered unlikely to be single-channel events because their unitary conductance would be much more than 20 pS, the value previously quoted for Ca^{2+} -activated channels in DRG neurons (Simonneau et al. 1987). Also, currents evoked by depolarizing voltage step commands confirmed that recordings were made with the use of the whole cell recording configuration. The peak amplitude of the inward current activity was -0.54 ± 0.13 (SE) nA, with a delay to initial activity of 23 ± 14 s. The principal conductances underlying these inward currents included both Ca^{2+} -activated Cl^- currents and Ca^{2+} -activated cation currents. Extracellular application of glutamate (10 μM) activated similar currents in 69% of DRG neurons (11 of 16; Fig. 1*Aii*). The peak amplitude of these currents was -0.63 ± 0.13 nA, after a delay of 33 ± 2 s. These results are summarized in Table 1.

The currents activated by glutamate in these cells were not blocked by application of the ionotropic glutamate receptor antagonists CNQX (20 μM) and MK 801 (20 μM). Under these conditions, glutamate activated inward currents in a similar proportion of cells (64%, 16 of 25; Fig. 1*Bi*). The peak amplitude of these currents was similarly unaffected (-0.85 ± 0.15 nA). The reversal potential of these glutamate-induced currents was estimated by extrapolating the current-voltage relationship of the difference current. A series of 100 ms duration voltage step commands was activated from a holding potential of -90 mV to command potentials of between -150 and -50 mV before and during the glutamate-induced currents. These voltage-step-induced currents were subtracted and the current-voltage relationship of the difference current was plotted and extrapolated to estimate reversal potential of the currents. Steps to more depolarized potentials than -50 mV activated voltage-gated Ca^{2+} currents and could therefore not be used to determine the absolute reversal potential. The mean reversal potential for glutamate activity in the presence of MK 801 and CNQX was -13 ± 9 mV ($n = 11$), which indicates the activation of nonselective cation currents, as has been previously reported with caffeine (Currie and Scott 1992). Direct evidence for the involvement of mGluRs in these responses was obtained with the use of the selective mGluR agonist (1*S*,3*R*)-ACPD. As with glutamate, application of (1*S*,3*R*)-*trans*-ACPD (10 μM) elicited inward currents in 67% (8 of 12) of cells, with a peak amplitude of -0.71 ± 0.14 nA developing after a delay of 52 ± 15 s (Fig. 1*Bii*). Application of *cis*-ACPD at a concentration of 20 μM failed to evoke any inward current activity in the eight cells studied (results not shown).

Ca²⁺-activated currents induced by intracellular photorelease of cGMP

Direct intracellular application of cADPribose has been shown to activate Ca^{2+} -dependent currents in DRG neurons (Currie et al. 1992). To determine whether these currents could also be activated by the formation of endogenous cADPribose, we examined the effects of intracellular application of its precursor βNAD^+ , and also photorelease of cGMP in the presence of βNAD^+ .

Neurons were voltage clamped at a holding potential of -90 mV and loaded with CsCl intracellular patch solution supplemented with either 10 or 100 μM βNAD^+ . Continuous whole cell recordings were then made for ~ 25 min. In five

of eight (63%) of neurons loaded with intracellular patch solution supplemented with 100 μM βNAD^+ , a small inward current (peak amplitude -0.52 ± 0.25 nA) developed after a mean delay of $1,104 \pm 125$ s (Fig. 2*A*). When the intracellular patch solution was supplemented with 10 μM βNAD^+ , an inward current still developed after a delay of $1,098 \pm 158$ s, but the peak amplitude (-0.28 ± 0.12 nA) of the inward current in these neurons was significantly ($P < 0.05$) lower than when 100 μM βNAD^+ was used. Additionally, with 10 μM βNAD^+ in place of 100 μM , inward currents were observed in a lower proportion of neurons (4 of 10, 40%). These Ca^{2+} -dependent inward currents were not seen over a 25-min recording period with the use of standard intracellular patch solution that did not contain βNAD^+ ($n = 5$).

In the next series of experiments, the effects of intracellular photorelease of cGMP from its caged axial isomer (200 μM ; estimated 10% release per 200 V flash) in the presence of 100 μM βNAD^+ were examined. In 77% of cells (17 of 22), intracellular photorelease of cGMP elicited inward currents whose mean amplitude was not significantly larger than that evoked by βNAD^+ alone (Fig. 2*B*). However, the delay to activation of these currents, 587 ± 84 s, was significantly shorter ($P < 0.05$), and the proportion of neurons responding was higher than that evoked by βNAD^+ alone (see Table 1). The currents activated by photorelease of cGMP, in the presence of βNAD^+ , consisted of both oscillatory and, in some neurons, sustained inward currents (Fig. 2*B*). Their amplitudes ranged from -0.21 to -1.55 nA, with a mean value of -0.98 ± 0.29 nA ($n = 17$). The estimated reversal potential of these currents, determined by measuring difference currents, was -2 ± 1 mV ($n = 10$) (Fig. 3). Cl^- tail currents were not seen in these cells, suggesting that Ca^{2+} -activated nonspecific cation channels were responsible for these cGMP-mediated events. Intracellular photolysis of caged cGMP (20–60 μM) without addition of βNAD^+ to the patch solution activated inward currents (peak amplitude -0.62 ± 0.9 nA) in five of six cells. The delay to initial current activation was 380 ± 64 s. In control experiments (duration 25 min), no Ca^{2+} -dependent inward currents were evoked by flashes of near-ultraviolet light applied to neurons in the absence of caged compounds ($n = 5$). Also, no responses were observed over 25 min when neurons were loaded with 100 μM caged cGMP but not flashed ($n = 6$), showing that currents were only evoked following photorelease of cGMP. Additionally, intracellular photorelease of cAMP from its caged precursor failed to evoke inward currents over a 15-min period ($n = 6$), showing that the cyclic nucleotide is critical for the response and not other byproducts of photolysis.

