# Modulation of Calcium Channels by Norepinephrine in Internally Dialyzed Avian Sensory Neurons

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ABSTRACT Modulation of voltage-dependent Ca channels by norepinephrine (NE) was studied in chick dorsal root ganglion cells using the whole-cell configuration of the patch-clamp technique. Cells dialyzed with $K^+$ and 2–10 mM EGTA exhibited Ca action potentials that were reversibly decreased in duration and amplitude by NE. Ca channel currents were isolated from other channel contributions by using: (a) tetrodotoxin (TTX) to block $g_{Na}$, (b) internal K channel impermeant ions (Cs or Na/N-methylglucamine mixtures) as K substitutes, (c) external tetraethylammonium (TEA) to block K channels, (d) internal EGTA to reduce possible current contribution from Ca-activated channels. A marked decline (rundown) of Ca conductance was observed during continual dialysis, which obscured reversible NE effects. The addition of 2–5 mM MgATP to the intracellular solutions greatly retarded Ca channel rundown and permitted a clear assessment of modulatory drug effects. The inclusion of an intracellular creatine phosphate/creatine phosphokinase nucleotide regeneration system further stabilized Ca channels, which permitted recording of Ca currents for up to 3 h. NE reversibly decreased both steady state Ca currents and Ca tail currents in Cs/EGTA/MgATP–dialyzed cells. A possible role of several putative intracellular second messengers in NE receptor–Ca channel coupling was investigated. Cyclic AMP or cyclic GMP added to the intracellular solutions at concentrations several orders of magnitude higher than the $K_d$ for activation of cyclic nucleotide–dependent protein kinases did not block or mask the expression of the NE-mediated decrease in $g_{Ca}$. Addition of internal EGTA to a final concentration of 10 mM also did not affect the expression of the NE response. These results suggest that neither cyclic AMP nor cyclic GMP nor Ca is acting as a second messenger coupling the NE receptor to the down-modulated Ca channel population.

## INTRODUCTION

The ubiquitous role $Ca^{++}$ plays in the regulation of intracellular metabolic events and the maintenance of cell-to-cell communication is just beginning to be under-

stood at the molecular level. Unlike $Na^+$ or $K^+$ currents, which serve mainly to convey electrical messages, $Ca^{++}$ currents carry biochemical messages as well. The inward flow of Ca ions through voltage-dependent channels is translated into biochemical effects through the interaction of Ca ions with specialized Ca-binding proteins. Important biological processes regulated by transient changes in intracellular Ca concentration include: secretion of neurotransmitters and hormones, activation of contractile processes, enzyme regulation, and control of membrane ion permeabilities (Cohen, 1982; Reuter, 1983).

Recently, it has been shown that the behavior of some voltage-dependent Ca channels can be influenced by receptor-specific ligand binding. Ca channels in vertebrate cardiac muscle and in both vertebrate and invertebrate neurons have been shown to be modulated by a host of substances, including putative neurotransmitters, hormones, enzymes, and drugs (Reuter, 1983; Tsien, 1983). Given the powerful influence that Ca influx may have on potentially widespread cellular processes, this modulation may represent a sensitive control mechanism through which Ca-dependent intracellular events can be regulated by systemic hormonal tone or direct synaptic inputs.

In contrast to agonist-gated channels, modulated voltage-dependent channels are not thought to be activated directly by agonist binding. Rather, receptor occupation appears to change the magnitude and/or kinetics of conductances that are activated exclusively by membrane potential changes. Although little is known about the coupling mechanisms that link modulatory receptors to their channel targets, evidence in some systems suggests that receptor occupation leads to changes in channel opening or closing probabilities. In cultured heart cells, cAMP has been implicated as a second messenger linking receptor occupation with increases in single Ca channel opening probability and functional Ca channel density (Reuter, 1983; Bean et al., 1984). The mechanisms underlying neuronal Ca channel modulation are less well understood.

Tissue-cultured embryonic chick dorsal root ganglion (DRG) cells exhibit $Ca^{++}$-dependent action potentials whose duration and amplitude are modulated by norepinephrine (NE) and several other neurotransmitter candidates (enkephalin, gamma-aminobutyric acid [GABA], serotonin, and dopamine) (Dunlap and Fischbach, 1978). All three amines appear to be acting on a single type of receptor, which is different from the GABA receptor population and which was initially thought to be an alpha-receptor. However, a recent study indicates that the amines may be acting on a novel receptor population pharmacologically distinct from the classically defined amine receptor types (Canfield and Dunlap, 1984).

Analysis of the modulatory effect of NE on DRG cells using microelectrode voltage-clamp techniques suggests that NE decreases action potential duration and amplitude by rapidly decreasing a Ca conductance (Dunlap and Fischbach, 1981). The whole-cell configuration of the gigohm-seal patch clamp provides certain advantages in addressing this question in DRG cells. This method allows solution access to the intracellular compartment and good spatial control of membrane potential, and provides an opportunity to clearly define the ionic and biochemical components necessary for neurotransmitter-induced channel modulation.

We have investigated modulation of Ca currents by NE in internally dialyzed DRG cells in vitro. Before examining the mechanism of receptor-channel coupling in a dialyzed cell, conditions that support a stable Ca channel conductance system had to be established. Therefore, the initial question we have addressed deals with Ca channel lability: can experimental conditions be found that will stabilize the Ca channel "rundown" or "washout" process enough to permit the study of agonist-induced channel modulation?

The dilution of soluble endogenous metabolic components resulting from the dialysis procedure creates a nonphysiological intracellular chemical environment that may complicate analysis of the mechanism of action of cellular events that rely directly or indirectly on metabolic pathways, such as second messenger systems for expression. Therefore, a second question addressed by this work is whether NE modulation of $Ca^{++}$ channels can be expressed and studied in an internally dialyzed cell. Ca channel modulation during intracellular dialysis has been examined, and conditions that support expression of NE-induced Ca channel modulation have been established. This study addresses these questions and describes methods suitable for studying receptor-channel coupling mechanisms at a molecular level in single neurons.

## METHODS

### Tissue Culture Procedures

Dorsal root ganglia were taken from 10-d-old chick embryos, dissociated into a single cell suspension, plated onto collagen-coated glass coverslips, and maintained at 37°C in a 4% $CO_2$ atmosphere in minimal essential medium (MEM) or Dulbecco's MEM supplemented with 10% heat-inactivated horse serum, 2% chick embryo extract, 40 $\mu$g/ml gentamycin, 600 mg/ml glucose, and an empirically determined amount of nerve growth factor (NGF) derived from a male mouse submaxillary gland extract. After 24 h in culture, cells were usually treated for 24–48 h with 1–5 $\mu$M cytosine arabinoside (ARA-C) to eliminate non-neuronal background cells. (The DRG dissociation protocol was kindly provided by K. Dunlap; cf. Fischbach, 1972; Fischbach and Dichter, 1974.) Cells were used for electrophysiology only during the period 5–14 d after plating. Cell diameters ranged from 20 to 35 $\mu$m.

