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Atrial Natriuretic Peptide Inhibits Evoked Catecholamine Release by Altering Sensitivity to Calcium[1]

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ABSTRACT

Natriuretic peptides are cyclized peptides produced by cardiovascular and neural tissues. These peptides inhibit various secretory responses such as the release of renin, aldosterone and autonomic neurotransmitters. This report tests the hypothesis that atrial natriuretic peptide reduces dopamine efflux from an adrenergic cell line, rat pheochromocytoma cells, by suppressing intracellular calcium concentrations. The L-type calcium channel inhibitor, nifedipine, markedly suppressed dopamine release from depolarized PC12 cells, suggesting that calcium entering through this channel was the predominant stimulus for dopamine efflux. Atrial natriuretic peptide maximally reduced depolarization-evoked dopamine release 20 ± 3% at a concentration of 100 nM and this effect was abolished by nifedipine, but not by pretreatment with the N-type calcium channel inhibitor, ω-conotoxin, or an inhibitor of calcium-induced calcium release, ryanodine. In cells loaded with Fura-2, atrial natriuretic peptide both augmented depolarization-induced increases of intracellular free calcium concentrations and accelerated the depolarization-induced quenching of the Fura-2 signal by manganese, findings consistent with enhanced conductivity of calcium channels. Dopamine efflux induced by either the calcium ionophore, A23187, or staphylococcal α toxin was attenuated by atrial natriuretic peptide. Additionally, a natriuretic peptide interacting solely with the natriuretic peptide C receptor in these cells, C-type natriuretic peptide, also suppressed calcium-induced dopamine efflux in permeabilized cells. These data are consistent with natriuretic peptides attenuating catecholamine exocytosis in response to calcium but inconsistent with the neuromodulatory effect resulting from a reduction in intracellular calcium concentrations within pheochromocytoma cells.

Atrial natriuretic peptide, the first member of the natriuretic peptide family to be identified (de Bold *et al.*, 1981), affects cardiovascular homeostasis by promoting diuresis, natriuresis and vasodilation, and by reducing both aldosterone secretion (Atlas and Maack, 1987) and adrenergic neurotransmitter release (Debinski *et al.*, 1990). The inhibitory effects of atrial natriuretic peptide on adrenergic neurotransmission have been reported in rat isolated mesenteric arteries (Nakamaru and Inagami, 1986), rabbit isolated vasa deferentia (Drewett *et al.*, 1989a), bovine adrenal chromaffin cells (Babinski *et al.*, 1995), the rat hypothalamus (Giridhar *et al.*, 1992) and nerve growth factor-treated PC12 cells (Drewett *et al.*, 1988). This inhibition of adrenergic neurotransmission, although widely observed, has not been explained mechanistically. We attempt to resolve the mechanism of natriuretic peptide neuromodulation by defining the following: 1) the calcium components accounting for neurotransmitter release from PC12 cells; 2) the involvement of the various calcium channels in neuromodulatory effects of atrial natriuretic peptides; 3) the influence of atrial natriuretic peptide on calcium homeostasis in depolarized cells; 4) the influence of atrial natriuretic peptide on the sensitivity of the exocytotic process to calcium and 5) the effect of a selective ligand for natriuretic peptide C receptors on exocytosis of dopamine in response to calcium.

Because calcium is typically the stimulus for neurotransmitter release from neurons (Kelly *et al.*, 1979), initial experiments sought to identify the calcium channel promoting neurotransmitter release in response to a depolarizing stimulus. We then tested the involvement of various calcium channels in neuromodulatory effects of natriuretic peptides by observing whether natriuretic peptide effects were sustained in the presence of selective calcium channel antagonists. The last group of experiments examined whether atrial natriuretic peptide attenuates catecholamine release in response to a depolarizing stimulus by suppressing either calcium entry into the cells or calcium effects on exocytosis. A suppression of calcium entry should be evidenced by reductions in both calcium conductance and intracellular calcium concentrations. Alternatively, a decreased sensitivity of do-

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ABBREVIATIONS: ANP, atrial natriuretic peptide; PC12, pheochromocytoma cells; EGTA, ethyleneglycol-bis-(β-amino-ethyl ether)N, N′-tetraacetic acid; ANOVA, analysis of variance.

pamine exocytosis to calcium should be indicated by an attenuation of dopamine release in cells permeabilized to calcium. Natriuretic peptides have been demonstrated to reduce intracellular calcium concentrations (Hassid, 1986; Cornwell and Lincoln, 1988; Barrett *et al.*, 1991; Nascimento-Gomes *et al.*, 1995), calcium conductance (Gisbert and Fischmeister, 1988; Sorbera and Morad, 1990; Pella, 1991; Le Grand *et al.*, 1992; White *et al.*, 1993) and calcium fluxes in a variety of cell types (Chiu *et al.*, 1986). Natriuretic peptides also suppress calcium concentrations in vascular smooth muscle by augmenting a calcium pump extruding calcium (Furukawa *et al.*, 1988). In contrast, natriuretic peptides augment L-type calcium channel conductance (McCarthy *et al.*, 1990; Dai and Quamme, 1993) and suppress calcium-induced aldosterone production from adrenal glomerulosa cells (Lotshaw *et al.*, 1991). Furthermore, the potentiative effect of natriuretic peptides on calcium conductance appeared to be mediated by the natriuretic peptide C receptor (Isales *et al.*, 1992), whereas the inhibitory effect was mediated by a different natriuretic peptide receptor that elevated guanylyl cyclase activity (Oda *et al.*, 1992). The majority of these studies examining natriuretic peptide effects on calcium homeostasis find an inhibitory effect on intracellular calcium concentrations. The exception to this generalization occurs in the adrenal glomerulosa, where natriuretic peptides attenuate calcium effects to augment aldosterone synthesis (Lotshaw *et al.*, 1991). Our study defines atrial natriuretic peptide effects on calcium concentrations, calcium conductance and the calcium sensitivity of neurotransmitter exocytosis in PC12 cells and the relevance of these actions to neuromodulatory effects of natriuretic peptides.