Involvement of intracellular Ca²⁺ in responses to glutamate and cGMP

To determine whether the mGluR- and cGMP-mediated events were dependent on mobilization of intracellular Ca^{2+} , the Ca^{2+} chelator BAPTA was used to buffer intracellular Ca^{2+} . Inclusion of BAPTA (10 mM) into the intracellular recording solution blocked the glutamate-induced inward currents in the majority of cells (Fig. 4*A*). Under these recording conditions, glutamate (10 μM), applied with MK 801 and CNQX, evoked a discrete inward current in only 1 of 10 cells examined, and this current had a reduced ampli-

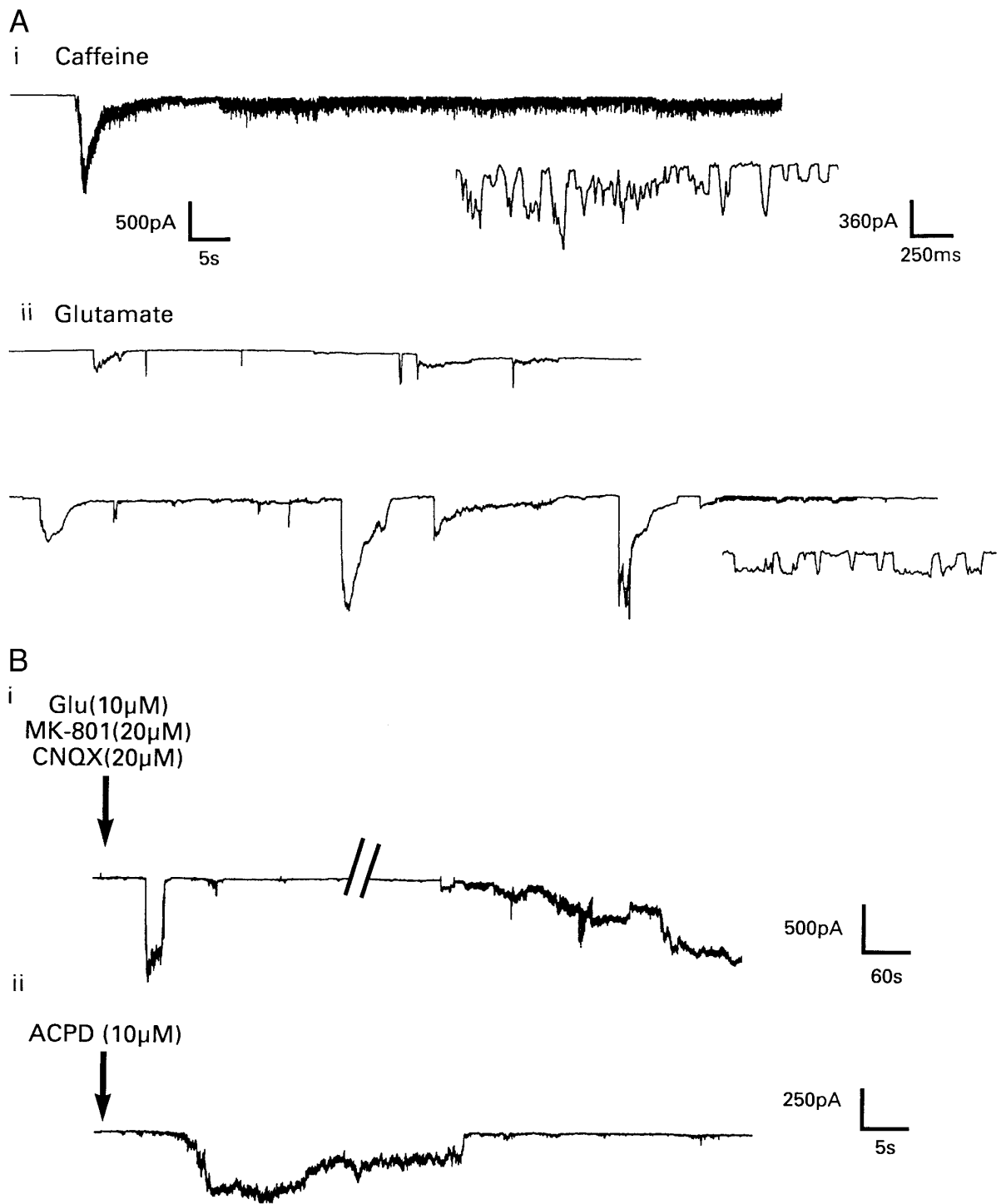


FIG. 1. *A*: inward currents induced by extracellular caffeine (5 mM) and glutamate (10 μ M) in dorsal root ganglion (DRG) neurons. *Ai*: whole cell current recorded from an individual cell to which 5 mM caffeine was applied. An inward current was elicited almost instantaneously, accompanied by smaller underlying transient inward currents (*inset*, at higher gain). *Aii*: extracts of records obtained from 2 separate cells in which glutamate (10 μ M) evoked similar inward current activity accompanied by short-duration spike like inward currents. *Inset*: low-amplitude transient events shown at a higher gain, thus emphasizing the similarity of the caffeine- and glutamate-evoked activity. In both cases the drugs were applied to the extracellular bathing solution via a pressure ejection perfusion pipette. In both cases the drugs were applied before the start of the traces shown here and were present in the bath for the duration of the recording. DRG neurons were voltage clamped at a holding potential of -90 mV. *B*: isolated metabotropic glutamate receptor (mGluR) activation evoked inward currents in DRG neurons. *Bi*: glutamate (Glu; 10 μ M) applied in the presence of ionotropic glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μ M) and dizocilpine maleate (MK 801, 20 μ M), was still able to evoke inward current activity similar to that shown in *A*. In this cell, both transient and sustained inward currents were observed. *Bii*: mGluR-specific agonist 1-amino-1,3-cyclopentanedicarboxylic acid [(1*S*,3*R*)-ACPD, 10 μ M] was also able to evoke similar inward current activity (note difference in time scale between *Bi* and *Bii*).

TABLE 1. Pharmacological modulation of calcium-activated currents in cultured DRG neurons

Condition	Number of Cells	Percent Responding	Peak Amplitude, nA	Latency, s
Normal (25-min recording)	5	0		
Caffeine (5 mM)	8/10	80	-0.54 ± 0.13	23 ± 14
Glutamate (10 μ M)	11/16	69	-0.63 ± 0.13	33 ± 2
Glutamate (10 μ M) + MK 801 (20 μ M) + CNQX (20 μ M)	16/25	64	-0.85 ± 0.15	45 ± 3
(1 <i>S</i> ,3 <i>R</i>)-ACPD (10 μ M)	8/12	67	-0.71 ± 0.14	52 ± 15
<i>cis</i> -ACPD (20 μ M)	8	0		
β NAD ⁺ (10 μ M)	4/10	40	-0.28 ± 0.12	$1,098 \pm 158$
β NAD ⁺ (100 μ M)	5/8	63	-0.52 ± 0.25	$1,104 \pm 125$
β NAD ⁺ (100 μ M) + cGMP (\cup 20–60 μ M)	17/22	77	-0.98 ± 0.29	587 ± 84

Values in right 2 columns are means \pm SE. Characteristics of the Ca²⁺-activated inward current activity seen in groups of cells exposed to varying stimuli are summarized. Peak amplitude: largest response exhibited by particular cells. Latency: time delay to initial activity seen. DRG, dorsal root ganglion; MK 801, dizocilpine maleate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; (1*S*,3*R*)-ACPD, *trans*-(1*S*,3*R*)-1-amino-1,3-cyclopentanedicarboxylic acid; *cis*-ACPD, (\pm)-1-aminocyclopentane-*cis*-1,3-dicarboxylic acid; β NAD⁺, nicotinamide adenine dinucleotide; cGMP, 3',5'-cyclic guanosine monophosphate.