### Voltage Clamp and Data Acquisition

Membrane currents and action potentials were recorded using a patch-clamp circuit incorporating either a 1-G$\Omega$ or 100-M$\Omega$ feedback resistor in the headstage. The "whole-cell" configuration (cf. Hamill et al., 1981) was employed in this study in order to control the intracellular ionic composition and permit the introduction of biochemical probe molecules to the cell interior. After the formation of membrane-pipette seals with resistances between 1 and 6 G$\Omega$, intracellular access was obtained by application of further suction to the patch pipette, which caused a rupture of the membrane circumscribed by the pipette. Occasionally, cellular debris would clog or obstruct the pipette tip, which resulted in increases in access (series) resistance as indicated by increases in the decay time constant of the capacitative current transient under voltage clamp.

Several procedures were employed to reduce errors arising from series resistance. (a) A slight negative pressure was often maintained in the pipette. This tended to keep the access resistance low and presumably helped maintain cell-pipette patency. (b) Low-resistance (1–2-M$\Omega$) patch electrodes were used to further minimize access resistance. (c)

Electronic compensation for series resistance ($R_s$) was routinely employed. Series resistance was determined in voltage clamp by eliciting a capacitative current transient with a hyperpolarizing step, fitting a single exponential to the early portion of the transient decay, and dividing the voltage-step magnitude by the extrapolated instantaneous current value. For 24 arbitrarily chosen cells, the average $R_s$ was 5.2 ± 1.6 MΩ. The average residual $R_s$ after compensation was 1.7 ± 1.4 MΩ (72% average compensation). ($d$) In later experiments, we employed analog nulling of cell capacitative currents, which routinely made 85–100% $R_s$ compensation possible without clamp oscillation (see Oxford, 1981). ($e$) The external permeant divalent cation concentration (Ca or Ba) was usually kept between 1 and 2 mM in order to restrict inward current magnitude. Lower current magnitudes helped to minimize series resistance errors and, in addition, reduced the load on the intracellular Ca buffering system.

A PDP-11/23 (Digital Equipment Corp., Marlboro, MA) laboratory computer interfaced to the patch clamp at 12-bit or 14-bit digital resolution was used for data acquisition, stimulation, and analysis. Membrane currents were usually low-pass-filtered (Bessel) at 10 kHz and then sampled at rates of 100–200 µs/point. Capacitative and linear leakage current components were digitally subtracted from all records via a P/−4 averaging process. A holding potential ($V_H$) of −50 mV was employed for all voltage-clamp experiments presented in this paper.

### Solutions

The experimental solution composition is indicated by the following convention in this paper: external//internal with all concentrations in millimolar units unless otherwise noted. Chloride and aspartate were used as principal external and internal anions, respectively; minor variations in anionic constituents are noted. 10 mM HEPES buffer was present in all solutions and the pH was adjusted to 7.35–7.40. D-Glucose was used to adjust osmolarity to 300 mosmol//285 mosmol. External tetrodotoxin (TTX) (300 nM) was employed to block Na channels unless otherwise noted. Solution compositions are described in the figure legends accompanying individual experiments. Drugs were either bath-applied or ejected under pressure onto the cell from a glass capillary micropipette with a 2–3-µm tip diameter located 50–100 µm from the cell. The drug pipette was driven by a timing circuit coupled to a solenoid valve that served to electronically gate pulses of compressed nitrogen at 2–5 psi into the micropipette. Na-ascorbate was present in all drug solutions at a 10-fold excess relative to the drug concentration to prevent oxidation of the amines. 100 µM Na-ascorbate alone had no effect on Ca currents and the NE effect was present when a 10-fold excess of Na-ascorbate was included in both control and drug treatment solutions. All experiments were done at room temperature (22°C). Internal solutions containing supplemental nucleotides were always kept on ice before use to help retard possible hydrolysis of the added biochemicals.

### RESULTS

#### $Ca^{++}$ Action Potentials Recorded from Dialyzed Neurons

Ca action potentials can be recorded from DRG cells dialyzed with K-aspartate solutions. A rapid rundown of their duration and amplitude is observed if an internal $Ca^{++}$ buffering system is not present. Fig. 1 illustrates this phenomenon and the retarding effect of 2 mM EGTA added to the dialysis solution. Internal EGTA at 2–10 mM helped to maintain a stable action potential amplitude. Current-clamped DRG cells bathed in a 10 mM Ca saline solution and dialyzed

with K-aspartate and 2 mM EGTA exhibited resting potentials between −35 and −60 mV that were stable for up to 1 h. These values are comparable to those previously reported for these cells using standard microelectrode techniques (Dichter and Fischbach, 1977). The action potential duration is a sensitive function of resting membrane potential and can vary substantially in response to small changes in resting potential. This phenomenon renders an assay of transmitter effects on Ca channels via measurement of spike duration ambiguous; thus, all but the initial drug effect studies were done on voltage-clamped neurons.
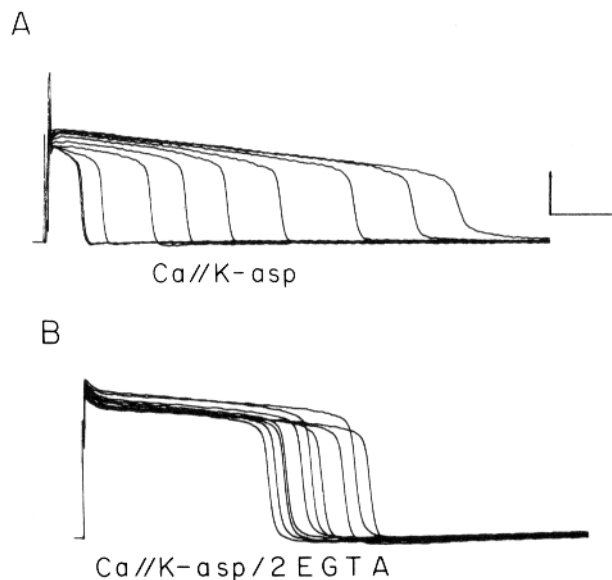
A

Ca//K-asp

B

Ca//K-asp/2 EGTA

FIGURE 1.   The effect of intracellular Ca buffering on Ca action potential rundown. (A) Rundown time course in a cell dialyzed with EGTA-free K-aspartate solution. Records were taken at 1-min intervals. Solutions: 10 Ca/2 Mg/125 Na/5 K/5 TEA//140 K/2 Mg/5 Cl. Experiment 83FEB14A. (B) Same conditions as in A but with the addition of 2 mM internal EGTA-KOH. Note the relative stabilization of action potential duration and spike amplitude in B. Calibrations: (A) 25 mV, 100 ms; (B) 25 mV, 50 ms. Experiment 83FEB14A.

*Adequacy of Internal Dialysis*

To document that internal dialysis could adequately control the internal ionic composition in DRG cell bodies, we carried out a series of experiments in which theoretical K current reversal potentials were set and tested by determining the instantaneous $I_K$-$V$ relation from K channel tail currents. We took care to block the Ca conductance system, as reversal potentials were invariably more positive than expected in the presence of functional Ca channels. This presumably reflects a slight Ca channel permeability to external Na and/or Mg. Thus, cells were dialyzed with a 130 mM KCl solution without EGTA or MgATP and bathed in a Ca-free Cd solution to block Ca channel contribution to the records.