Materials and Methods

Cell culture. PC12 cells were subcultured in 25-cm^3 flasks or 25-mm coverslips coated with rat tail collagen and bathed in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum and 5% heat inactivated horse serum. Cells were plated at a density of 6×10^5 per 25-cm^3 culture flask for catecholamine release experiments and 3.6×10^5 cells per coverslip for measurement of intracellular free calcium concentration. After 24 hr, the growth media was replaced by differentiating media consisting of Dulbecco's modified Eagles medium containing 200 ng/ml 7S-nerve growth factor and 1% fetal calf serum. Cells were allowed to differentiate over a period of 8 to 10 days in an incubator at 37°C and an atmosphere containing 5% CO_2. Cells received fresh differentiating media every 3 days. Cells from passages 18 to 35 were used for experiments.

Catecholamine release and measurement. Catecholamine release was induced by exposure to either high potassium chloride concentrations (40 mM), caffeine (40 mM), the calcium ionophore, A23187 (10 μM) or staphylococcal α toxin (100 U/ml), in the presence of 2 mM extracellular calcium concentrations. The high potassium buffer contained 76 mM NaCl, 40 mM KCl, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM glucose. Catecholamines were measured by high performance liquid chromotography with electrochemical detection, as previously described (Trachte *et al.*, 1995). The participation of the various calcium channels in mediating catecholamine release in response to the high potassium buffer was assessed using either the L-type calcium channel inhibitor, nifedipine (20 nM), or an inhibitor of N-type calcium channels, ω-conotoxin (500 nM). The contribution of calcium released from the intracellular ryanodine-sensitive calcium store toward catecholamine release was assessed by the ability of ryanodine (10 μM) to block catecholamine secretion. The importance of extracellular calcium in inducing dopamine efflux was defined by eliminating calcium from the high potassium buffer and substituting 2 mM EGTA. Nifedipine was dissolved in 95% alcohol and diluted in Krebs-bicarbonate buffer. ω-Conotoxin and ryanodine were dissolved and diluted in Krebs-bicarbonate buffer. This buffer was identical to the high potassium buffer except that the concentration of sodium chloride was adjusted to 112 mM, and that of potassium chloride, to 4.5 mM. Cells were pretreated with nifedipine, ω-conotoxin or ryanodine for 5 min before treatment with the high potassium buffer. The medium then was discarded and each flask was treated with the high potassium buffer in the presence of the above test agents or their vehicles for 5 min. The effectiveness of ryanodine was ascertained by its ability to block dopamine efflux promoted by caffeine (40 mM).

The effect of atrial natriuretic peptide on high potassium-induced catecholamine release was examined by exposing each culture to the high potassium buffer plus atrial natriuretic peptide concentrations of 1, 10 or 100 nM or the atrial natriuretic peptide diluent (Krebs-bicarbonate buffer). The involvement of calcium channels or the intracellular ryanodine-sensitive calcium pool in natriuretic peptide effects on high potassium-induced catecholamine release was assessed by repeating the above experimental sequence in cultures pretreated with nifedipine, ω-conotoxin or ryanodine for 5 min and these test agents were also present in the high potassium buffer during the 5-min treatment period.

The influence of natriuretic peptides on exocytosis induced by calcium was assessed using either the calcium ionophore, A23187 (10 μM), or the membrane permeabilizing agent, staphylococcal α toxin (100 units/ml in a final volume of 3 ml), in the presence of 2 mM calcium. A23187 was dissolved in dimethyl sulfoxide and diluted in Krebs-bicarbonate buffer. Cultures were treated with Krebs-bicarbonate buffer containing A23187 and either the natriuretic peptide vehicle (Krebs-bicarbonate buffer) or one of the three concentrations of atrial natriuretic peptide (0.1–10 nM). Staphylococcal α toxin was dissolved in distilled water and diluted in calcium-free Krebs-bicarbonate buffer to achieve a final concentration of 100 units/ml. Calcium-free Krebs-bicarbonate buffer was prepared by excluding $CaCl_2$ and chelating residual calcium with 4 mM EGTA. Cells were exposed to calcium-free buffer containing staphylococcal α toxin for 30 min. The medium then was discarded and each flask was treated with Krebs-bicarbonate buffer containing 2 mM $CaCl_2$ and either the atrial natriuretic peptide vehicle or one of the three concentrations of atrial natriuretic peptide (0.1–10 nM) for 5 min. The potential for atrial natriuretic peptide to shift the sensitivity of the exocytotic machinery to calcium was investigated further in cells permeabilized with staphylococcal α toxin. The calcium concentration was buffered by altering the ratio of EGTA to calcium, as described by Portzehl *et al.* (1964).

Digitonin (10 μM for 4 min) was used to permeabilize cells in six experiments involving C-type natriuretic peptide. After permeabilization, cells were exposed to an intracellular buffer consisting of potassium glutamate (137 mM), HEPES (10 mM), magnesium chloride (5 mM), adenosine triphosphate (5 mM), EGTA (4 mM) and calcium chloride (4 mM) containing either one of four concentrations of C-type natriuretic peptide (0.01 to 10 nM) or its diluent (distilled water). Catecholamines in the effluent and cells then were extracted and analyzed as described above.

