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NORADRENALINE MODULATES CALCIUM CHANNELS IN AVIAN DORSAL ROOT GANGLION CELLS THROUGH TIGHT RECEPTOR–CHANNEL COUPLING

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SUMMARY

1. Averaged ensemble Ba currents were recorded from tissue cultured embryonic chick dorsal root ganglion (d.r.g.) cells using the cell-attached patch-clamp technique.

2. Noradrenaline (NA) applied to extrapatch membrane had no clear consistent effect on drug-free patch currents. This finding supports a previous suggestion that second messengers may not be involved in NA-mediated decreases in Ca currents in sensory neurones (Forscher & Oxford, 1985).

3. Cell-attached patch currents sometimes increased slowly after extrapatch application of NA, but were not reversibly decreased by drug treatment.

4. Large patch currents were used to trigger cellular action potentials. NA reversibly decreased action potential duration as reflected in extracellularly recorded patch action currents. Simultaneously recorded inward patch currents were not affected.

5. D.r.g. cell adenylate cyclase activity was assayed. NA did not affect intracellular cyclic AMP levels at concentrations which cause 30–70 % decreases in g_{Ca} in dialysed cells (Forscher & Oxford, 1985). Treatment with forskolin (50 μM) or isoprenaline (10 μM) resulted in 60- and 2-fold increases respectively in adenylate cyclase activity over basal levels.

6. These results suggest that NA decreases Ca currents by direct NA interactions with the Ca channel or a molecule tightly coupled to channel function in d.r.g. cells.

INTRODUCTION

The activity of voltage-dependent Ca channels in neurones and heart cells can be modulated by neurotransmitters (neuromodulators). The mechanism of β-adrenergic modulation of Ca channels in cardiac cells has been extensively studied and it is now clear that coupling of receptor occupation to increases in Ca currents in these cells involves increases in intracellular cyclic AMP and probably protein phosphorylation (Reuter, 1983). Cell-attached patch recordings of single cardiac Ca channels have shown that exposure to isoprenaline or 8-bromo-cyclic AMP results in increases in both the opening probability and the number of functional channels (Cachelin, de Peyer, Kokobun & Reuter, 1983; Tsien, Bean, Hess & Nowycky, 1983; Bean, Nowycky & Tsien, 1984).

Modulation of Ca currents in neurones is a less well understood process. In contrast to heart cells, voltage-dependent Ca channel activity is often depressed in the presence of neuromodulators. For example, Ca action potential duration is decreased by several neuroactive substances, including noradrenaline (NA), γ-aminobutyric acid (GABA), serotonin, dopamine, and opioid peptides in tissue cultured chick or mouse sensory neurones (Dunlap & Fischbach, 1978, 1981; Wertz & MacDonald, 1982). Little is presently known about (1) how modulatory receptors are coupled to Ca channel targets in neurones or (2) how neuronal receptor occupation affects the behaviour of single Ca channels.

In a previous study we established stable whole-cell voltage-clamp conditions that allowed investigation of Ca channel modulation by NA in internally dialysed chick dorsal root ganglion (d.r.g.) cells (Forscher & Oxford, 1985). In addition, we presented preliminary evidence that changes in intracellular cyclic AMP, cyclic GMP, or Ca ions were not likely to be involved in the NA-mediated effects.

In this study we present further evidence derived from cell-attached patch Ca channel experiments that a second messenger is not involved in noradrenergic neuromodulation. We have also assayed stimulation of adenylate cyclase by NA, isoprenaline and forskolin in d.r.g. cells to determine directly whether changes in intracellular cyclic AMP levels could be a mechanism for NA effects on Ca channels.

Second-messenger-mediated modulation of Ca channels in heart cells has served as a model system for approaching modulation of ionic channels by neurohormones in other cell types. In the work presented here we examine possible second messenger involvement in the mechanism of neuronal Ca channel modulation. The results suggest that different types of processes may be responsible for modulation of Ca channel activity in heart cells and sensory neurones.

METHODS

Tissue culture

Cells were taken from 10 or 11 day chick embryos and maintained at 37 °C in a 4% CO_2 atmosphere in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated horse serum, 2% chick embryo extract, 40 μg gentamycin/ml, 600 mg glucose/ml and nerve growth factor as previously described (Forscher & Oxford, 1985). Voltage-clamp experiments were performed between day 5 and day 18 *in vitro*.

Voltage clamp and data acquisition

Membrane currents were recorded and analysed using a PDP-11/23 laboratory computer interfaced to the patch clamp at 14-bit resolution as previously described (Forscher & Oxford, 1985).

Cell-attached patch currents were recorded using a patch clamp with a 1 gigohm head-stage feed-back resistor. Patch currents were low-pass filtered (Bessel) at 2–5 kHz and then sampled at rates of 100–200 μs/point. Capacitive and linear leakage current components were digitally subtracted from all patch current records via a '-P/4' averaging process. Patch current records represent averaged ensemble currents from five to forty sweeps (noted in Figure legends). Patch membrane potential is defined with respect to the cell resting potential (r.p.). In all experiments the pipette potential was initially set equal to the bath potential. After gigaseal formation a negative patch holding potential (V_h) was sometimes achieved by making the pipette positive with respect to the bath. Patch depolarizations were obtained by driving the pipette negative with respect to V_h.