Fig. 2 illustrates a typical experiment in which $E_K$ was set to $-50$ mV. Fig. 2$A$ shows the method used to assess the K channel reversal potential. A prepulse to $+20$ mV was followed by test pulses to various potentials. The $I$-$V$ relationship (Fig. 2$B$) was generated by plotting tail current values 100 $\mu$s after the voltage transition. Similar results were obtained from cells in which $E_K$ was set to either $-20$ or $-80$ mV. Reversal potential values did not vary >3 mV from the predicted value ($n = 7$) under these conditions.

Fig. 2$C$ shows a K current family from the same cell used to generate the $I$-$V$ plot. Note the absence of inward tail currents after repolarization to the holding potential ($-50$ mV), which indicates elimination of Ca channel activity and adequacy of dialysis.

### Measurement of Macroscopic Ca Channel Currents

Isolation of the $Ca^{++}$ channel component from total membrane currents in DRG cells requires the elimination or reduction of several opposing outward currents representing voltage- and Ca-activated K channel populations and possibly a Ca-activated nonselective cation channel (Yellen, 1982). Tetraethylammonium ($TEA^+$) blocks K channels in a variety of excitable cells and has been used successfully at high concentrations to block K channels in DRG cells (Dunlap and Fischbach, 1981). The results of an experiment to test the effectiveness of 20 mM external $TEA^+$ in blocking opposing outward currents at $+10$ mV in a K-dialyzed cell are shown in Fig. 3. The initial current (record $a$) is clearly inward and does not decay substantially during the pulse. To a first approximation, the record appears to reflect a pure inward Ca current. However, after addition of 1 mM Cd to block Ca channels, a large opposing outward current (record $b$) is unmasked and the tail current after repolarization becomes outward. The lower record results from digital subtraction of record $b$ from record $a$ and represents the $Cd^{++}$-sensitive current present in the initial record. Note that although the Ca current in this cell masks a large opposing outward K current, the net inward current does not decay significantly over the 20-ms current time course.

Interpreting the ionic mechanism of drug effects on Ca currents in the presence of such opposing K currents is ambiguous. Thus, alternative conditions, which would eliminate opposing K currents and also support healthy Ca currents, were investigated. Because of the deleterious effects of using higher doses of external TEA (>20 mM) to block K channels (see section below on Ca action potentials), experiments were done to determine the extent to which outward K currents could be suppressed by the substitution of internal $K^+$ with impermeant cations in DRG cells.

With $Cs^+$ or $N$-methylglucamine ($NMG^+$) replacing internal $K^+$, noninactivating inward currents carried by Ca or Ba could be elicited that were sensitive to Ca channel antagonists (Co, Cd, or verapamil). In addition, $TEA^+$ was usually added to the external bath. EGTA at 2–10 mM was also routinely added to the pipette solution to reduce the intracellular Ca concentration and thereby reduce the contribution of $Ca^{++}$-activated currents. The adequacy of Ca buffering with intracellular EGTA has recently been examined in dialyzed neurons by Byerly and Moody (1984). They determined that the use of Ca buffers at the concen-
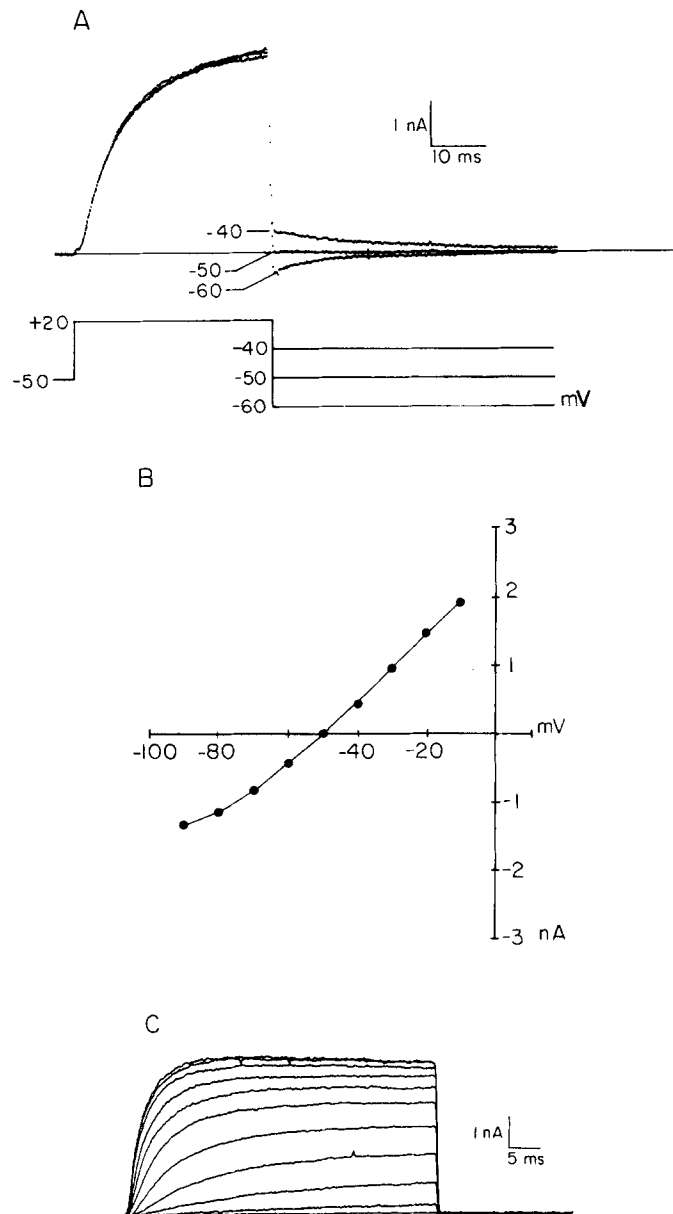
A



B



C



FIGURE 2.   Ionic reversal potentials can be predictably controlled in dialyzed DRG cells. (*A*) With $E_K$ set to −50 mV, the K$^+$ tail current reversal potential was assessed after a 35-ms conditioning pulse to +20 mV. Repolarization values are noted to the left of corresponding tail currents. (*B*) K$^+$ tail currents measured 100 μs after the repolarization step are plotted as a function of the repolarization potential for the cell in *A*. (*C*) A family of K currents from the same cell in response to 70-ms voltage steps from −20 to +90 mV in 10-mV increments. Solutions: 0.2 Cd/2 Mg/17.9 K/ 122 Na//130 KCl. Experiment 84JAN13B.

trations we employed with the addition of ATP to the internal solution (see below) permitted cells to maintain intracellular Ca at or near the predicted levels.