Measurement of cytosolic free calcium concentration. The effect of natriuretic peptides on depolarization-induced alterations in intracellular calcium concentrations was determined by monitoring fluorescence of the calcium sensitive dye, Fura-2 (Grynkiewicz *et al.*, 1985). Cells grown on coverslips were incubated with 1 μM Fura-2 acetoxymethyl ester in serum free Dulbecco's modified Eagle's medium (pH 7.2) containing 0.05% bovine serum albumin for 30 min at room temperature. Subsequently, the above medium was discarded and cells were incubated for an additional 30 min in medium lacking Fura-2 acetoxymethyl ester to allow for the deesterification of the intracellular dye. After incubation, cells were rinsed once with serum free media and twice with physiological salt solution containing 118 mM NaCl, 5.0 mM KCl, 1.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM

Na_2HPO_4, 24 mM Hepes and 10 mM glucose (pH 7.5). The coverslips were then set in Bellco chambers. After an initial resting period in physiological salt solution, cells were treated with 40 mM KCl in physiological salt solution to increase intracellular calcium concentrations over basal levels. In identical experiments, cells were treated with 100 nM atrial natriuretic peptide in the depolarizing physiological salt solution to test for its effects on intracellular calcium concentrations. Intracellular calcium concentrations were determined by measuring the ratio of the Fura-2 fluorescence detected at 510 nm when excited at either 340 or 380 nm (Grynkiewicz *et al.*, 1985). Fluorescence ratios were converted to calcium concentrations, as described by Grynkiewicz *et al.* (1985). The calibration values for R_{max} and R_{min} were obtained by permeabilization of the cells with digitonin (10 μM) followed by the addition of EGTA (4 mM). The K_D value was taken as 135 nM since these experiments were performed at room temperature (Grynkiewicz *et al.*, 1985).

Fluorescence measurements with manganese quenching. Calcium conductance in PC12 cells was assessed using the manganese technique described by Merritt *et al.* (1989). Changes in intracellular manganese concentrations were measured by taking advantage of the isobestic point of Fura-2, wavelength 357 nm, where the intensity of the fluorescence signal is independent of intracellular calcium concentrations. Cells were incubated for 5 min in a nominally calcium-free physiological salt solution containing 500 μM manganese chloride before application of a nominally calcium-free physiological salt solution containing 40 mM potassium chloride. Either atrial natriuretic peptide (100 nM) or its vehicle was included in the depolarizing calcium-free physiological salt solution to assess its effect on manganese quenching. The excitation wavelength employed was 357 nm, while fluorescence was measured at an emission wavelength of 510 nm.

Materials. Human atrial natriuretic peptide, ω-conotoxin GVIA, nifedipine, caffeine, fetal calf serum, heat inactivated horse serum, 7S-nerve growth factor, norepinephrine, dihydroxybenzylamine, dopamine, digitonin and A23187 were purchased from Sigma Chemical Co. (St. Louis, MO). Staphylococcal α toxin and Dulbecco's modified Eagles medium were obtained from GIBCO BRL (Gaithersburg, MD). Rat tail collagen was purchased from Collaborative Biomedical Products (Bedford, MA). Fura-2 acetoxymethyl ester was obtained from Molecular Probes, Inc. (Eugene, OR). C-type natriuretic peptide was obtained from Peninsula Laboratories (Belmont, CA).

Statistical analysis. Concentration-response curves were compared by analysis of variance (ANOVA) for repeated measures and individual values were compared by Student's paired t test with Dunnett's correction for multiple comparisons. $P \leq .05$ was considered statistically significant.

Results

Involvement of specific calcium channels in neurotransmitter release. As shown in figure 1, dopamine efflux induced by the high potassium buffer averaged 12.4 ± 0.6% of total dopamine content in the presence of vehicles for nifedipine, ω-conotoxin, EGTA or ryanodine. Nifedipine (20 nM) and EGTA (2 mM) markedly inhibited dopamine release to 7.1 ± 1.2% (P = .0004) and 2.6 ± 1.0% (P = .04), respectively. Neither ω-conotoxin (500 nM) nor ryanodine (10 μM) significantly affected dopamine efflux, which averaged 11.2 ± 1.3% (P = .14) and 13.1 ± 0.8% (P = .48), respectively, in their presence. These results are consistent with L-type calcium channels being the major conduit for calcium inducing dopamine release in these cells; however, neither ω-conotoxin-sensitive channels nor intracellular ryanodine-sensitive calcium stores appear to participate in catecholamine release evoked by high potassium concentrations in PC12 cells. The effectiveness of the ryanodine concentration was confirmed by a 62 ± 2% attenuation of dopamine efflux evoked by 40 mM caffeine ($P < .05$; fig. 2). The concentrations of nifedipine and ω-conotoxin have been demonstrated to be effective by prior investigators (Shafer and Atchison, 1991; Hirning *et al.*, 1988). The pronounced reduction in catechol-

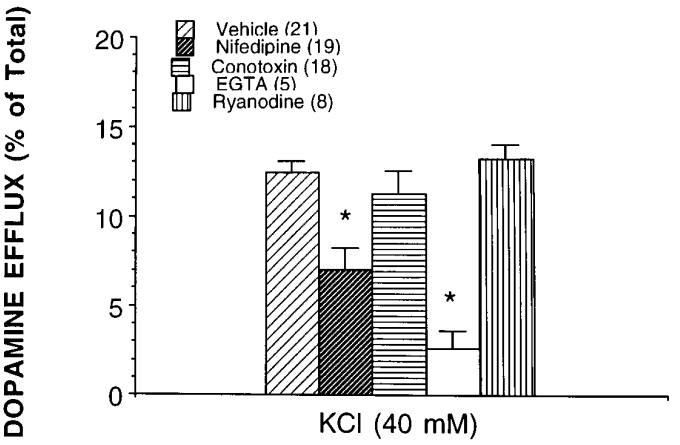

Fig. 1. Dopamine release after exposure to high potassium (40 mM) buffer. Cells were incubated with one of the following agents in high potassium buffer for 5 min: 20 nM nifedipine, 500 nM ω-conotoxin, 2 mM EGTA, 10 μM ryanodine or their vehicles. Results with the vehicles were combined because no vehicle affected dopamine release evoked by the high potassium buffer. Numbers in parentheses indicate the number of experiments per group. All values are means + S.E. The asterisk indicates a significant difference from cultures receiving vehicles (*$P < .05$ by paired t test with Dunnett's correction for multiple comparisons).