In order to obtain a large, reliable, Ca channel signal and to ensure that a representative sample of channels were tested for sensitivity to NA, we measured ensemble Ca channel currents from relatively large patches of membrane. Patch pipettes fabricated out of soft flint soda-ash glass

(Blue-Tips, Fisher Scientific) with 2–4 μm tip diameters after fire-polishing were used to achieve this end.

Patch currents recorded in 100 mM-$BaCl_2$ ranged from 20 to 450 pA in peak magnitude. D.r.g. cell Ca channel densities estimated from whole-cell voltage-clamp experiments were in the range 13–51 channels/μm^2, assuming a peak single-channel current magnitude of 0·1–0·4 pA in 10 mM-Ca (cf. Reuter, 1983; Tsien, 1983). The corresponding range of patch-current magnitudes for 2–4 μm diameter patches is 41–640 pA assuming a single-channel current magnitude of about 1 pA in isotonic $BaCl_2$ at a membrane potential near 0 mV (see Tsien, 1983). The predicted current magnitudes thus agree reasonably well with those observed.

Solutions and biochemicals

The bath solution used for all cell-attached patch experiments contained (mM): $CaCl_2$, 10; $MgCl_2$, 2; NaCl, 140; KCl, 5; plus 300 nM-tetrodotoxin (TTX). The pipette solution used for cell-attached patch recording contained: 100 mM-$BaCl_2$ and 10 mM-HEPES–$Ba(OH)_2$. Ensemble Ba patch currents recorded in the presence of pipette TTX did not differ from those in which TTX was absent; thus, TTX was not usually included in this solution. Significant Na channel contribution to the records is not likely because Ba permeability through Na channels is low and inward currents were not observed in control cell-attached patch experiments using pipette solutions containing (mM): NaCl, 135; $MgCl_2$, 2; $CaCl_2$, 10; KCl, 5. Whole-cell records were obtained using an internal solution containing (mM): Na aspartate, 94; *N*-methylglucamine chloride (NMG chloride), 26; Mg ATP, 5; cyclic AMP, 5; cyclic GMP, 5; EGTA–NMG hydroxide, 10. Sodium was chosen as the principal internal cation in the whole-cell Ca channel experiments for several reasons: (1) Na ions do not permeate K channels well, (2) a convenient test potential for Ca channel experiments can readily be set to E_{Na} in the presence of 140 mM-external Na, (3) Ca channels and their negative modulation do not appear to be adversely affected by high internal Na ion concentrations. Intracellular Ca ion concentration was buffered to a low level as a precaution against contribution of Ca-activated currents to the records with 10 mM-EGTA. All solutions contained 10 mM-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) buffer and pH was adjusted to 7·35–7·40 External and internal osmolarity was adjusted to 300 and 340 mosM respectively with D-glucose. The solution notation employed in the Figure legends is external solution//internal solution. Concentrations are given in mM units unless otherwise noted.

NA and isoprenaline were ejected under pressure onto cells from a micropipette positioned 20–50 μm from the cell as previously described (Forscher & Oxford, 1985). The HCl salt of noradrenaline was used (Sigma) and all NA solutions contained Na ascorbate in 10-fold excess relative to NA to prevent oxidation of the amine. We have shown previously that ascorbate alone has no effect on Ca channels at the concentrations employed here. The monosodium salt of dibutyryl cyclic AMP (Sigma) was added directly to test solutions. A stock solution (50 mM in dimethylsulphoxide (DMSO)) of forskolin was diluted into test solutions. Cyclic AMP, Mg ATP and cyclic GMP were obtained from Sigma. Internal solutions were kept on ice to retard nucleotide hydrolysis. All voltage-clamp experiments were performed at room temperature (22 °C).

Measurement of adenylate cyclase activity

Intact cells. Cells were plated directly onto a collagen substrate using polystyrene twelve-well cluster dishes (Costar). After 7–8 days, the effects of various compounds on cyclic AMP accumulation were measured using a modification (Su, Cubbedu & Perkins, 1976) of the procedures of Shimizu, Daly & Creveling (1969). The cells were washed with Dubellco's Minimum Essential Medium (DMEM)–25 mM-HEPES, and the plates were then incubated at 37 °C for 1 h with DMEM-HEPES containing 10 μCi [^{3}H]adenine (ICN). The cells were then washed twice with DMEM-HEPES, and 0·95 ml fresh medium was added. After 10 min, 50 μl aliquots from stock solutions of NA, forskolin or ascorbic acid vehicle were added, yielding final concentrations of 10 or 50 μM. The reaction was terminated by aspiration followed by immediate addition of 1 ml 5% tricholoroacetic acid. Chromatographic separation of [^{3}H]ATP utilized a modification of the method of Salomon, Londos & Rodbell (1974), and the levels of these two nucleotides were determined by scintillation counting. Cyclic AMP (0·5 mM) was included in the samples for quantification of recovery, which was determined after chromatography using a Beckman spectrophotometer to be 35–70%. Cyclic AMP formation is expressed as the percentage converted from [^{3}H]ATP precursor. These experiments were performed twice; the total number of culture dishes per condition is shown in Table 1.