tude of -0.4 nA, compared with -0.85 nA in 64% of cells under control conditions. Likewise, after application of (1*S*,3*R*)-ACPD in the presence of BAPTA, only one neuron of five responded with a transient and submaximal inward

current of -0.16 pA after a delay of 304 s. Chelation of intracellular Ca²⁺ with BAPTA (10 mM) also reduced the proportion of responding neurons from 77% to 45% (5 of 11) after intracellular photolysis of caged cGMP in the pres-

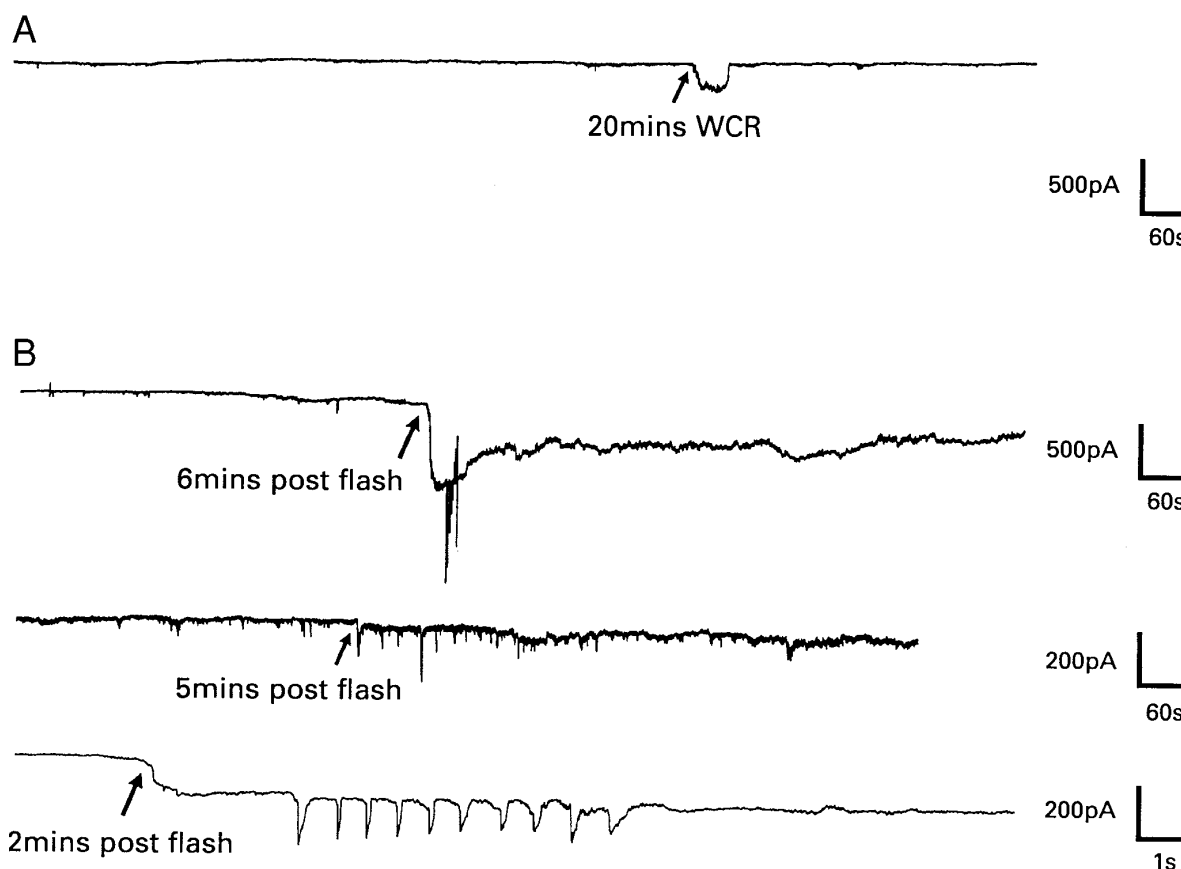


FIG. 2. Effects of supplementing the intracellular patch solution with 100 μ M nicotinamide adenine dinucleotide (β NAD⁺). *A*: whole cell current record obtained from a cell in which β NAD⁺ (100 μ M) was included in the intracellular patch solution. Only 1 small isolated current was observed after a delay of 20 min. *B*: intracellular photolysis of "caged" 3',5'-cyclic guanosine monophosphate (cGMP) in the presence of β NAD⁺ (100 μ M) caused comparatively more inward current activity after a shorter delay (WCR, whole cell recording). Traces are from 3 separate cells, illustrating the variable nature of the responses elicited by photorelease of cGMP. *Top trace*: relatively sustained event similar to those evoked by caffeine and mGluR activation. Currents activated during the response were used to construct a current-voltage relationship plot to estimate the reversal potential. *Middle trace*: short-duration spikelike inward current activity. *Bottom trace*: more prolonged event with rhythmic oscillations superimposed on top. All records were obtained from DRG neurons voltage clamped at a potential of -90 mV.