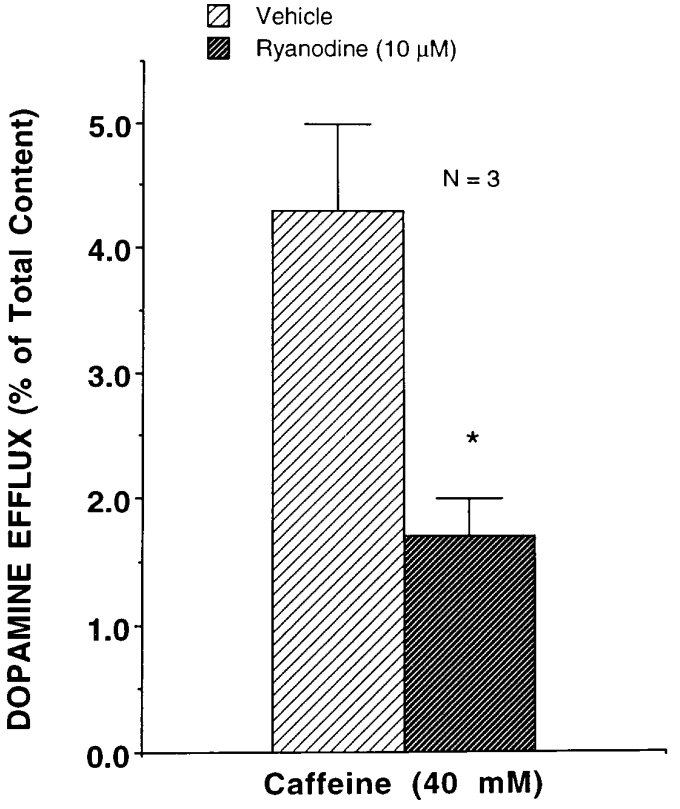

Fig. 2. Effect of ryanodine (10 μM) on dopamine efflux evoked by 40 mM caffeine. The asterisk indicates a significant effect of ryanodine ($P < .05$ by Student's paired t test). All values are means ± S.E. The number of experiments is indicated by the N.

amine release in the presence of EGTA and nifedipine indicates that extracellular calcium entering through L-type calcium channels is the predominant stimulus for dopamine efflux initiated by depolarization in these PC12 cells.

Involvement of specific calcium channels in neuromodulatory effects of natriuretic peptides. Figure 3 illustrates the effects of atrial natriuretic peptide on dopamine release evoked by the high potassium buffer. Atrial natriuretic peptide (100 nM) maximally suppressed dopamine efflux 20 ± 3% of control release (P = .004). The effect of atrial natriuretic peptide was nearly maximal at concentrations as low as 1 nM, therefore, no EC_{50} could be calculated. Attempts to better define the control curve were frustrated by the finding that a lower concentration of atrial natriuretic peptide, 0.1 nM, failed to influence neurotransmitter efflux in six experiments.

The potential role of calcium channels in the neuromodulatory effect of atrial natriuretic peptide was assessed using inhibitors of the various channels. The presence of nifedipine (20 nM) in the high potassium buffer prevented atrial natriuretic peptide from reducing dopamine efflux (fig. 3). In fact, atrial natriuretic peptide tended to elevate dopamine efflux in the presence of nifedipine, although this effect was not statistically significant. Thus, treatment with nifedipine completely eliminated inhibitory neuromodulatory effects of atrial natriuretic peptide (P = .008 by ANOVA). In contrast, neither ω-conotoxin nor ryanodine altered the neuromodulatory effect of atrial natriuretic peptide (P = .60 and .80, respectively, data not shown for ω-conotoxin because they obscure the control curve). These results are consistent with atrial natriuretic peptide reducing dopamine secretion either by modulating L-type calcium channels or by affecting a process initiated by calcium entering through L-type calcium channels.

Atrial natriuretic peptide effects on calcium homeostasis. Figure 4 depicts the effect of 100 nM atrial natriuretic peptide on intracellular calcium concentrations in the presence of high potassium concentrations (40 mM) as a depolarizing stimulus over a time course of 2.9 min. The high potassium solution raised the level of intracellular calcium concentrations from a basal value of 75 ± 3 nM to 179 ± 13 nM. When the cells were challenged with 100 nM atrial natriuretic peptide in the presence of the high potassium solution, intracellular calcium concentrations increased to 631 ± 197 nM. This effect was statistically significant com-