Broken cells. Tissue was scraped from the bottom of 35 mm culture dishes, pooled and

homogenized manually in 50 mM-HEPES (pH 7·4) containing 2 mM-EGTA. A 20 μl aliquot (*ca.* 20 μg protein) of the crude homogenate was added to a prepared reaction mixture (Schulz & Mailman, 1984) which included 0·25 mM-ATP, [α-^{32}P]ATP (4 μCi), 1 mM-cyclic AMP and 0–100 μM-dopamine, NA, isoprenaline or forskolin. Samples were incubated for 10 min at 30 °C. [^{32}P]cyclic AMP was separated from [^{32}P]ATP using reverse-phase high performance liquid chromatography as described previously (Schulz & Mailman, 1984), and recovery was monitored by analysing peak amplitudes of unlabelled cyclic AMP. Adenylate cyclase activity is expressed as pmol cyclic AMP formed/mg protein.min.

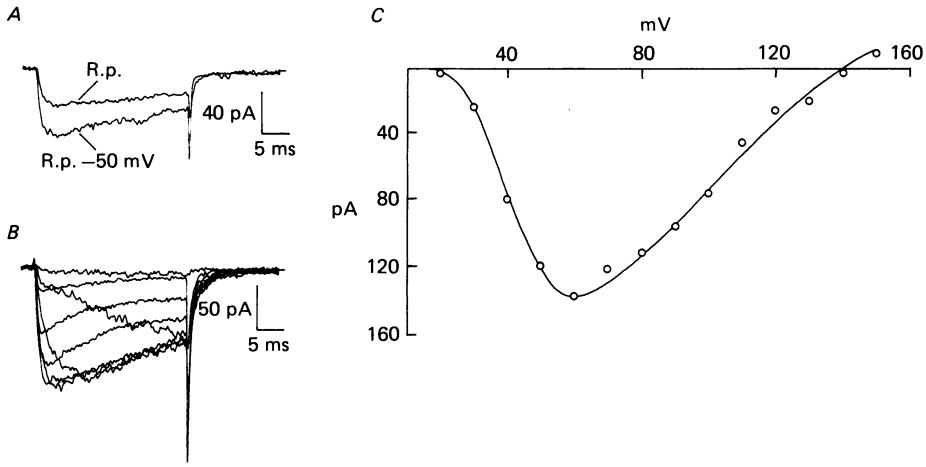

Fig. 1. Representative ensemble Ca channel currents recorded from cell-attached patches. *A*, Ba currents elicited by a 30 ms patch depolarization to r.p. + 60 mV from patch holding potentials of r.p. and r.p. − 50 mV. Each record is the average of five sweeps. *B*, cell-attached patch Ba current family elicited by 30 ms patch depolarizations from r.p. + 20 to r.p. + 60 mV by 10 mV increments and from r.p. + 80 to r.p. + 120 by 20 mV increments. The patch was held at the cell r.p. Records are the average of two sweeps. Different cells were used in *A* and *B*. *C*, *I–V* relationship for the cell in *B*. *Y*-axis values correspond to absolute voltage displacements from the cell r.p. Assuming the cell r.p. = − 50 mV, peak currents occurring at r.p. + 60 mV would correspond to a patch membrane potential of about + 10 mV. Currents were measured isochronally at 2 ms.

RESULTS

Ensemble Ba currents in large cell-attached patches

We have recorded ensemble Ca channel currents from cell-attached patches in intact d.r.g. cells using patch pipettes containing 100 mM-$BaCl_2$. Peak current magnitudes depended on patch size and holding potential. Patch currents often ran down over time; however, on a good cell currents could be recorded for up to an hour with adequate stability to assess the effects of drug applications. Representative Ba current records elicited by a 30 ms test pulse to a patch potential of resting potential (r.p.) + 60 mV are illustrated in Fig. 1*A*. When the patch holding potential was shifted from the cell r.p. to r.p. − 50 mV the patch currents increased. Negative shifts in holding potential away from the cell r.p. always resulted in an increase in patch current at the same command potential. A family of cell-attached patch Ba currents is illustrated in Fig. 1*B*. The current–voltage (*I–V*) relationship obtained from this

patch is also shown (Fig. 1*C*). Assuming an average resting potential of about –50 mV, the *I–V* relationship shown here is similar to those obtained from whole-cell recordings. It should be noted that a time-dependent decay of inward patch currents was observed in most instances. The degree of this decay varied in different cells. We have not examined the nature of this phenomenon in detail, but a component of the decay probably represents activation of an opposing outward K current as evidenced by the rather large tail current amplitudes following some voltage steps (cf. Fig. 4*B*).