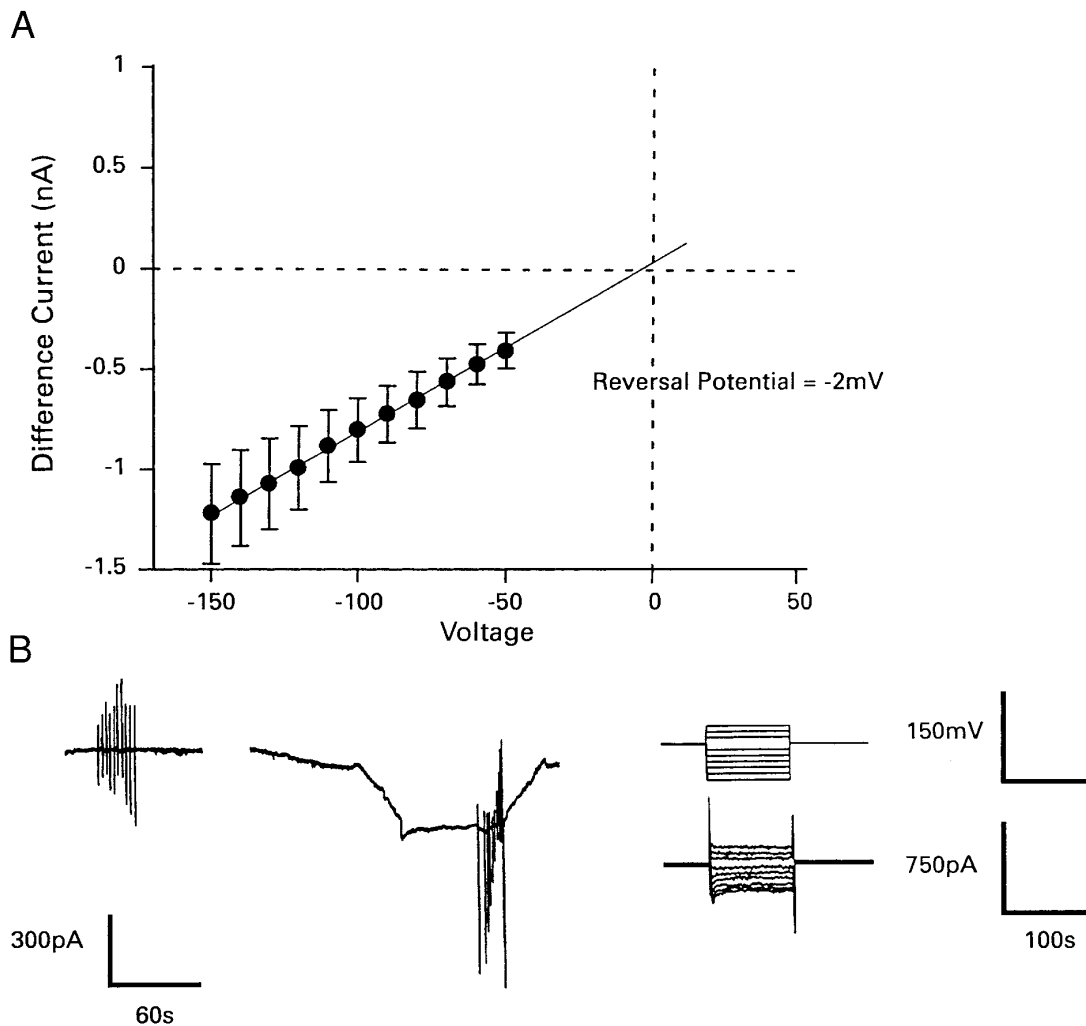


FIG. 3. Estimated reversal potential of cGMP-mediated activity on DRG neurons. *A*: difference current-voltage relationship for the inward current activity mediated by photorelease of cGMP in the presence of βNAD^+ . A family of hyperpolarizing and depolarizing step commands was subtracted from each other to obtain a difference current for each test potential and these were used to generate the current-voltage plot. Relationship was linear and extrapolated to give an estimated reversal potential of -2 mV ($n = 10$). *B*, bottom traces: difference currents resulting from voltage steps carried out during the response in 1 particular cell.

ence of βNAD^+ (100 μM ; Fig. 4*B*). The peak amplitude of these currents, -0.68 ± 0.11 nA, was not significantly reduced, nor was their delay to activation, 639 ± 109 s. However, these currents were transient, and the frequency at which they were seen was suppressed by BAPTA.

To assess the importance of extracellular Ca^{2+} in the mediation of the responses to both mGluR activation and flash release of cGMP, Ca^{2+} -free extracellular recording solution was applied during the activation of observed Ca^{2+} -dependent responses (Fig. 5*A*). After the application of (1*S*,3*R*)-ACPD (20 μM), 8 of 12 cells responded with inward current activity. In seven cells, the application of Ca^{2+} -free solution did not cause a decline in the inward current activity (Fig. 5*Ai*). Likewise, after the flash release of cGMP (200 μM , with 10% release per 200 V flash), 9 of 16 cells responded with inward currents and none showed a reduction on addition of Ca^{2+} -free solution during the response (Fig. 5*Aii*). The application of Ca^{2+} -free solution alone (i.e., no ACPD application, or no photorelease of cGMP) was not seen to produce any inward currents or

have any adverse effects on the cultured DRG neurons ($n = 5$). The importance of release of Ca^{2+} from intracellular stores was determined by pretreating the cultured neurons with 10 mM caffeine in a modified Ca^{2+} -free extracellular recording medium containing 2 mM barium (Ba^{2+}) in place of Ca^{2+} . The caffeine was applied twice for 5 min each time, followed by 5 min of washout, and then subsequent experiments were performed with the use of the modified Ca^{2+} -free extracellular recording medium. Under these conditions, in which internal caffeine-sensitive Ca^{2+} stores are depleted (Stapleton et al. 1992), (1*S*,3*R*)-ACPD was unable to elicit a response in 14 of 15 cells tested (Fig. 5*Bi*). Caffeine-sensitive Ca^{2+} stores can be refilled with Ca^{2+} by applying Ca^{2+} to the extracellular environment and repeatedly activating voltage-dependent Ca^{2+} currents. In each of these neurons (1*S*,3*R*)-ACPD was also applied in a Ca^{2+} -containing solution, during refilling of the stores, and in seven of the cells, a subsequent Ca^{2+} -dependent inward current was observed. Correspondingly, flash release of cGMP under the same conditions was unable to