To assess the purity of the Ca channel currents under these conditions, currents were recorded from a Cs-dialyzed cell and then all external Ca was replaced with 1 mM $Cd^{++}$/3 mM $Co^{++}$ to block Ca channel activity. Fig. 4A illustrates currents elicited by a voltage step to +10 mV before and after Cd/Co substitution. At this membrane potential, a small net inward current remains in Ca-free Cd/Co solution that is eliminated by total replacement of external $Na^+$ with $TEA^+$. Other evidence suggests that the residual Na permeability described above is probably through Ca channels and does not represent a novel TTX-resistant Na channel population (Kostyuk et al., 1981; Hess and Tsien, 1984). There is no
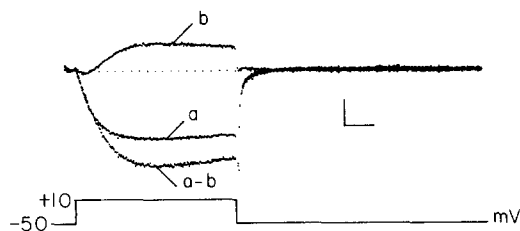


FIGURE 3.    Currents elicited by a 20-ms pulse to +10 mV from a cell dialyzed with $K^+$ and exposed to 20 mM $TEA^+$. Records were taken before (a) and after (b) addition of 1 mM external Cd to block Ca channel currents. The elimination of $g_{Ca}$ unmasks a residual $TEA^+$-insensitive outward K current component. Record a − b is the result of digital subtraction of the residual outward component (b) from the total current (a). Solutions: 2 Ca/20 TEA/120 Na//140 K/5 MgATP/0.5 cAMP/2 EGTA. Experiment 83OCT21A.

channel population contributing residual outward current to the records at this membrane potential. A Ca-activated outward current component in the initial record would not be revealed by the Cd/Co substitution procedure; however, a significant contribution of Ca-activated current is unlikely because of the presence of 2–10 mM EGTA in all intracellular solutions.

The I-V relations for this cell (Fig. 4B) show that blockade of the Ca channel

FIGURE 4.    (opposite) Isolation and control of Ca channel currents in Cs-dialyzed cells. (A) Currents at +10 mV before (lower record) and after (middle record) 1 Cd/2 Co// substitution for external Ca and Mg. The upper record is the result of further replacement of 120 Na// with 120 TEA//. Residual inward current in 1 Cd/2 Co/120 Na// solution was eliminated by TEA substitution. Ca currents under these conditions are free from opposing outward current contributions at this test potential. (B) Current-voltage plots for the cell in A, where the data points represent Ca currents before (circles) and after (triangles) substitution of 1 Cd/2 Co// for Ca and Mg. Control solutions were 1 Ca/2 Mg/120 Na/20 TEA//140 Cs/20 Cl/2 EGTA-CsOH/2 MgATP/0.5 cAMP. Experiment 84JAN31A. (C) Ca currents in response to 30-ms voltage steps ranging from −20 to +10 mV in 5-mV increments. Solutions were the same as in A. (D) Peak current-voltage relationship for the cell in C. Experiment 84FEB3A-1.
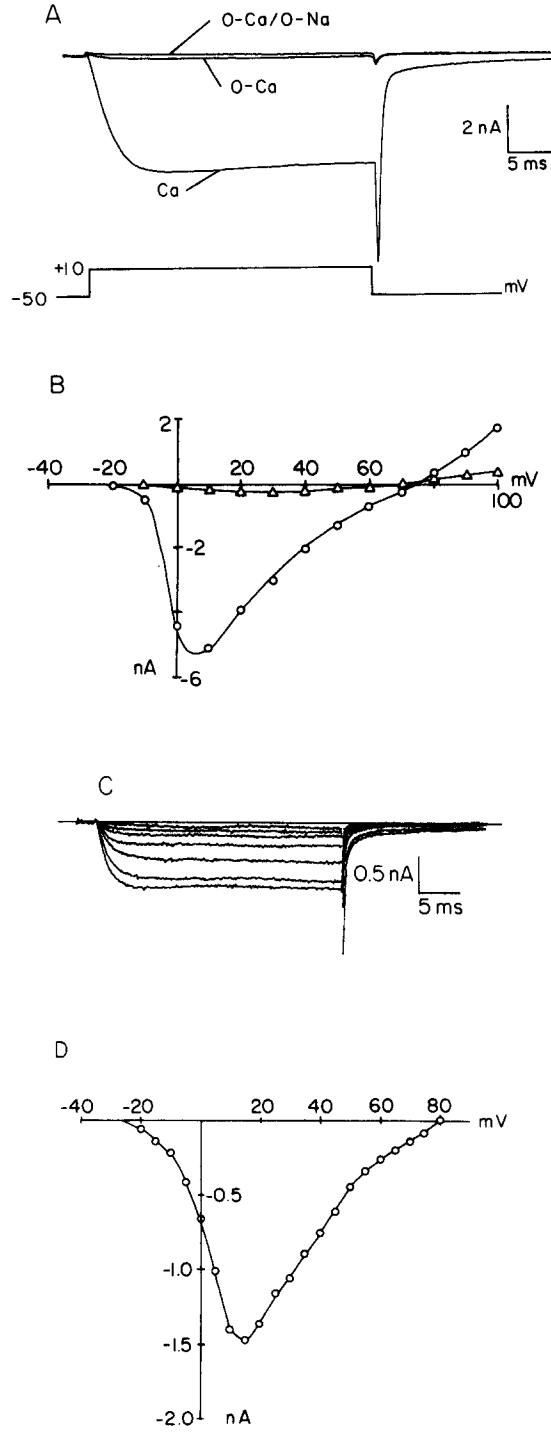
FIGURE 4.

population (triangles) produced no significant change in the reversal potential and a decrease in outward currents at membrane potentials above +70 mV. This suggests that Cs ions are passing outward through Ca channels at membrane potentials positive to +70 mV and that other channel populations are not contributing significantly to the records under these conditions. Cs$^+$ currents passing outward through Ca channels have been observed and analyzed in other preparations (Fenwick et al., 1982; Lee and Tsien, 1982, 1983, 1984).

Fig. 4C is an example of Ca currents elicited by voltage steps in 5-mV increments from another Cs-dialyzed cell. The currents in C illustrate adequate control of membrane potential in the negative limb of the I-V relationship. Fig. 4D shows the current-voltage relationship for the same cell from −20 to +80 mV.

### Ca Channel Rundown

Ca channel "rundown" or "washout" has been described in virtually every study of macroscopic Ca channel currents in internally perfused or dialyzed neurons and has been ascribed to cytoplasmic depletion of a soluble component necessary for Ca channel activity (cf. Fenwick et al., 1982; Kostyuk et al., 1981; see also Yazejian and Byerly, 1984). In support of this notion, we have often observed a slowing of the Ca channel rundown process that accompanies spontaneous increases in pipette access resistance. Pipette clogging may decrease the rate of diffusional loss of any cytoplasmic component(s) essential for Ca channel viability.

Fig. 5A is an example of Ba currents at +10 mV recorded from a dialyzed cell at successive time points following rupture of the patch membrane. This cell exhibited a rapid loss of Ca channel activity typical of the magnitude of the rundown problem often encountered under these particular experimental conditions. We observed no marked difference in rundown between inward Ba and Ca currents. This spontaneous rundown process and the expected NE modulatory effect both result in decreases in Ca conductance, which makes interpretation of drug studies difficult. One important difference between the two phenomena is that rundown is not readily reversible (Doroshenko et al., 1982), whereas the NE effect is. Therefore, in order to document a reversible drug effect, it was necessary to stabilize Ca channel activity.