A factor to be considered when using the cell-attached patch configuration is depolarization of the cell resulting from large inward currents in the patch. In order to estimate this potential change, the patch current can be approximated as a step function. The average capacitance of a 25 μm diameter cell is 34 ± 6 pF ($n = 5$) and the range of input resistances for d.r.g. cells obtained from whole-cell patch-clamp experiments was 300–600 MΩ. Therefore, the membrane charging time constant for a typical cell with a 500 MΩ input resistance is about 17 ms. The maximum current in Fig. 1*C* is 136 pA. A current step of this magnitude would have caused a 7–8 mV depolarization of the cell after 2 ms when the measurements in Fig. 1*C* were made. Cell depolarization may thus contribute slightly to inward current decay because of the resulting reduction of inward current driving force.

Although large patch currents may cause cell depolarization, this should not prohibit assessment of NA effects on patch currents given the following considerations. Measurements were made at times that are short relative to the charging time constant of the cell, minimizing the depolarizing effects of injected patch current. In addition, changes in currents due to shifts in membrane potential during or after NA treatment are predictable. For example, NA-induced decreases in patch current could be obscured by an opposing increase in current arising from a negative shift in patch potential. However, if such were the case, one would predict that activation kinetics of patch currents would be slowed, yet this was never observed. All NA experiments were performed at the peak current patch potential; thus, *any* shift in patch potential would decrease patch current. In addition, NA itself has no appreciable effect on resting membrane potentials in d.r.g. cells (Dunlap & Fischbach, 1978); therefore it should be possible to assess reliably whether bath-applied NA affects Ca channels isolated in drug-free cell-attached patches.

NA does not reversibly decrease cell-attached Ca channel currents

A previous study suggested that NA modulation of Ca channels in dialysed d.r.g. neurones does not involve acute changes in intracellular cyclic AMP, cyclic GMP or Ca ion concentration (Forscher & Oxford, 1985). To test further for the presence of any second-messenger-mediated NA effects on Ca channels, experiments were performed on cell-attached patches. NA was introduced into the solution bathing the cell, but was absent from the pipette solution. Drug effects on channels in the cell-attached patch in response to externally applied NA would presumably reflect actions mediated through indirect receptor–channel coupling mechanisms such as second messengers. The extracellular (bath) ionic conditions chosen are known to support NA modulation of Ca channels (Dunlap & Fischbach, 1978, 1981).

Examples of typical results obtained for long (2 min) exposures to 10 and 100 μM-NA are illustrated in Fig. 2*A* and *B*, respectively. Ca channel currents in the patch were

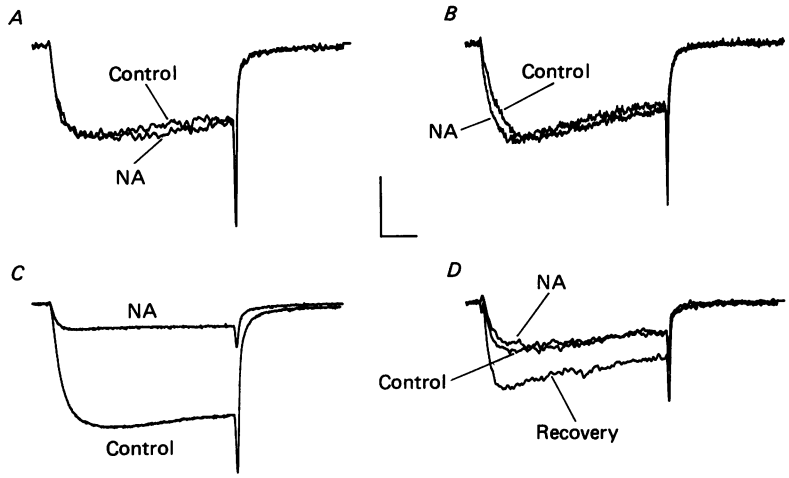

Fig. 2. NA does not modulate cell-attached patch Ba currents, whereas macroscopic currents are strongly affected. *A*, patch currents recorded at r.p. + 90 mV before (control) and after (NA) a 2 min exposure to 10 μM-NA. V_h = r.p.; records are the average of five sweeps. *B*, patch currents records at r.p. + 60 mV before and after a 2 min exposure to 100 μM-NA. V_h = r.p. − 50 mV; records are the average of sweeps. *C*, the effect of 100 μM-Na on macroscopic Ca currents recorded at + 10 mV from a cell in the same dish as *B*. Solutions: 10 mM-Ca, 2 mM-Mg, 140 mM-Na//94 mM-Na, 26 mM-NMG, 5 mMMg ATP, 5 mM-cyclic AMP, 5 mM-cyclic GMP, 10 mM-EGTA–NMG hydroxide. V_h = −50 mV. Ca currents were recorded at E_{Na}. *D*, patch currents recorded at r.p. + 60 mV before and after a 1 min exposure to 10 mM-NA. The recovery record was taken after an 11 min wash in NA-free control solution. V_h = r.p. − 50 mV, records are the average of five sweeps. Four different cells were used for *A*, *B*, *C* and *D*. Calibrations: *A*, 13 pA, 5 ms; *B*, 37 pA, 10 ms; *C*, 1·8 nA, 5 ms; *D*, 25 pA, 5 ms. Note the change in time base in *B*.