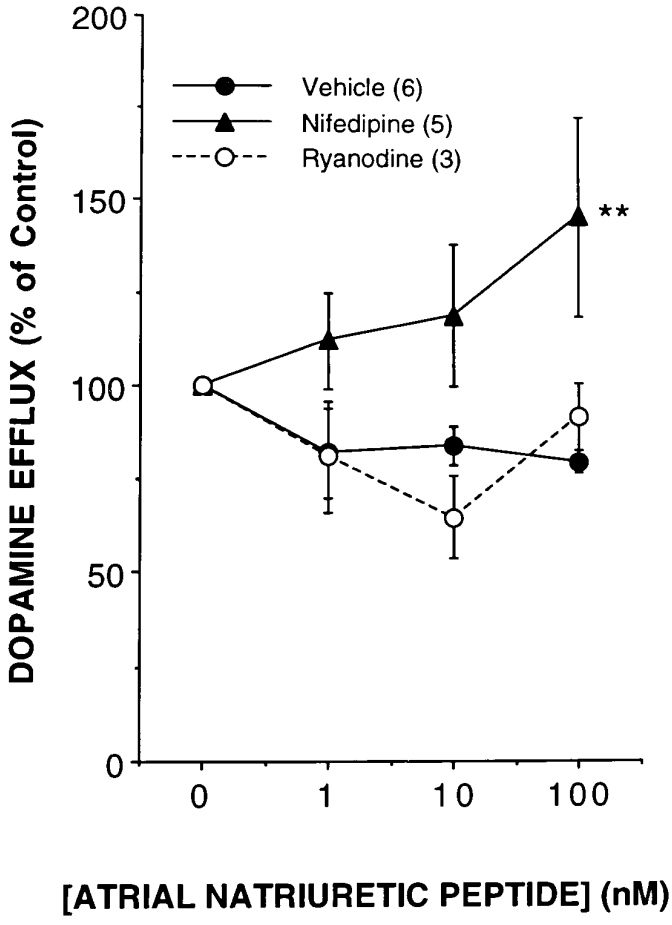

Fig. 3. Effect of atrial natriuretic peptide on dopamine release evoked by high potassium (40 mM) in the presence of nifedipine (20 nM), ryanodine (10 μM) or their vehicle. Control release is taken as 100%. All values are means ± S.E. and are expressed as % of control. Atrial natriuretic peptide reduced dopamine efflux in the presence of the vehicle or ryanodine but did not suppress dopamine release in the presence of nifedipine. The asterisks indicate a significant difference from cultures receiving the vehicle (P < .01 by ANOVA). The number of experiments performed in each group is indicated in parentheses.

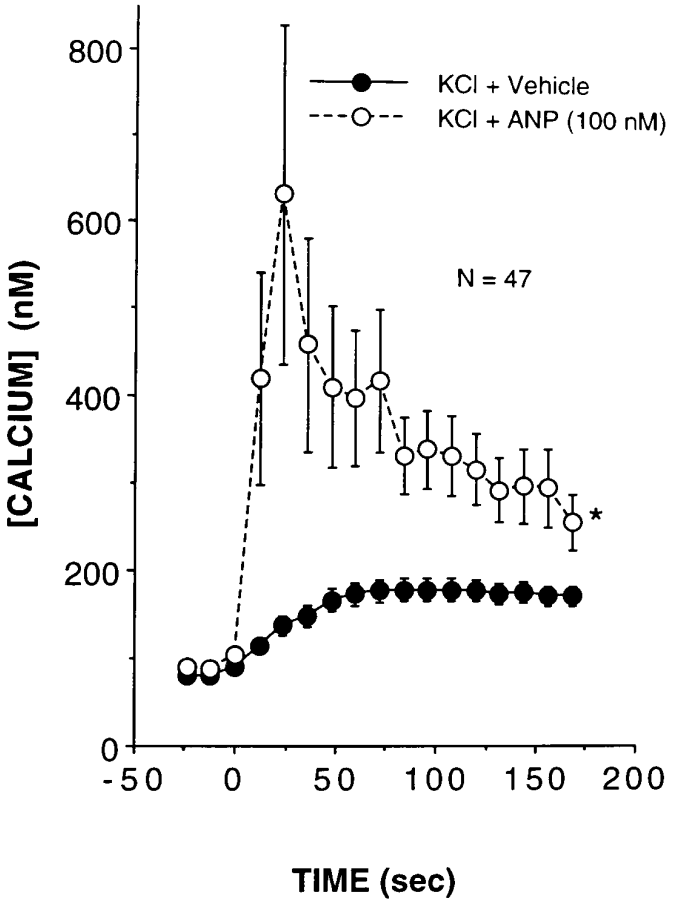

Fig. 4. Effect of high potassium (KCl; 40 mM) in the presence of atrial natriuretic peptide (ANP 100 nM) or its diluent on cytosolic free calcium concentrations. Atrial natriuretic peptide augmented increases in cytosolic free calcium concentrations (*P < .05 by ANOVA) resulting from exposure to high potassium. All values are means ± S.E. obtained from 47 cells in three separate experiments.

pared to the response observed in cells exposed to the high potassium solution containing the atrial natriuretic peptide vehicle (P = .02). These surprisingly potentiative effects on intracellular calcium concentrations were observed at atrial natriuretic peptide concentrations as low as 0.1 nM (P = .02 by ANOVA, data not shown). Atrial natriuretic peptide failed to alter calcium concentrations within cells that were not depolarized (data not shown).

Atrial natriuretic peptide could augment the effect of high potassium concentrations on intracellular calcium concentrations by enhancing calcium conductance through the plasma membrane. This possibility was investigated by monitoring the intensity of Fura-2 fluorescence at 357 nm in the presence of extracellular manganese. Figure 5 shows that addition of the calcium-free physiological salt solution containing 40 mM potassium resulted in a time-dependent decrease in fura-2 fluorescence. Atrial natriuretic peptide (100 nM) in the presence of the depolarizing calcium-free physiological salt solution accelerated the rate of Fura-2 quenching, as was revealed by a significantly greater slope in the cells treated with atrial natriuretic peptide (P = .0001). The time required to reduce fluorescence 50% averaged 42 ± 4 sec in the presence of atrial natriuretic peptide and 72 ± 6 sec in the presence of the atrial natriuretic peptide diluent (P = .0001 by Student's *t* test). These data suggest that atrial natriuretic peptide increases intracellular calcium levels by increasing a calcium conductance in the plasma membrane.