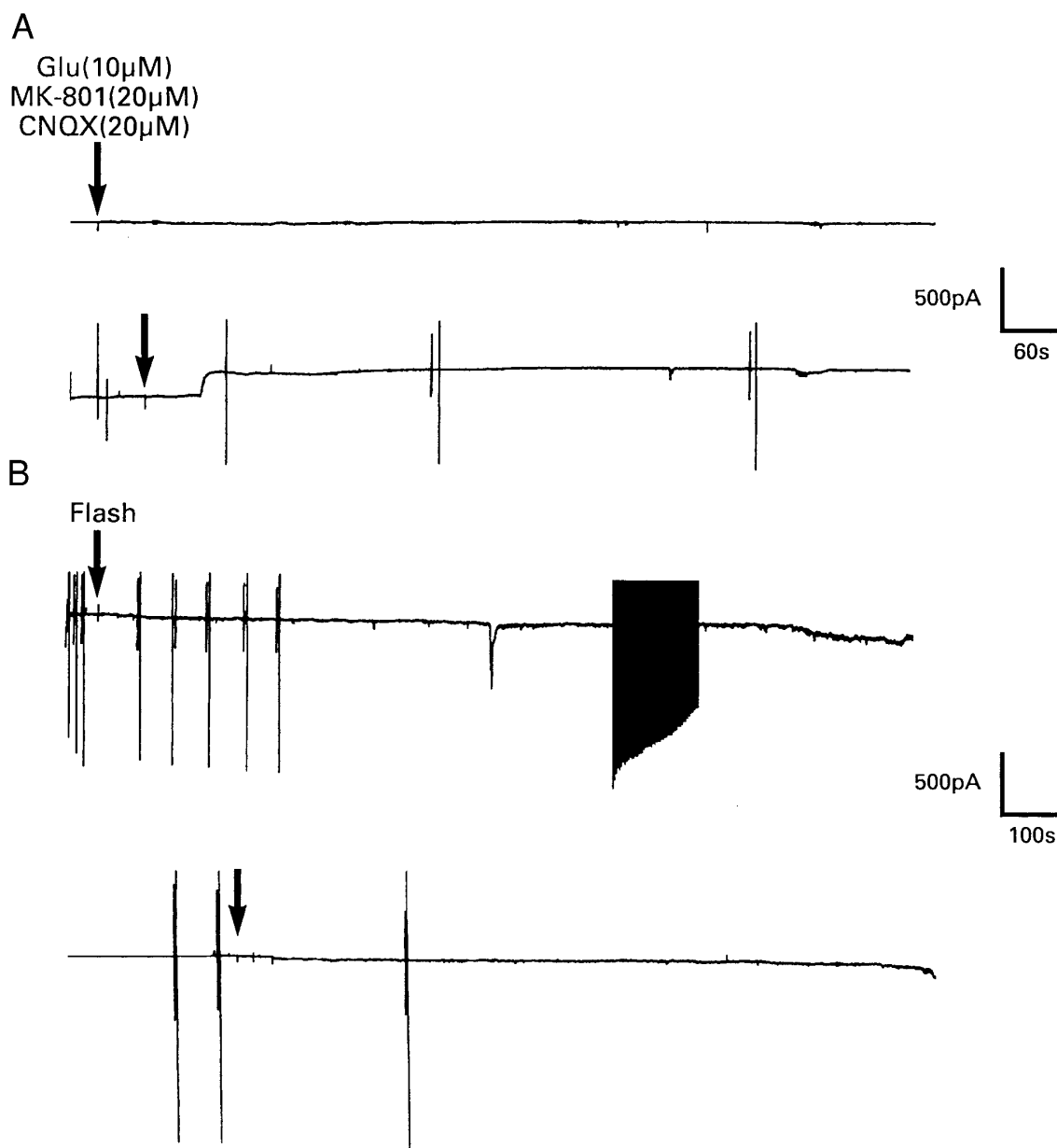
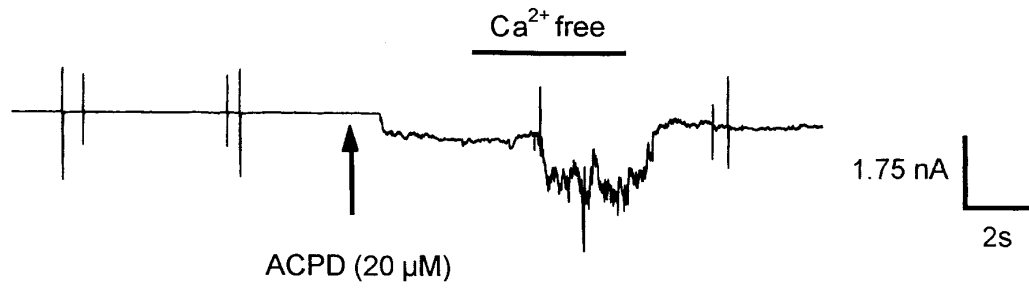


FIG. 4. Effects of intracellular bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA; 10 mM) on mGluR activation and cGMP-mediated inward currents. *A*: raising intracellular buffering capacity of DRG neurons, with 10 mM BAPTA, suppressed inward currents activated by glutamate application. Recordings were made in the presence of CNQX (20 μ M) and MK 801 (20 μ M). *Top trace*: single cell in which no significant mGluR-mediated inward currents were observed. *Bottom trace*: another cell in which directly following glutamate application, a small outward current was observed, possibly due to the block of leak channels. Enhancement of the high-voltage-activated Ca^{2+} currents was also observed, but no significant inward current activity was seen. *B*: after intracellular photorelease of cGMP in the presence of βNAD^+ , inward current activity was also attenuated. *Top trace*: record from 1 cell in which a single small inward current was observed. No modulation of the high-voltage-activated Ca^{2+} current was observed, and loading the cell with Ca^{2+} (created by the activation of voltage-dependent Ca^{2+} influx) did not trigger any further activity. *Bottom trace*: cell in which all inward current activity was abolished and no effect on the high-voltage-activated Ca^{2+} current was seen.

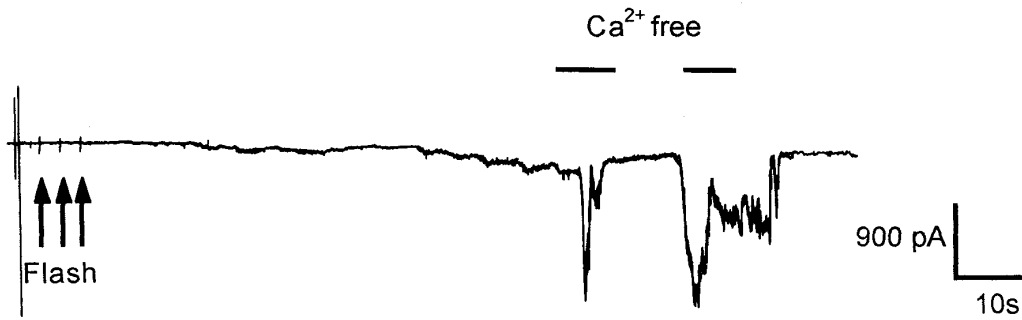
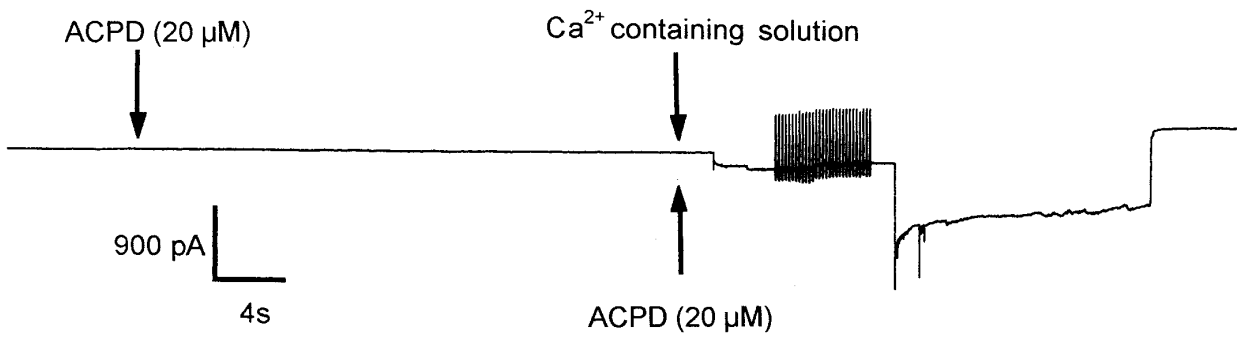
produce any Ca^{2+} -dependent inward current activity in six cells (Fig. 5*Bii*). In three of these cells, refilling of the Ca^{2+} stores and/or a second attempt at flash release of cGMP in the presence of a Ca^{2+} -containing solution did lead to a small amount of inward current activity.