TABLE 1.

Patch V_h	n (experiments)	% change during NA*
R.p.	7	−5·0 ± 9
R.p. − 50 mV	10	+0·7 ± 15
Total	17	−2·0 ± 13

* Mean ± S.D.

largely unaffected by bath-applied NA. In contrast, in whole-cell preparations, 10 μM-NA typically reduced Ca channel currents by 41·0 ± 16·6 % (S.D., n = 32) within 1–2 s of drug application. A typical NA (100 μM) effect observed under whole-cell clamp is illustrated in Fig. 2*C*. This experiment was performed using the same dish of cells as in Fig. 2*B*, and thus the large NA effect contrasts sharply with the results of the cell-attached patch experiments. In seventeen experiments where cells (n = 13) were exposed to 10–100 μM-NA for up to 3 min at patch holding potentials of r.p. or r.p. − 50 mV, no clear, consistent effects on cell-attached patch Ba currents were ever observed. A two-tailed Student's t test was performed on data obtained at each patch holding potential and also on the cumulative data. Currents recorded in the presence of NA did not differ significantly from control currents ($P > 0·10$) under any

of the patch holding potential conditions. The results from all cell-attached patch experiments are given in Table 1, expressed as average percentage changes in patch currents during NA exposure. Records obtained in NA were bracketed by control records taken before NA and 2–3 min after NA wash-out. The averages of two controls were used to determine percentage NA responses to reduce any influence of spontaneous changes. Patch currents increased in four cases, decreased in six cases, and in seven cases did not change in the presence of NA. There was no apparent correlation of observed changes with patch holding potential.

In some patches Ba currents increased during recovery from NA exposure. In Fig. 2*D* the patch was held at r.p. −50 mV and currents were recorded before and after a 1 min exposure to 10 μM-NA. There was no significant change in current magnitude due to acute NA exposure; however, after an 11 min wash the inward currents had almost doubled. This phenomenon could result from a secondary NA effect mediated by a second messenger. Another possible explanation is that Ca channel conductance slowly increased during the wash period as a result of slow, holding-potential-dependent relief from inactivation (cf. Nowycky, Fox & Tsien, 1984). We have also observed slow increases in Ca currents after negative shifts in holding potential in whole-cell preparations. Patch-current increases in this example are not likely to have been caused by a shift in patch potential as the test potential was chosen to be that yielding maximum current.

NA decreases action potential duration without affecting patch currents

In order to document the absence of NA effects on patch currents in a cell with demonstrated NA sensitivity, we sought to assess simultaneously NA effects on Ca channels both in the patch and in the surrounding cell membrane of a single cell. To this end we attempted to record cellular action currents following stimulation by inward patch currents as a monitor of extrapatch Ca channel activity. Patch currents (200–400 pA) could trigger Ca action currents when the cell was bathed in Ca–TTX medium. This phenomenon is illustrated in Fig. 3*A* which shows a family of currents from a cell in which inward currents at some potentials stimulated a Ca spike in the cell soma. The action current wave form can be seen as a transient decrease in inward patch current and represents the decrease in inward driving force experienced by the patch during the Ca spike. The current injected by the patch at r.p. +30 mV was subthreshold whereas patch currents at r.p. +50 and r.p. +70 mV elicited action currents. As the patch membrane potential was made even more positive, inward patch currents decreased and thus regenerative Ca channel behaviour is not evident on the positive limb of the *I–V* curve above r.p. +90 mV.

Fig. 3*B* illustrates the peak inward *I–V* relationships for the cell described above at two holding potentials. No regenerative activity was evident when the patch was held at the cell's r.p. (□); however, when the patch holding potential was shifted to r.p. −50 mV, (circles) regenerative activity was observable at some potentials (●). The 30 mV negative shift of the maximum-current voltage at the more negative holding potential is due to cell (and thus patch) depolarization by the larger peak currents at this holding potential. This shift is predictable and can be accounted for by calculating the expected membrane potential changes produced by peak patch currents at the two holding potentials. The calculated depolarizations for maximum