We have found that inclusion of 2–10 mM MgATP in the intracellular solution stabilizes the rundown process to a degree that allows a clear assessment of drug effects on Ca channel currents (Forscher and Oxford, 1984). Ca currents recorded at +30 mV from a cell dialyzed with Cs and 2 mM MgATP are shown in Fig. 5B at 0, 10, and 30 min. In contrast to the loss of 95% of the Ca conductance after 10 min of dialysis by the cell in Fig. 5A, Ca currents in this cell decreased by only 20% in 30 min. The use of various internal monovalent cations (Cs$^+$, NMG$^+$, Na$^+$, or K$^+$) did not seem to grossly affect the rate of rundown. Cells dialyzed with various mixtures of these cations and 2–10 mM EGTA, but without supplemental MgATP, typically retained only 10–20% of their initial Ca conductance after 20 min. The average time course of Ca current rundown from two cells dialyzed without ATP is shown in Fig. 5C (open circles). The average magnitude of Ca currents monitored in five other cells dialyzed with supplemen-
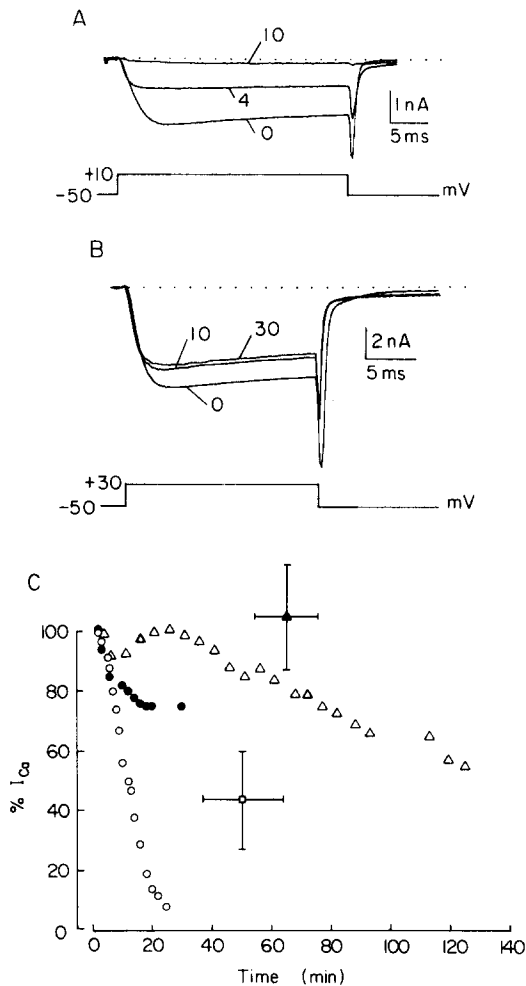
FIGURE 5. Ca channel rundown is attenuated by addition of internal ATP. (A) Ba current rundown at +10 mV in a cell dialyzed with //140 NMG/2 Mg/5 EGTA-TEAOH. Numbers indicate time points in minutes with $t_0$ taken ~1 min after gaining access to the intracellular compartment. Experiment 83APR26B. (B) Attenuated Ca current rundown in a cell dialyzed with //140 Cs/2 EGTA/2 MgATP. 20-ms pulse to +30 mV. Experiment 84MAR25A. (C) Effects of internal MgATP and a nucleotide regeneration system on Ca current rundown. Closed circles: average rundown time course from five cells dialyzed with 2–5 mM MgATP/10 EGTA; open circles: average rundown time course from two cells dialyzed with 10 EGTA; open triangles: rundown time course from a single cell dialyzed with 20 CP/50 U·ml⁻¹ CPK/5 MgATP/40 $\mu$M GTP/10 EGTA. Experiment 84OCT8A. The average percentage of Ca current remaining after ~50 min of dialysis with MgATP- and EGTA-supplemented solutions is depicted by the open square (± SD, $n$ = 7). The filled triangle represents the percentage of remaining Ca current (± SD, $n$ = 7) after ~1 h of dialysis with solutions containing the nucleotide regeneration system. All solutions contained Na and/or Cs as K substitutes.

tal MgATP (Fig. 5C, filled circles) decayed much more slowly during dialysis. Ca channel rundown was determined in other ATP-supplemented cells used in drug studies to assess Ca channel rundown under typical experimental conditions. The routine addition of 1–5 mM internal MgATP attenuated loss of $g_{Ca}$ to the extent that 43 ± 16% ($n = 7$) of the initial Ca conductance remained 50 min after initiation of dialysis (Fig. 5C, open square).

Occasionally, a slow increase in Ca current after ~15 min of dialysis was observed. Slow increases in Ca conductance were never observed in any ATP-free internal solutions after the initial rapid (1–2 min) loss of K channel currents was complete. Internal EGTA augments the Ca channel stabilization effect of MgATP, whereas the addition of other internal nucleotides (e.g., cAMP or cGMP) does not appear to further affect the stability of Ca currents if 2–5 mM ATP is already present.

In our most recent experiments, we have found that the inclusion of a nucleotide regeneration system comprised of 20 mM disodium creatine phosphate (CP) and 50 U/ml creatine phosphokinase (CPK) greatly prolongs the survival of Ca currents beyond that seen with the addition of ATP alone. Under these conditions, we have been able to perform experiments on Ca currents for as long as 3 h on a single DRG cell. We specifically examined the rundown time course in a cell dialyzed with CP, CPK, and MgATP, during which time no other experimental interventions were imposed. The rundown of Ca currents in this cell (Fig. 5C, open triangles) is clearly attenuated relative to those cells dialyzed with additional MgATP alone. The stabilization of Ca currents under these conditions during other experiments was generally better than in this example. Under typical experimental conditions, Ca currents remaining after 1 h of dialysis with this solution were 105 ± 17% of their initial value (Fig. 5C, filled triangle). A systematic study of the mechanism by which internal ATP or the nucleotide regeneration system preserves Ca channel activity has not yet been undertaken. The level of Ca channel stability provided by 2 mM MgATP alone in the internal Cs or Na/NMG solutions proved sufficient to allow experiments on Ca channel modulation by neurotransmitters.

### Agonist-induced Modulation of Ca Channels in Dialyzed DRG Cells

MODULATION OF $Ca^{++}$ ACTION POTENTIALS    Having ensured the adequacy of dialysis and Ca channel stability, we turned to the second question of this study: can agonist-induced modulation of Ca channels be studied in an internally dialyzed cell? We chose to first examine NE and dopamine effects on Ca action potentials in cells dialyzed with a K-aspartate/2 mM EGTA solution. External TEA (5 mM) was present in these experiments to partially block K currents and thus increase the action potential duration, which would facilitate observation of the drug effects. Exposure to higher doses of TEA was avoided, since it caused the cells to depolarize and appear granular after ~20 min.

Fig. 6, A and B, illustrates the effects of bath-applied 1 μM NE and 10 μM dopamine, respectively, on DRG cell action potentials. Both agonists reversibly decreased the duration and amplitude of the action potentials. These results are qualitatively similar to those previously described for chick DRG cells studied

with conventional microelectrode techniques (Dunlap and Fischbach, 1978), and demonstrate that the dialysis procedure itself does not disrupt the expression of the modulatory effect of NE on Ca action potentials.