Ryanodine was used as a pharmacological tool to assess whether the effects of glutamate and cGMP were due to their actions on Ca^{2+} -induced Ca^{2+} release from ryanodine-sensitive intracellular Ca^{2+} stores. At high concentrations,

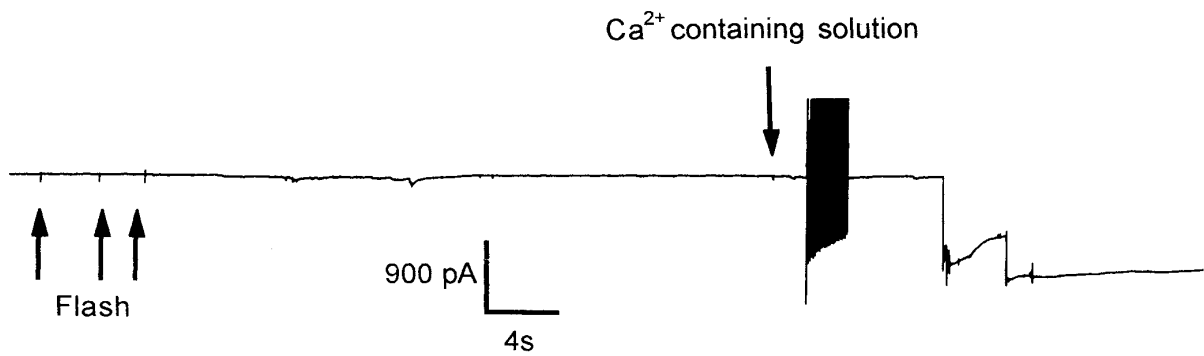
ryanodine has been shown to irreversibly block RyRs, whereas at lower concentrations, ryanodine modulates receptor function by a mechanism that could involve a transition into a subconductance state and may lead to a depletion of internal stores with the subsequent prevention of any further release of intracellular Ca^{2+} . Initially, 10 μ M ryanodine was applied extracellularly. Under these conditions, ryanodine applied during a glutamate-mediated response inhibited the inward current in 70% of cells (7 of 10). Surprisingly, on

A
i

ii

B
i

ii



removal and washout of the ryanodine, the inward current developed again in six of these cells. This was not in concordance with previous literature, in which the inhibitory actions of ryanodine have been reported to be irreversible (Marrion and Adams 1992); therefore it was possible that extracellular ryanodine was acting directly on the Ca^{2+} -activated channels on the cell membrane rather than the intracellular Ca^{2+} release channel (RyR). We thus supplemented the intracellular patch solution with $10 \mu\text{M}$ ryanodine and recorded continuously for 25 min from neurons clamped at -90 mV . A 5-min equilibration period after whole cell configuration was gained was allowed before the experiment was undertaken. During this period ryanodine alone induced inward current activity in 3 of 19 neurons (see Fig. 6). After intracellular treatment with ryanodine, extracellular glutamate ($10 \mu\text{M}$) in the presence of MK 801 ($20 \mu\text{M}$) and CNQX ($20 \mu\text{M}$) did not elicit any whole cell currents ($n = 7$; Fig. 6A). Likewise, after treatment with ryanodine, the proportion of cells responding with currents activated by photorelease of cGMP in the presence of βNAD^+ ($100 \mu\text{M}$) was also attenuated (Fig. 6B). The peak inward current under these conditions was $-0.83 \pm 0.45 \text{ nA}$, and was observed in only 33% of neurons (4 of 12) compared with 77% under control conditions. Furthermore, the delay to initial activity was $868 \pm 217 \text{ s}$, which was significantly ($P < 0.05$) longer than when no ryanodine was present in the patch solution.

DISCUSSION

The present study demonstrates that mGluR activation can stimulate Ca^{2+} -dependent inward currents in cultured sensory neurons via mechanisms that are likely to involve Ca^{2+} stores that are sensitive to cGMP/ βNAD^+ and that can be modulated by ryanodine.

Neuronal RyRs have previously been shown to function as caffeine- and ryanodine-sensitive Ca^{2+} release channels that are distinct from IP_3 receptors (McPherson et al. 1991); however, their physiological significance remains uncertain. Ca^{2+} is well known for its central role in regulating neuronal function, by either altering membrane excitability directly or indirectly via Ca^{2+} -activated enzyme cascades and Ca^{2+} -activated ion channels. Caffeine can also stimulate Ca^{2+} release from intracellular Ca^{2+} stores, in both peripheral (Thayer et al. 1988a,b) and central neurons (Glaum et al. 1990; Murphy and Miller 1989), and we have recently shown, with the use of the fluorescent Ca^{2+} indicator Fura 2, that caffeine can mobilize intracellular Ca^{2+} in cultured DRG neurons (Crawford et al. 1996). In the present study we investigated the functional role of ryanodine-sensitive Ca^{2+} stores in DRG neurons, with the use of the activation

of Ca^{2+} -dependent ion channels on the cell membrane as a physiological index of intracellular Ca^{2+} release. Caffeine elicited inward currents in these neurons, an effect that was mimicked by mGluR activation, supplementation of the intracellular patch solution with βNAD^+ , and photorelease of caged cGMP in the presence of βNAD^+ . These events were not only similar to each other, but also to those obtained by Currie et al. 1992 following the direct intracellular application of cADPribose to cultured DRG neurons. These currents were also shown to be insensitive to the application of a Ca^{2+} -free extracellular solution. Further evidence that these mGluR- and cGMP-mediated inward currents were reliant on the release of intracellular Ca^{2+} from internal stores was provided in two ways. First, supplementation of the intracellular patch solution with the Ca^{2+} chelator BAPTA (10 mM) suppressed Ca^{2+} -dependent inward currents. Additionally, when Ca^{2+} stores were depleted by pretreatment of the cells with 10 mM caffeine, and prevented from refilling by the use of a barium-containing extracellular recording solution, Ca^{2+} -dependent inward currents following mGluR activation or flash release of cGMP were not observed. This indicates that in both cases it is release of Ca^{2+} from intracellular stores and not influx from the extracellular environment that plays the critical role in the mediation of these Ca^{2+} -dependent responses. A recent study has also illustrated increases in intracellular Ca^{2+} , in cultured DRG neurons, directly measured with the use of the Ca^{2+} fluorescent dye Fura 2 AM and Ca^{2+} imaging techniques following mGluR activation (Crawford et al. 1996).