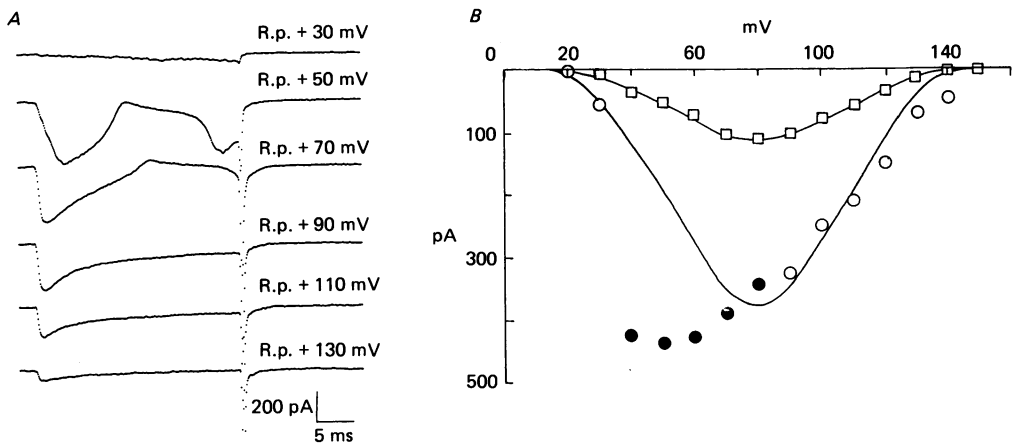

Fig. 3. Patch currents can be used to stimulate cellular action potentials. *A*, patch current family recorded from a cell in which peak patch currents triggered regenerative Ca action potentials. Currents were elicited by 30 ms step patch depolarizations from a patch holding potential of r.p. −50 mV. Patch membrane potentials are indicated above the records. Patch action current wave forms corresponding to cellular action potentials are evident in records taken at r.p. +50 and r.p. +70 mV. *B*, peak patch *I–V* relationship for the same cell taken at patch holding potentials of r.p. −50 mV (circles) and r.p. (squares). Filled circles indicate records in which regenerative activity was present. The upper curve was fitted to the data (V_h = r.p.) by eye and scaled by 3·4 to generate the lower curve. The cell was the same as that used in the experiment of Fig. 4.

currents were +46 and +12 mV for currents obtained at V_h = r.p. −50 mV and r.p. respectively. The predicted 34 mV shift to the left for the larger currents is close to the observed shift. When the currents recorded at r.p. are scaled by 3·4, the resulting curve fits the curve taken at r.p. −50 mV reasonably well for currents where regenerative activity was absent, suggesting that cell depolarization is not significant in the absence of regenerative Ca channel activity.

D.r.g. cells typically maintain resting potentials of −40 to −50 mV under the ionic conditions used in these experiments and exhibit Ca action potentials that are usually 80–90 mV in amplitude and roughly 10 ms in duration (Dichter & Fischbach, 1977). If a Ca spike with these characteristics were to be recorded through an isolated cell-attached patch held at r.p. −50 mV (pipette potential = +50 mV), the cell would depolarize to a peak value of about +50 mV and therefore no net current would be driven through the pipette near the peak of the spike. The two action currents shown in Fig. 3*A* peak near the null-current level, supporting this prediction. Furthermore, with a calculated maximum patch conductance of 5·0 nS, a cellular action potential 80–90 mV in magnitude would correspond to a 400–450 pA action current measured through the patch. This is close to the measured action current value of 432 pA.

The effects of NA on the Ba patch currents and cellular Ca action currents described above are illustrated in Fig. 4. Similar results were observed in two other cells. Currents in Fig. 4*A* were recorded using a command voltage of r.p. +50 mV. A 30 s application of 10 μM-NA reversibly decreased the action current duration. In contrast, peak inward patch currents did not appear to be affected by the NA

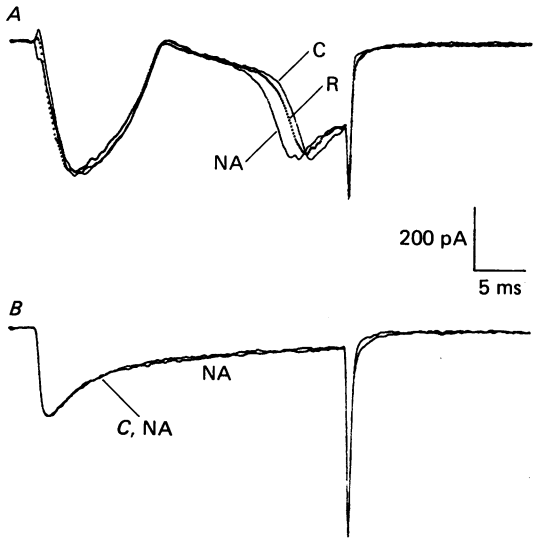

Fig. 4. NA decreases action potential duration without affecting patch currents. *A*, patch currents elicited by a 30 ms step to r.p. + 50 mV from a patch holding potential of r.p. − 50 mV before (C), immediately after a 30 s application of 10 μM-NA (NA), and after a 3 min wash in NA-free control solution (R). NA reversibly shortened the cellular action potential duration (monitored by the corresponding extracellulary recorded patch action current) and had no discernible effect on peak inward patch currents. *B*, the protocol in *A* was repeated at a patch potential on the positive limb of the patch *I–V* curve (r.p. + 90 mV) where cellular regenerative activity was absent. The cell was the same as that used in the experiment of Fig. 3.

challenge, illustrating that extrapatch NA has little effect on isolated patch currents in a d.r.g. cell with demonstrated NA sensitivity. The magnitude of NA-induced action potential shortening we observed in these experiments was not as large as has been observed in dialysed cells (Forscher & Oxford, 1984, 1985); however, this may be due to the absence of tetraethylammonium (TEA) ions in the bath solutions employed here. TEA was added in previous whole-cell recording experiments to increase action potential duration via partial K channel blockade. TEA was not used in the present study because d.r.g. cell membranes become granular in appearance after TEA exposure and maintenance of membrane integrity was essential for obtaining reliable membrane-pipette seals for cell-attached patch recordings.