MODULATION OF Ca CURRENTS    Fig. 7A shows the effects of NE on a cell dialyzed with Cs/MgATP. The equilibrium potentials for $Cs^+$ ($E_{Cs}$) and $Cl$ ($E_{Cl}$) have been set equal to the holding potential (−50 mV) by adjusting the ionic concentrations in the external and internal solutions. 5 $\mu$M NE reversibly decreased the steady state Ca current elicited by a step to +10 mV and the inward tail currents recorded at −50 mV. Similar results were obtained when
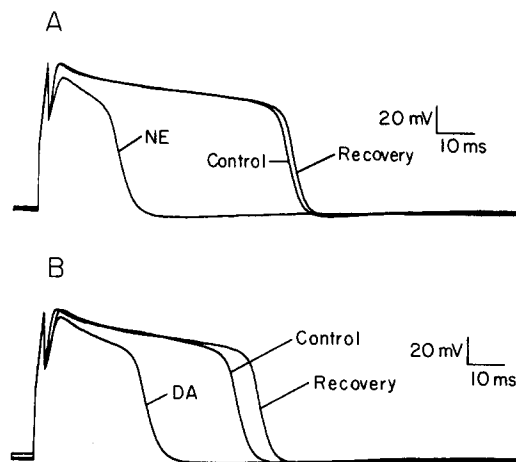


FIGURE 6.    Drug-induced action potential shortening in $K^+$-dialyzed neurons. Reversible effects of 1 $\mu$M NE (A) or 10 $\mu$M dopamine (B) in two different cells. Both drugs were bath-applied. Solution in A and B contained 10 Ca/2 Mg/125 Na/5 K/ 5 TEA//140 K/2 Mg/9 Cl/2 EGTA-KOH. Note that in both cases the recovery traces are slightly longer in duration than the controls and there is full recovery of spike amplitude. Experiment 83FEB25A.

Ba ions carried the current. The recovery record in Fig. 7A was taken ~8 min after removal of NE. Ca current recovery in excess of control levels was observed after NE treatment in several dialyzed cells. The current-voltage relation for another cell dialyzed with Cs/MgATP before (open circles) and after (open triangles) exposure to 5 $\mu$M NE is illustrated in Fig. 7B. Recovery from NE is indicated by the solid circle at +10 mV. A small depolarizing shift of the potential corresponding to maximum inward current occurred in NE. The observed shift is just that expected (+7 mV) from the current magnitude and the 1.5-M$\Omega$ residual series resistance in this cell. In most experiments, current magnitudes were kept below 2 nA to reduce this error to insignificant levels.

Ca currents recorded from five cells bathed in Na-isethionate (Na-2-hydroxy-ethanesulfonate) instead of NaCl also responded to NE application. This indicates that activation of inward Cl current does not contribute to the observed effect. An example is shown in Fig. 7C, where the calculated $E_{Cl}$ was positive (+29 mV).
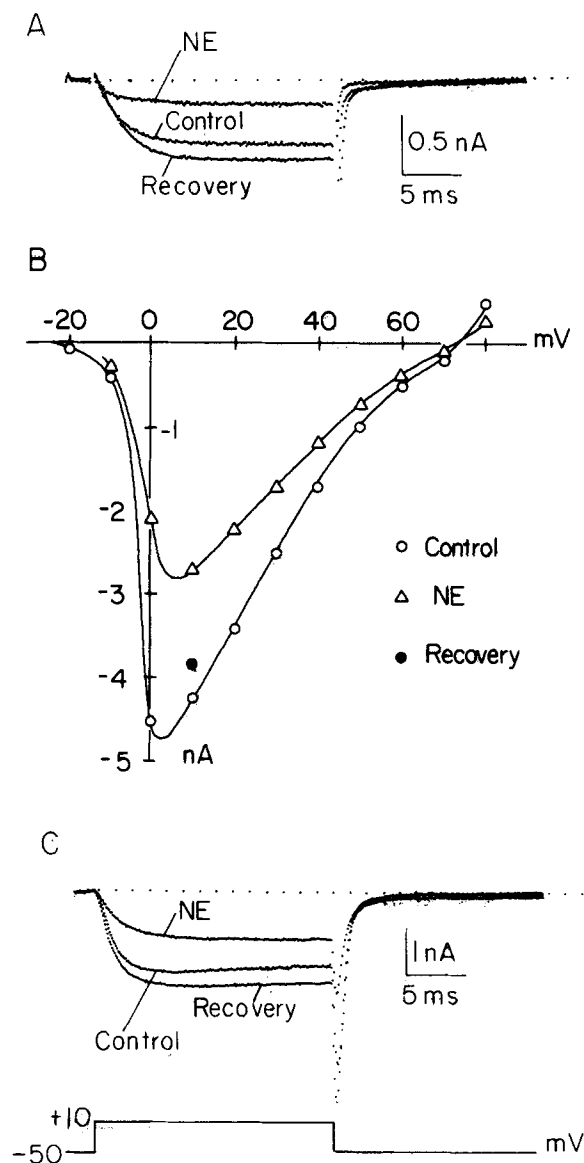
FIGURE 7. NE reversibly reduces Ca currents in cells dialyzed with K⁺-free solutions. (A) Effect of 5 μM NE on Ca currents at +10 mV from a Cs⁺/ATP-dialyzed cell. $E_{Ca}$ = −50 mV. Solutions: 1 Ca/2 Mg/20 TEA/100 Na/19.2 Cs//140 Cs/2 EGTA/2 MgATP/0.5 cAMP. Experiment 84JAN20B. (B) Peak current-voltage relationship plotted for a Cs⁺/MgATP-dialyzed cell. Data points represent Ca currents before (open circles), during (open triangles), and after (solid circle) pressure application of 5 μM NE. Solutions: 2 Ca/2 Mg/20 TEA/110 Na/5 K//140 Cs/2 EGTA/5 MgATP/0.5 cAMP. (C) Cl currents are not involved in the NE effect. Effect of pressure-applied 10 μM NE on Ca currents at +10 mV from a cell bathed in Na-isethionate. $E_{Na}$ = +10 mV in this experiment. Solutions: 2 Ca/2 Mg/ 140 Na-HOE + SO₃//94 Na/26 NMG/26 Cl/5 MgATP/5 cAMP/5 cGMP/10 EGTA. Experiment 84APR25B.

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Any NE-induced increase in Cl conductance would have increased inward current at +10 mV, an effect opposite to the observed actions.

These results provide strong evidence that Ca channels are directly down-modulated by NE in DRG cells, in agreement with previous conclusions to that effect in intact, nondialyzed preparations. However, our results do not rule out possible additional NE effects on other ionic channels specifically removed from our records.

### Are Second Messengers Involved in Receptor-Channel Coupling?

The involvement of intracellular cyclic nucleotides and associated protein kinase activity have been previously implicated in neuronal modulation of Ca channels (Reuter, 1983). Although in some of our early experiments on Ca currents stabilized with internal ATP, 0.5–1.0 mM cAMP was also added to the pipette solution, supplementary cAMP was not necessary for stabilization of the Ca currents or for expression of the NE modulation effect. Since cAMP-dependent protein kinase activity is normally half-maximally activated at submicromolar levels of cAMP (Walsh et al., 1968), it seemed plausible that the concentration of cAMP in our pipette solutions would be sufficient to buffer changes in intracellular cAMP concentration induced by receptor occupation. Specifically, expression of the NE effect in cAMP/ATP-loaded cells suggests that a change in cAMP concentration does not mediate the NE response.