Atrial natriuretic peptide effects on exocytosis in response to calcium. The potentiative effect of atrial natriuretic peptide on intracellular calcium concentrations led us to examine whether atrial natriuretic peptide lowered calcium sensitivity of the exocytotic process. We first measured the atrial natriuretic peptide effect on dopamine release in response to calcium influx induced by the calcium ionophore A23187 (10 μM), which released 11.4 ± 2.0% of the total cellular dopamine content. Dopamine efflux in the presence of A23187 was reduced by atrial natriuretic peptide to approximately 40% of control (P < .05 relative to control release)(fig. 6). Staphylococcal α toxin released 5.3 ± 0.2% of the total cellular dopamine content and atrial natriuretic peptide reduced dopamine efflux in a concentration-dependent manner, with a maximal inhibition of approximately 60% occurring at a concentration of 10 nM (fig. 6). Inasmuch as atrial natriuretic peptide reduced both A23187- and staph-

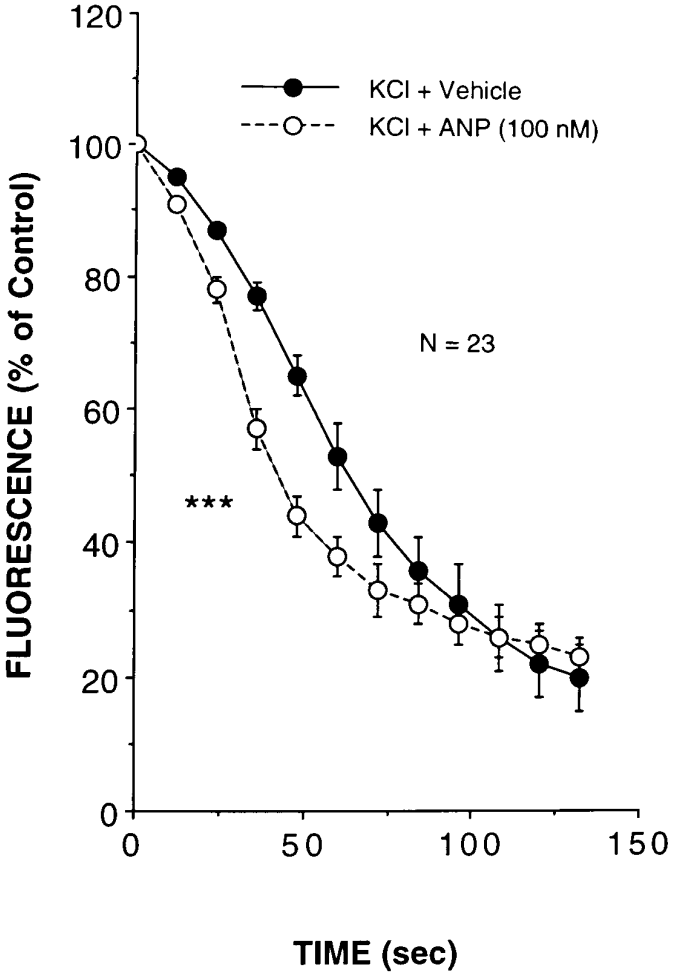

Fig. 5. Effects of high potassium concentrations (KCl) on manganese entry in the presence of atrial natriuretic peptide (KCl + ANP (100 nM)) or its vehicle. The addition of atrial natriuretic peptide significantly accelerated the slope of the curve (***P < .0001). Values are expressed as means ± S.E. and the N indicates the number of cells in which fluorescence was measured.

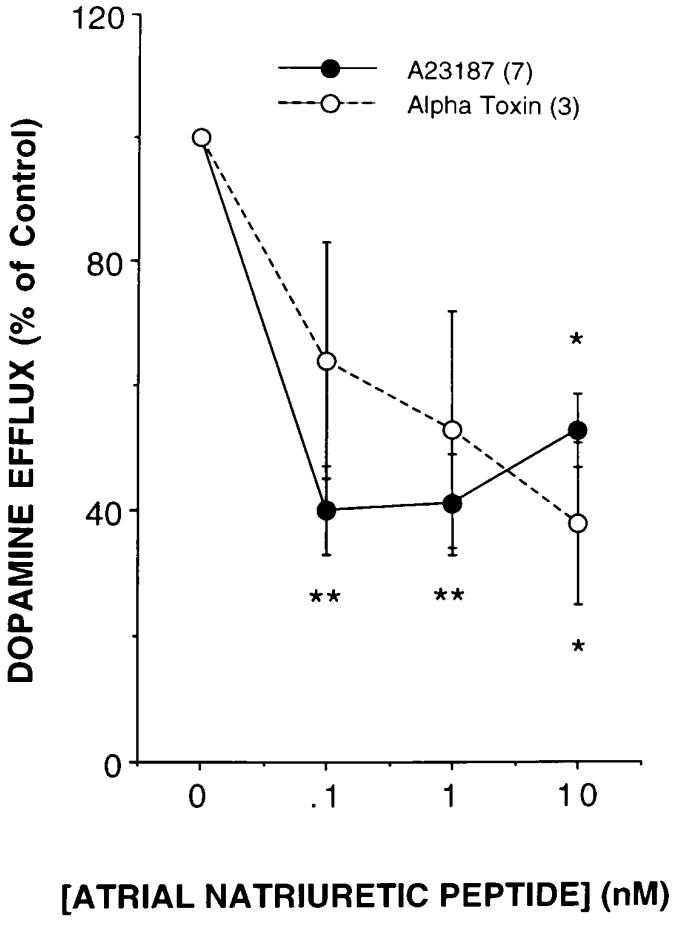

Fig. 6. Effect of atrial natriuretic peptide on dopamine efflux resulting from calcium influx initiated by either the ionophore, A23187 (10 μM), or cell permeabilization with staphylococcal α toxin (Alpha toxin; 100 units/ml). Values are expressed as means ± S.E. and the number of experiments is indicated in parentheses. Asterisks indicate significant differences from the control value in the absence of atrial natriuretic peptide (*P < .05, **P < .01 by paired *t* test with Dunnett's correction for multiple comparisons).

ylococcal α toxin-induced dopamine release, we conclude that atrial natriuretic peptide modulates a calcium sensitive process to reduce dopamine efflux.

An attempt was made to define further the interaction between calcium and atrial natriuretic peptide by testing if atrial natriuretic peptide altered the potency of calcium in promoting dopamine efflux. As shown in figure 7, elevations in calcium concentrations augmented dopamine efflux to a maximum of 6.50 ± 0.58% of total dopamine contents. Atrial natriuretic peptide (100 nM) suppressed the efflux of dopamine in response to calcium without significantly shifting the curve. The EC_{50} values averaged 312 ± 120 and 308 ± 82 nM for the curves representing either vehicle or atrial natriuretic peptide treatment, respectively. Thus, the alteration in calcium sensitivity represents a generalized attenuation of the response and not a displacement of the curve to the right.