In order to document the lack of NA effect on Ca channels in this patch under more controlled conditions another NA application was performed while currents were monitored on the positive limb of the patch current *I–V* relationship. Patch currents recorded at a command potential of r.p. + 90 mV in the presence and absence of NA superimpose (Fig. 4*B*). There was no evidence of uncontrolled regenerative activity present in the records at this patch potential. As discussed above, the inward currents in this example probably depolarized the cell. However, the close match in current kinetics between control and NA records would be unlikely if NA had caused a significant decrease in patch current and consequent negative shift in patch potential. These results support previous evidence suggesting that negative modulation of Ca

channels by NA involves a direct effect on the Ca channel itself or a molecule tightly coupled to channel function (Forscher & Oxford, 1985).

Adenylate cyclase activity in d.r.g. cells

NA has little effect on intracellular cyclic AMP levels in d.r.g. cells

In order to test directly whether changes in cyclic AMP levels might accompany and thus contribute to any form of NA-mediated modulation of Ca channels, we have assayed the effects of NA, isoprenaline and forskolin on adenylate cyclase activity in intact and broken d.r.g. cell preparations. Isoprenaline effects were examined to assess whether d.r.g. cells respond to β-adrenergic receptor stimulation. Forskolin was tested to get an estimate of the level of adenylate cyclase stimulation we might expect to achieve during biophysical experiments on d.r.g. cells using bath application of this compound and as a probe for the presence of adenylate cyclase in these cells.

Adenylate cyclase assays were performed on d.r.g. cells grown in the presence of cytosine arabinoside which eliminated all visible background cells. Exposure of intact d.r.g. cells to 10 μM-NA for 10 min had little effect on adenylate cyclase activity. In contrast, isoprenaline (10 μM) and forskolin (50 μM) stimulated adenylate cyclase, resulting in 2- and 60-fold increase in intracellular cyclic AMP, respectively, over basal levels (Table 2).

TABLE 2.

Treatment	*n* (dishes)	% conversion ATP to cyclic AMP*
Control	5	0·12 ± 0·01
10 μM-NA	5	0·14 ± 0·01
10 μM-isoprenaline	4	0·24 ± 0·04
50 μM-forskolin	2	7·1 ± 0·50

* Mean ± S.D.

These results indicate that : (1) NA does not cause significant increases in cyclic AMP levels at concentrations which produce large decreases in Ca channel currents, (2) intact d.r.g. cells display weak adenylate cyclase stimulation mediated by β-adrenergic receptor activation and (3) forskolin has a large stimulatory effect on adenylate cyclase in d.r.g. cells similar to that seen in other systems (Seamon, Padgett & Daly, 1981).

Experiments performed using broken d.r.g. cell preparations revealed a basal adenylate cyclase activity of 115 pmol cyclic AMP formed/mg protein.min. Interestingly, stimulation of adenylate cyclase activity by isoprenaline was much weaker than in the intact cells, a finding which is consistent with discrepancies observed previously between brain slice and brain homogenate preparations (Daly, 1977). However, stimulation of adenylate cyclase by forskolin remained intact (10 μM-forskolin treatment caused a 12-fold increase in cyclic AMP formed/mg protein . min over basal levels). Forskolin is thought to activate adenylate cyclase through direct interactions with the catalytic subunit and guanine nucleotide binding proteins and thus does not rely on intact receptor–adenylate cyclase coupling for its effects (Seamon *et al.* 1981; Green & Clark, 1982).

It should be emphasized that although forskolin at concentrations of 1–50 μM strongly stimulated adenylate cyclase in d.r.g. cells (Table 2 above), treatment with forskolin or a cyclic AMP analogue (dibutyryl cyclic AMP) did not appear to affect NA-mediated Ca channel modulation in whole-cell recording preparations (data not shown). Dunlap (1984) has also reported that forskolin treatment does not interfere with action potential modulation by NA in intact cells.

DISCUSSION

Currents recorded from drug-free cell-attached patches were never reversibly decreased by external application of 10–100 μM-NA. In contrast, macroscopic Ca channel currents were rapidly decreased under the same external conditions. In addition, Ca action currents recorded from d.r.g. membrane exposed to NA were decreased in duration by bath-applied NA whereas cell-attached patch currents recorded from the same cell remained unaffected. These results suggest that NA is not decreasing Ca channel currents in d.r.g. cells through pathways which employ diffusable second messengers. Rather the NA receptor is tightly coupled to the channel either directly or via another membrane-associated molecule.

Alternative explanations for our observations include the possibilities: (1) that patch formation somehow mechanically disrupts receptor–channel coupling via a second messenger pathway despite the care taken to form gigaseals gently or (2) that the isotonic Ba solution used in the pipette might disrupt channel modulation. Other studies of Ca channel modulation in heart cells however indicate that such explanations are unlikely. For example, the number of functional Ca channels in heart cells is increased following exposure to β-adrenergic agonists. This effect, mediated by an increase in intracellular cylic AMP and activation of a cyclic-AMP-dependent protein kinase, has been demonstrated in single-channel recordings of Ba currents from cell-attached patches in dissociated frog ventricular cells (Reuter, 1983; Tsien *et al.* 1983).