Accumulating evidence suggests that cADPribose acts to mobilize intracellular Ca^{2+} via modulation of Ca^{2+} -induced Ca^{2+} release and RyRs (Galione 1992; Lee 1993). However, some controversy exists as to the nature of the interaction between cADPribose and the RyR channel complex that results in Ca^{2+} mobilization (Sitsapesan et al. 1995). Since the demonstration of the Ca^{2+} -mobilizing activity of cADPribose in sea urchin eggs homogenates (Clapper et al. 1987), cADPribose has now been shown to be active in a variety of other cell types (Galione 1994). Recent studies report that it may also be acting to release intracellular Ca^{2+} in neurons (Currie et al. 1992; Hua et al. 1994; Ishizaka et al. 1995). Additionally, it has been implicated in the receptor-mediated inhibition of voltage-gated K^+ currents in neuroblastoma hybrid cells (Higashida et al. 1995). A functional role for cADPribose as an endogenous modulator of the RyR has not yet been established in many tissues in which the necessary precursors for cADPribose production are present. However, convincing evidence for a role of cADPribose as a Ca^{2+} -mobilizing second messenger in sea urchin eggs (Clapper et al. 1987; Lee et al. 1994), pancreatic islets

FIG. 5. A: inward current responses persisted after the application of " Ca^{2+} -free" external solution. Ai: application of *trans*-(1*S*,3*R*)-ACPD [(1*S*,3*R*)-ACPD] ($20 \mu\text{M}$) evoked an inward current response that was not blocked by the perfusion of Ca^{2+} -free solution (black bar). Aii: flash release of cGMP led to an inward current response that was also unaffected by the perfusion of Ca^{2+} -free solution. B: depletion of caffeine-sensitive intracellular Ca^{2+} stores led to inhibition of Ca^{2+} -dependent responses. Bi: intracellular Ca^{2+} stores were depleted by pretreatment of the cells with 10 mM caffeine and prevented from refilling by the use of a barium-containing external recording medium. Under these conditions, (1*S*,3*R*)-ACPD was unable to elicit an inward current response until the stores were refilled, and (1*S*,3*R*)-ACPD was applied in the presence of a Ca^{2+} -containing solution. Attempts were made to refill the internal stores by creating a Ca^{2+} influx into the cell through the repeated activation of voltage-dependent Ca^{2+} channel on the cell membrane. Bii: similarly, flash release of cGMP ($200 \mu\text{M}$; 10% release per 200-V flash) was unable to cause inward current activity until the perfusion of a Ca^{2+} -containing extracellular solution was carried out. In each case, traces are taken from individual cells representing records from DRG neurons clamped at -90 mV .

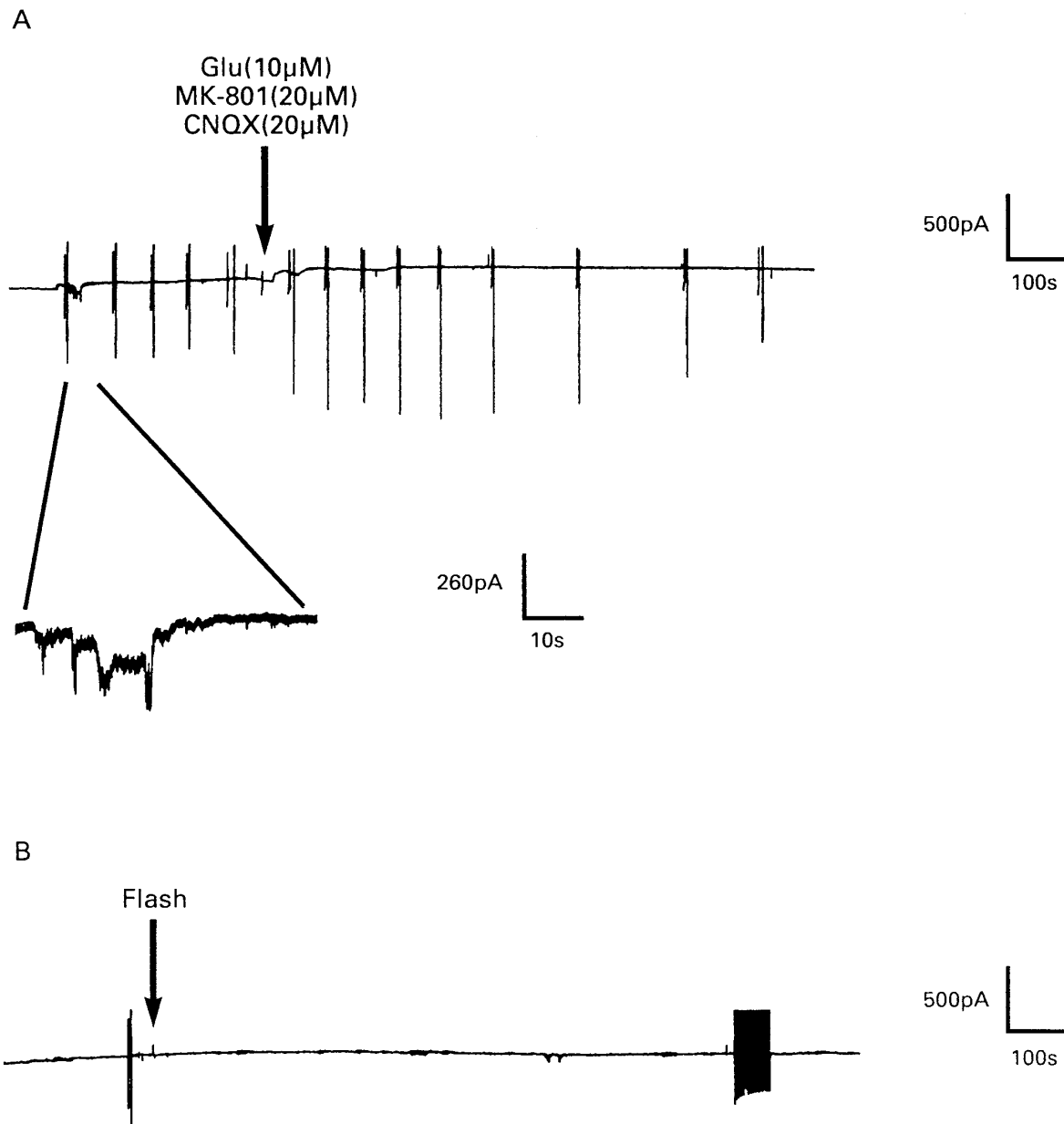


FIG. 6. Effects of intracellular ryanodine ($10 \mu\text{M}$) on mGluR- and cGMP-mediated inward currents. *A*: supplementing intracellular patch solution with $10 \mu\text{M}$ ryanodine suppressed mGluR-evoked inward currents. *Inset*: at higher gain, small amount of ryanodine-induced activity that occurred during the 5-min equilibration period before glutamate was applied in the presence of CNQX and MK 801. High-voltage-activated Ca^{2+} current was monitored every min and showed a degree of enhancement followed by some rundown. Note a small outward current was once again observed directly following glutamate application. *B*: inward currents caused by the intracellular photorelease of cGMP in the presence of βNAD^+ were also suppressed by ryanodine. Repeated activation of the high-voltage-activated Ca^{2+} current, in the attempt to produce a Ca^{2+} influx into the cell, did not trigger any inward current activity.