To further test this hypothesis and extend the question to other putative "second messengers," we examined NE modulation of Ca channels under conditions designed to buffer changes in intracellular cAMP, cGMP, or Ca levels. In a series of experiments on separate cells, cyclic nucleotides were cumulatively added to the pipette solutions in concentrations that would be expected to saturate any nucleotide-regulated processes at cell-pipette equilibrium. 10 mM EGTA was used to test for effects dependent on changes in intracellular Ca concentration (e.g., Ca-calmodulin interactions).

Fig. 8 illustrates that cumulative sequential addition of cAMP, cGMP, and high concentrations of EGTA did not block or mask the expression of NE modulation in a series of cells obtained from the same dissociation. A mixture of $Na^+$ and $NMG^+$ was used as an internal cation substitute for $K^+$. $E_{Na}$ was set at a test voltage of +10 mV in order to assess drug effects on steady state Ca currents with the major permeant cation driving force restricted to $Ca^{++}$.

Under control conditions, the internal solution contained 2 mM EGTA and 5 mM MgATP. In Fig. 8A, 10 $\mu$M NE reversibly decreased the peak Ca current under these conditions. Fig. 8, B and C, illustrates that cumulative addition of 5 mM cAMP and 5 mM cGMP, respectively, does not disrupt expression of Ca channel modulation by NE. In addition, increasing the internal EGTA to a final concentration of 10 mM also did not affect expression of the drug response (Fig. 8D). In other experiments (data not shown), 10 mM NaF was added to the pipette solution used in Fig. 8A in an attempt to activate adenylate cyclase. NaF decreased the typical magnitude of Ca current observed under these conditions by ~25% (Kostyuk et al., 1975), but had no discernible effect on the expression of the NE-mediated decrease in $g_{Ca}$. These results suggest that changes in cAMP,

cGMP, or cytosolic Ca concentration are not critical for expression of this NE response.

A persistent inward tail current like that in Fig. 8$D$ was observed in some cells. Slow tails of this sort were only observed under conditions where $g_K$ had been largely reduced or eliminated by the use of external $Ba^{++}$ and/or $TEA^+$ and internal $Cs^+$ or $NMG^+$. Prolonged $Co^{++}$-sensitive depolarizing afterpotentials have been described by Dichter and Fischbach (1977) in tissue-cultured DRG cells. These depolarizations were observed in ~5% of their cells and ranged from
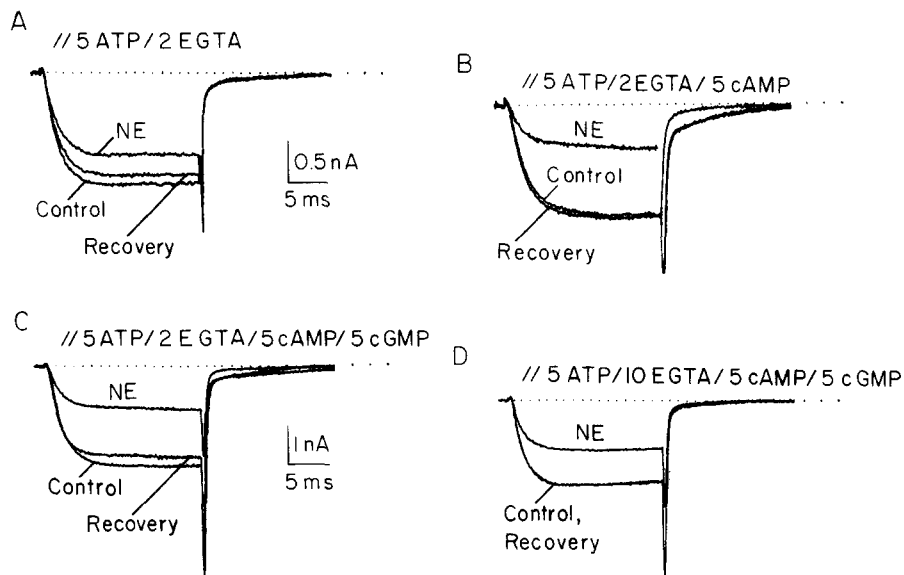


FIGURE 8. Elevated putative second messenger concentrations do not affect expression of Ca channel modulation. $E_{Na}$ was set to the test potential of +10 mV for all cells to facilitate observation of steady state Ca currents. (A) Control conditions: 2 Ca/2 Mg/140 Na//94 Na/26 NMG/26 Cl/5 MgATP/2 EGTA-KOH. Effect of 10 $\mu$M pressure-applied NE on Ca current at +10 mV. (B–D) Results from the same procedure on different cells after sequential addition of 5 mM cAMP (B), 5 mM cGMP (C), and 10 mM EGTA-CsOH (D). Experiments 84APR20A–22A and MAY10A.

50 to 300 ms in duration. We have observed similar slow depolarizations in K-dialyzed cells under current-clamp conditions. It is possible that the slow tail currents we observed correspond to a distinct population of slowly deactivating Ca channels, as observed in other preparations (Matteson and Armstrong, 1984), since these tail currents are blocked by external $Co^{++}$ or $Cd^{++}$. Competing outward K currents may mask these slow tails in some cases. This is suggested by the fact that the slow tail component develops over time as the $Cs^+$ or $NMG^+$ in the pipette enters the cell (for example, compare tail current kinetics at the 0- and 10-min time points in Fig. 5, $A$ and $B$). Initially, tails turn off rapidly; after 3–4 min, they turn off more slowly. This behavior appeared to be unrelated to

increases in the pipette access resistance and was not affected by attempts to clear the pipette tip with pulses of suction or pressure.

It has been reported that membranes of DRG cell neurites do not contain enough Ca channels to propagate Ca action potentials (Dichter and Fischbach, 1977). This property should allow the cell body to be approximated as a sphere for voltage-clamp purposes if Na and K channels in the neurites are blocked. However, a gradient of Ca channels may extend out from the soma into the neurites. In some cells, it may be difficult to adequately control the membrane potential at the spatially distant edges of a Ca channel gradient. Thus, slow tail current turn-off may in part represent a loss of complete space clamp in these regions. We have performed preliminary experiments measuring Ba currents from "on-cell" patches where the membrane potential is spatially uniform. Averaged Ba current records often exhibit a similar slow tail component, which suggests that the slow turn-off kinetics are an intrinsic property of some Ca channels and are unrelated to poor spatial control of membrane potential. Appropriate caution should be exercised, however, before drawing conclusions about Ca channel kinetics from DRG tail current data.

### DISCUSSION

We have shown that NE and dopamine reversibly decrease the Ca action potential duration in K-dialyzed cells and that one unambiguous direct effect of NE receptor occupation is a decrease in the voltage-dependent Ca conductance. These results confirm a previous study of NE modulation effects in nondialyzed DRG cells (Dunlap and Fischbach, 1981). In addition, we have defined artificial intracellular conditions that will support the NE effect in the absence of internal K using either Cs or Na/NMG mixtures as K substitutes. This work thus demonstrates the feasibility of studying agonist-induced modulation of Ca channels in dialyzed vertebrate neurons. Such preparations enable us to control the intracellular biochemical environment and allow investigations of the molecular events that mediate the receptor-channel coupling.