The rapid and fully reversible NA effects characteristic of whole-cell recording experiments were never observed in the cell-attached patch experiments described in this paper; however, patch currents both increased and in some cases decreased slowly subsequent to NA exposure. These changes had time constants of the order of minutes, whereas NA modulation is normally a rapid event reaching maximum Ca channel inhibition within seconds after agonist application. NA effects are also rapidly reversible in whole-cell preparations. We cannot rule out the possibility that the cell-attached patch configuration in some way drastically slowed the kinetics of the response or decreased the likelihood of obtaining a response (Dunlap & Fischbach (1978) reported that 92% ($n = 52$) of chick d.r.g. cells tested respond to NA). If NA receptors and/or modulatable Ca channels are asymmetrically distributed on the cell surface, experiments performed on patches distant from receptor clusters might respond slowly to remote receptor occupation, i.e. second messenger action could be delayed by diffusion barriers.

Recent evidence has appeared suggesting the existence of three distinct classes of Ca channels in d.r.g. neurones (Nowycky, Fox & Tsien, 1985). These channels can be distinguished on the bases of inactivation behaviour, single-channel conductance,

voltage dependence, and pharmacological sensitivity. It is conceivable that only one class of Ca channel is NA sensitive and is clustered over the cell surface in discrete patches. This would lead to a lowered probability of obtaining NA-sensitive cell-attached patches and could result in a false conclusion regarding second messenger involvement. These interpretations cannot be definitively ruled out without cell-attached patch mapping studies employing drug changes in the pipette and/or outside-out patch recordings with external NA application. However, in view of the essential absence of NA effects in all seventeen patches examined, we regard our negative findings as representative.

We have tentatively attributed increases in cell-attached patch current after NA treatment to potential-dependent relief from channel inactivation, since this type of result was most often observed at patch holding potentials negative to the cell r.p. An alternative explanation could be that NA triggers a slow second-messenger-mediated effect that is normally masked by the larger more rapid NA-induced Ca conductance decreases observed in whole-cell recording experiments. In accord with this hypothesis we have sometimes observed slow increases in whole-cell Ca channel currents after complete recovery from a high dose of NA (100 μM). This NA dose is two orders of magnitude higher than the ED_{50} for the rapid inhibitory effect (Canfield & Dunlap, 1984). If d.r.g. cells have a population of lower-affinity amine receptors that trigger increases in Ca channel conductance, they might contribute preferentially to the Ca channel effects we have observed at high NA doses. Canfield & Dunlap (1984) reported that amine application (NA, 5-hydroxytryptamine or dopamine) at concentrations greater than 10 μM caused smaller decreasees in action potential shortening than lower, maximally effective doses. They suggested that the amines might have partial antagonist effects in the higher dose range. Their observations could alternatively be explained by the presence of an opposing effect on action potential duration triggered at the higher agonist concentrations.

This study demonstrates that NA has no effect on adenylate cyclase activity in a dose range where where NA treatment results in large decreases in Ca channel currents in internally dialysed sensory neurones. We have demonstrated previously that internal cyclic AMP at concentrations that would saturate cyclic-AMP-dependent processes such as protein kinase activation does not inhibit expression of NA modulation (Forscher & Oxford, 1985). We found that incubation of cells in forskolin or dibutyryl cyclic AMP did not inhibit expression of NA modulation (data not shown) although forskolin increased intracellular cyclic AMP levels in both intact and broken cell preparations. High levels of intracellular cyclic AMP (or dibutyryl cyclic AMP) would tend to buffer effects due to acute decreases in cyclic AMP and mimic effects due to cyclic AMP increases. These observations provide further evidence that acute changes in intracellular cyclic AMP are not involved in mediation of NA-induced modulatory effects. The lack of NA effects in the cell-attached patch experiments presented above also suggest that NA may decrease Ca currents in d.r.g. cells through a direct effect on the Ca channel itself or on a molecule tightly coupled to Ca channel function.

An example of such mechanisms may be reflected in recent observations in cardiac muscle which suggest the direct coupling of a guanine nucleotide binding protein (G protein) to an inwardly rectifying K channel and to a muscarinic receptor during

cholinergic activation of K currents (Pfaffinger, Martin, Hunter, Nathanson & Hille, 1985; Breitweiser & Szabo, 1985). If, on the other hand, a second message is somehow involved in the NA response, it is mediated over short distances relative to those observed in cardiac cells (Reuter, 1983) and is unlikely to employ cytosolic diffusable molecules. In this regard, Rane & Dunlap (1985) have recently presented evidence that diacylglyerol activation of protein kinase C, a membrane-associated, hydrophobic messenger system, could function as a link between NA binding and Ca channel modulation in d.r.g. neurones. Because bath-applied NA does not have any clear effects on Ca channel activity in drug-free cell-attached patches, an unambiguous answer to the question of NA effects on single Ca channels and the biochemical mechanisms involved may have to await demonstration of NA modulation in excised outside-out membrane patches.

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