C-type natriuretic peptide effects on exocytosis to calcium. The identity of the receptor mediating these actions of natriuretic peptides was investigated using C-type natriuretic peptide, a natriuretic peptide with selectivity for the natriuretic peptide C receptor in PC12 cells (Trachte *et al.*, 1995). Evoked release in the presence of A23187 (10 μM) or digitonin pretreatment was similar; therefore, the results were pooled and averaged 14.9 ± 2.4%. The C-type natriuretic peptide suppressed this evoked release to a maximum of 44 ± 13% at a concentration of 1 nM, as depicted in Figure 8. The C-type natriuretic peptide acted similarly to atrial natriuretic peptide, as presented in Figure 6. Furthermore, the C-type natriuretic peptide also tended to augment intracellular calcium concentration increases in response to depolarization, although the elevations were neither as large as those in the presence of atrial natriuretic peptide nor were they statistically significant (data not shown). Finally, we ascertained that C-type natriuretic peptide failed to influence cyclic guanosine monophosphate concentrations at peptide concentrations altering dopamine efflux (data not shown). These last experiments are consistent with the natriuretic peptide C receptor mediating the effects of natriuretic peptides to suppress catecholamine efflux.

Discussion

These results indicate that atrial natriuretic peptide suppresses catecholamine efflux in response to depolarizing stimuli (fig. 2) although augmenting both calcium conductance (fig. 5) in the cell membrane and calcium concentrations within the cell (fig. 4). Furthermore, natriuretic peptides suppressed calcium-dependent catecholamine efflux

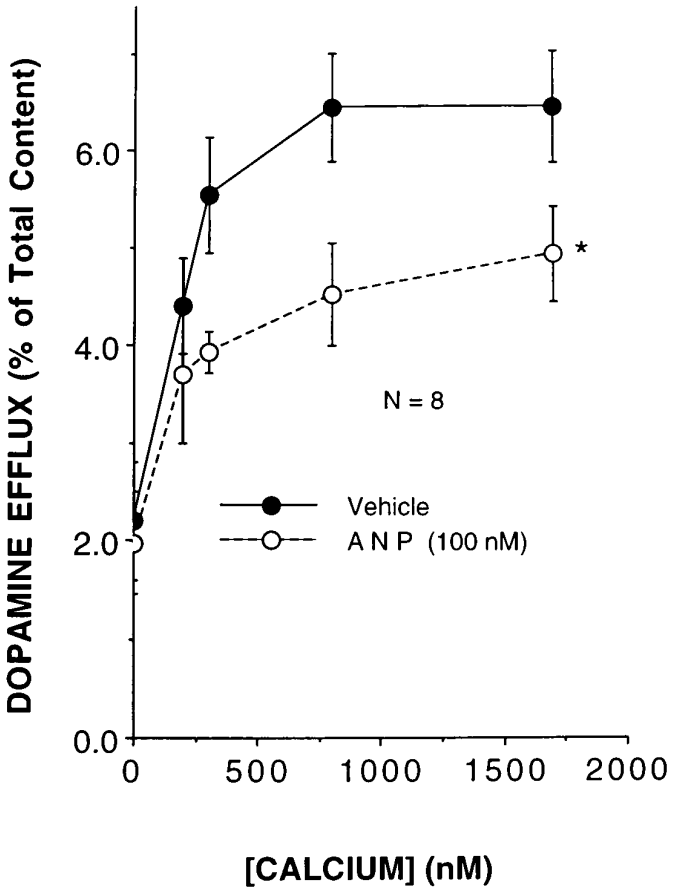

Fig. 7. The effect of atrial natriuretic peptide on the potency and efficacy of calcium in promoting dopamine efflux from cells permeabilized with staphylococcal α toxin. All data points are means ± S.E. The number of experiments is indicated by the N. Atrial natriuretic peptide (ANP; 100 nM) significantly suppressed the response to calcium (*P < .05 by ANOVA) without altering its potency.

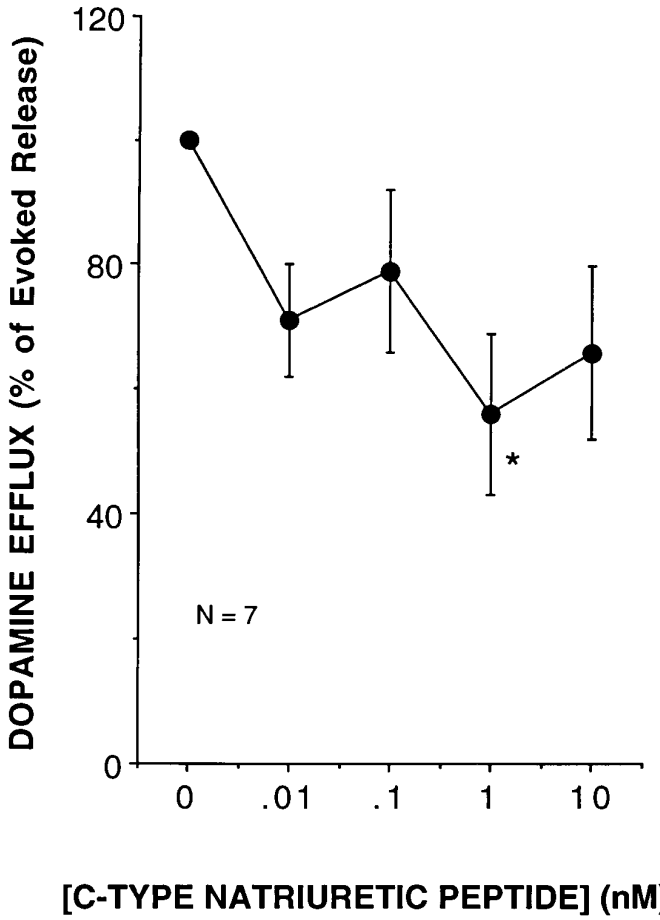

Fig. 8. The effect of C-type natriuretic peptide on dopamine efflux from cells permeabilized to calcium using either A23187 (10 μM) or digitonin (10 μM). All data points are means ± SE. The number of experiments is indicated by the N. The asterisk indicates a statistically significant effect of C-type natriuretic peptide at a concentration of 1 nM (P < .05 by Student's *t* test with Dunnett's correction for multiple comparisons).