(Takesawa et al. 1993), and, more recently, in intestinal (Kuemmerle and Makhlof 1995) and pancreatic acinar cells (Thorn et al. 1994), provides further support for this theory. Endogenous levels of cADPribose in brain tissue are ~ 3 pmol/mg, suggesting that cADPribose is also a naturally occurring nucleotide in neurons (Walseth et al. 1991). In the present study, the sensitivity of inward currents to ryanodine suggests that, like cADPribose in other studies (Takesawa et al. 1993), cGMP and mGluR activation can mobilize intracellular Ca^{2+} stores by activation of RyRs.

mGluR1/5 activation can release intracellular Ca^{2+} (for

review see Pin and Duvoisin 1995) via an increase in phosphoinositide turnover in some cell types, and has also been shown to exhibit a degree of heterologous desensitization in cerebellar granule neurons (Simpson et al. 1995). However, previous studies in which DRG neurons were used have shown that neither 1,4,5-trisphosphorothioate, a nonhydrolyzable analogue of IP_3 , nor photolysis of caged IP_3 is able to produce Ca^{2+} transients of a magnitude sufficient to activate Ca^{2+} -dependent currents in these cultured neurons (Currie et al. 1992, 1995). These data suggest that the effects of glutamate seen in the present study are likely to be mediated by

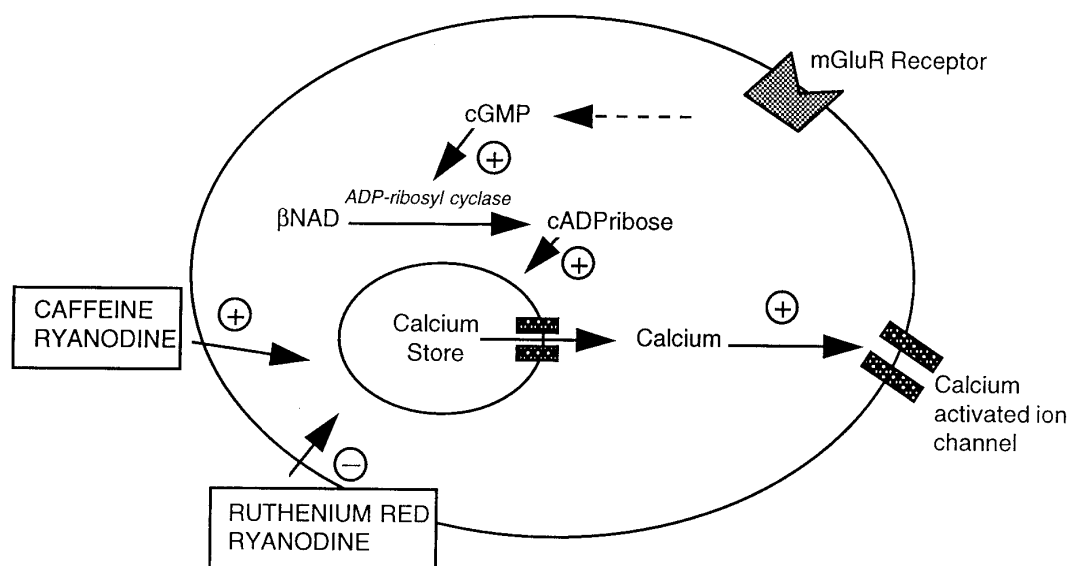


FIG. 7. Novel receptor-mediated mechanism of intracellular Ca^{2+} release from ryanodine-sensitive intracellular Ca^{2+} stores in DRG neurons. Evidence from this investigation has shown that mGluR activation and cGMP release Ca^{2+} from a ryanodine-sensitive store. A possible mechanism is that mGluR activation leads to the synthesis of cGMP, which subsequently enhances ADP-ribosyl cyclase activity. This may lead to the formation of cyclic adenosine 5'-phosphoribose (cADPribose), from βNAD^+ , which mobilizes intracellular Ca^{2+} stores either by binding directly to the ryanodine receptor, or to a separate associated protein that then interacts with the ryanodine receptor.

Ca^{2+} release from ryanodine-sensitive stores rather than those gated by IP_3 . In cerebellar neurons, mGluR activation has been suggested as a pathway of cGMP production via Ca^{2+} -dependent activation of nitric oxide synthase (Okada 1992). In the present study, it could therefore be argued that mGluR activation may be producing concentrations of IP_3 that may not be high enough to activate the Ca^{2+} -dependent ion channels on the cell membrane, but may be causing enough Ca^{2+} release to cause activation of nitric oxide synthase. However, in this study, the precise mechanism by which mGluR activation may stimulate the production of cGMP remains to be elucidated, but is presently under investigation.

The delay before current activation following photorelease of cGMP was comparable with the time lag before the Ca^{2+} transient seen following application of cGMP, in the presence of βNAD^+ , in sea urchin egg homogenates (Galione et al. 1993). This time delay would suggest that a critical level of cADPribose must be reached before its Ca^{2+} -mobilizing properties become apparent. The ability of flash-released cGMP by itself to elicit inward currents implies that there is sufficient βNAD^+ within some cells to allow the formation of this critical level of cADPribose. Likewise, the ability of βNAD^+ alone to evoke currents in a small proportion of neurons suggests the presence of endogenous cGMP, which may lead to the formation of cADPribose. Alternatively, βNAD^+ itself may have a direct effect on intracellular Ca^{2+} stores, although this remains to be proven in this preparation. Following mGluR activation, the delay to activation of the inward currents was significantly shorter than that following photorelease of cGMP. This anomaly probably reflects the lipophilic character of nitrophenyl caged cGMP, which may cause it to partition into cell membranes rather than into the cytosol where cGMP elicits its effects. Consequently an artificial, and potentially rate-limiting, diffusion step may be introduced into the signaling pathway.

The variable nature of the currents evoked by mGluR

activation and intracellular photorelease of cGMP is in agreement with data obtained by Currie et al. (1992) with the use of direct intracellular application of cADPribose. This may relate to the diversity of neuronal subtypes in DRG neuron cultures. The types of responses seen did, however, show recovery to baseline current levels in the majority of cells. Furthermore, the proportion of cells responding may be affected by differences in the expression of the relevant receptor/channel mRNA, different basal levels of βNAD^+ between cells, and the possible requirement of soluble proteins such as calmodulin (Lee et al. 1994) for cADPribose modulation of Ca^{2+} -induced Ca^{2+} release.

In conclusion, the results of the present study demonstrate that mGluRs can activate Ca^{2+} -dependent currents in a sub-population of cultured sensory neurons, by a mechanism that involves modulation of RyRs and mobilization of intracellular Ca^{2+} . Direct intracellular application of cADPribose also activates ryanodine-sensitive Ca^{2+} stores, an effect that is mimicked by flash photolysis of cGMP in the presence of βNAD^+ , the precursor of cADPribose (Fig. 7). These data suggest that mGluRs may be either linked to a pathway that involves cGMP production or Ca^{2+} -induced Ca^{2+} release. The precise mechanism by which this occurs and the mGluR subtype that is involved remain to be determined.

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Address for reprint requests: J. H. Crawford, Merck Sharp and Dohme, Neuroscience Research Centre, Terlings Park, Harlow, Essex CM20 2QR, UK.

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