Reversible NE-mediated decreases in Ca action potential duration were obtained in K-aspartate/EGTA–dialyzed cells with minimal technical difficulties, whereas under similar intracellular conditions, voltage-clamped cells showed no appreciable NE response that could be distinguished from Ca channel rundown. Furthermore, under voltage clamp, cells dialyzed with Cs/EGTA or NMG/ EGTA solutions but without added ATP did not appear to respond to even high doses of NE (up to 100 $\mu$M). Given that Ca action potential modulation need only involve a very small conductance change (Dunlap and Fischbach, 1978), it is perhaps not surprising that the NE effect was more readily observable under current-clamp conditions. Under voltage clamp, a small conductance decrease in response to NE would be difficult to measure, especially during continual Ca channel rundown. Thus, reversible NE effects involving small conductance changes may have been observable under current-clamp conditions but obscured in voltage clamp.

Large, reversible decreases in Ca currents in response to NE were observed in these experiments after supplementing our pipette solutions with MgATP. The

addition of MgATP and cAMP to the internal perfusion or dialysis solutions has been reported to stabilize and sometimes increase Ca currents in adult rat DRG cells and snail neurons (Fedulova et al., 1981; Doroshenko et al., 1982; Yazejian and Byerly, 1984; see also Irisawa and Kokubaun, 1983). The molecular mechanism underlying this stabilization effect is not known. Doroshenko et al. (1982) have suggested that functional Ca channels must be maintained in a phosphorylated state by a cAMP-dependent protein kinase. Depletion of cAMP as a result of the internal perfusion or dialysis procedure could lead to dephosphorylation of the channel and subsequent loss of conductance. Our results are consistent with this possibility, but an investigation of the effects of cAMP-dependent protein kinases on Ca channel function and phosphorylation state is clearly required to test this hypothesis.

Another possible effect of ATP supplementation could be the maintenance of energy-dependent Ca transport systems. Ca-ATPases are coupled to Ca pumps in mitochondrial, endoplasmic reticulum (ER), and plasma membranes. These Ca transport systems are responsible for maintaining low intracellular Ca levels in cooperation with plasma membrane Na-Ca exchange and direct Ca sequestration by intracellular Ca-binding proteins. The lack of an energy source to maintain the Ca gradient across mitochondrial and ER membranes could conceivably result in elevated cytoplasmic Ca concentrations and subsequent Ca-dependent channel inactivation (Rasmussen and Goodman, 1977; Tillotson, 1979; Brown et al., 1981). An involvement of intracellular Ca buffering in the rundown process is suggested by the observation that internal EGTA alone stabilizes Ca action potentials. In addition, 10 mM internal EGTA augments the ATP-induced Ca channel stabilization effect in voltage clamp.

We hope that studies of Ca channel modulation in cells dialyzed with an artificial biochemical medium will lead to an understanding of the metabolic pathways involved in maintaining the Ca channel in a functional state. The Ca channel rundown phenomenon makes an unambiguous study of channel modulation difficult in dialyzed cells. On the other hand, investigating the mechanism of Ca channel rundown may yield clues about the molecular mechanism(s) involved in channel modulation by hormones and neurotransmitters.

The details of how amine receptors are functionally coupled to down-modulated Ca channels are unknown. By analogy with beta-receptor–mediated increases in Ca current in heart cells (Reuter, 1983; Tsien et al., 1983), the involvement of cyclic nucleotides might be suspected. In DRG cells, however, pipette concentrations of cAMP and cGMP sufficient to saturate cyclic nucleotide–dependent protein kinase activity by several orders of magnitude do not block or mask the expression of NE modulation of Ca channel activity. Also, addition of 10 mM EGTA or 10 mM NaF to the pipette solutions had no discernible effect on the response. Preliminary experiments on DRG cells dialyzed with a calmodulin inhibitor, trifluoperazine (TFP), were performed. A typical down-modulation response to NE remained in the presence of 50 $\mu$M internal TFP (Fig. 9). The $IC_{50}$ for TFP as a specific calmodulin inhibitor is 10 $\mu$M (Weiss, 1983). TFP concentrations of >50 $\mu$M run the risk of generating nonspecific membrane effects. Taken together, these observations suggest that

increases in intracellular cAMP, cGMP, or $Ca^{++}$ are unlikely to serve as a second message in the NE-mediated decrease in Ca channel conductance. While the high concentrations of cyclic nucleotides used in our experiments should be sufficient to buffer against receptor-mediated changes in their concentrations, we cannot rule out the possibility of rapid reductions in cAMP or cGMP localized to the immediate vicinity of the Ca channel protein. The involvement of such local changes in second messenger concentrations would suggest a relatively tight coupling between individual amine receptors and channels, a possibility that we are currently investigating. Our experiments do not rule out an alternative role for protein kinase activity in the maintenance of Ca channel function, and cyclic nucleotides may be necessary co-factors in this process.

In most cases where cyclic nucleotide second messengers have been implicated in a receptor-mediated modulatory effect or ionic permeability change, the
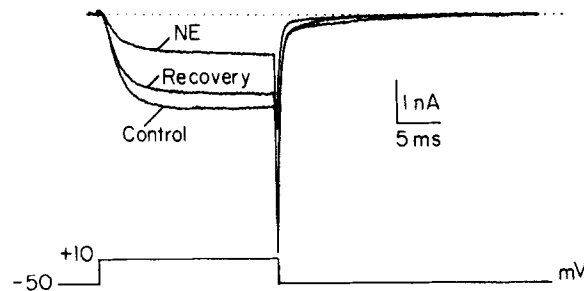


FIGURE 9.   Introduction of an intracellular calmodulin inhibitor does not block Ca channel modulation. 50 $\mu$M TFP was added to the pipette solution as a probe for calmodulin dependence of the NE effect. Effect of pressure-applied 10 $\mu$M NE on Ca currents at +10 mV. Solutions: 2 Ca/2 Mg/140 Na//94 Na/26 NMG/26 Cl/5 MgATP/5 cAMP/5 cGMP/10 EGTA-CsOH/50 $\mu$M TFP. Experiment 84MAY1A.

effects have a relatively slow onset and persist for several minutes and sometimes hours after removal of the agonist (Kehoe and Marty, 1980). In contrast, DRG cell Ca currents are reduced by rapidly applied NE within 1 s and recover within 30 s of drug removal under favorable conditions. The recovery time course is probably limited by our chamber washout rate.

The macroscopic conductance decrease we observe in response to NE could be the result of a decrease in the probability of a channel being open or a decrease in single channel conductance. Experiments are planned to address this question. Recent work suggests that more than one Ca channel population exists in DRG somata (Nowycky et al., 1984; Carbone and Lux, 1984). A Ca channel population in chick DRG cells recently described by Carbone and Lux is selectively activated at membrane potentials near the resting potential. These channels are largely inactivated at a holding potential of −50 mV. Thus, they probably do not contribute significantly to the Ca current records presented in this paper. However, the biophysical characterization of multiple Ca channel types raises the possibility that NE down-modulates a subpopulation of Ca channels that have

biophysical properties different from nonmodulated channels or channels that are coupled to other types of receptors via cAMP-dependent protein kinase activity (Reuter, 1983; Cachelin et al., 1983).

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