(figs. 6–8). These data are consistent with opposing effects of natriuretic peptides to increase intracellular calcium concentrations while simultaneously desensitizing exocytotic processes to calcium. This pattern of activity is relatively unique for natriuretic peptides and somewhat similar to their effects in the adrenal glomerulosa, in which natriuretic peptides both augment L-type calcium channel activity (McCarthy *et al.*, 1990) and suppress aldosterone synthesis in response to calcium (Lotshaw *et al.*, 1991). Most investigators in other tissues find natriuretic peptides to suppress intracellular calcium concentrations and none attribute natriuretic peptide actions to an altered sensitivity to calcium (Chiu *et al.*, 1986; Hassid, 1986; Appel *et al.*, 1987; Nascimento-Gomes *et al.*, 1995). Therefore, this report supports the relatively novel concept that atrial natriuretic peptide modifies the sensitivity of cellular functions to calcium, in addition to its more widely recognized effects to alter intracellular calcium concentrations.

This investigation also found that L-type calcium channels appear to account for nearly 50% of the calcium entry resulting in dopamine efflux in PC12 cells (fig. 1). Furthermore, atrial natriuretic peptide was inactive as an inhibitory neuromodulator in the presence of nifedipine (fig. 3). The persistence of atrial natriuretic peptide neuromodulatory effects in the presence of ω-conotoxin, and their abolition in the presence of nifedipine, suggests that atrial natriuretic peptide reduced catecholamine release by suppressing either calcium entry through L-type calcium channels or a process affected by calcium influx through L-type calcium channels. The natriuretic peptides are characteristically negative modulators of several biological functions. A classical pathway invoked to explain this negative modulatory effect involves a reduction of cytosolic calcium levels. The vasodilatory effects of natriuretic peptides are partly mediated by decreased intracellular calcium concentrations in vascular smooth muscle cells (Hassid, 1986; Cornwell and Lincoln, 1988). Natriuretic peptides also reduce intracellular calcium concentrations in renal tissues (Appel *et al.*, 1987; Nascimento-Gomes *et al.*, 1995). An unexpected finding of this study is that atrial natriuretic peptide increased intracellular calcium concentrations in depolarized cells (fig. 4) although reducing dopamine release (fig. 2). Atrial natriuretic peptide also accelerated the quenching of fura-2 fluorescence in the presence of extracellular manganese (fig. 5), suggesting that it elevated intracellular calcium concentrations by augmenting calcium influx through channels in the plasma membrane. Atrial natriuretic peptide inhibits the low threshold T-type calcium channel but enhances calcium current through the high threshold L-type channel in the bovine adrenal glomerulosa (McCarthy *et al.*, 1990). Atrial natriuretic peptide not only reduces aldosterone release when the glomerulosa cells are weakly depolarized, but also at strongly depolarized potentials when it enhances L-type channel activity (Barrett *et al.*, 1991). A similar situation appears to exist in PC12 cells, wherein atrial natriuretic peptide reduces catecholamine release while simultaneously increasing intracellular calcium concentrations by enhancing extracellular calcium entry.

The mechanism accounting for the increased conductivity of calcium channels in the presence of atrial natriuretic peptide could involve any of the calcium channels. Previous reports observed natriuretic peptides to increase calcium conductivity by L-type channels in bovine adrenal glomerulosa (McCarthy *et al.*, 1990; Barrett *et al.*, 1991) and porcine renal cells (Dai and Quamme, 1993). This mechanism could also be functioning in PC12 cells. Another report indicated that atrial natriuretic peptide increased calcium conductance by sodium channels in rodent cardiac tissue (Sorbera and Morad, 1990). This represents one of many possibilities capable of accounting for the large increase in calcium conduction caused by atrial natriuretic peptide in our study.

The reduction of A23187- and staphyloccocal α toxin-induced dopamine release by atrial natriuretic peptide suggests that atrial natriuretic peptide affects a calcium-dependent process to suppress exocytosis (fig. 6). This result is in agreement with a previous study in which aldosterone secretion induced by A23187 was concentration-dependently inhibited by atrial natriuretic peptide in rat adrenal glomerulosa cells (Lotshaw *et al.*, 1991). In contrast to intact cells, where atrial natriuretic peptide maximally reduces dopamine release by 20 to 40% (fig. 2), a 40 to 60% reduction in dopamine release was observed in permeabilized cells (fig. 6). The enhancement of a calcium conductance by atrial natriuretic peptide in intact cells may oppose the inhibitory neuromodulatory action of atrial natriuretic peptide; however, the stabilized calcium concentrations present in permeabilized cells may allow the inhibitory neuromodulatory effect of atrial natriuretic peptide to proceed unopposed. Such a scenario involving both potentiative and inhibitory effects of atrial natriuretic peptide on neurotransmission could explain the disparate magnitude of the inhibitory neuromodulatory effect of atrial natriuretic peptide in intact and permeabilized cells.

Atrial natriuretic peptide interacts with both the natriuretic peptide A and C receptors within PC12 cells (Drewett *et al.*, 1988; Drewett *et al.*, 1989b). Thus, the data obtained with atrial natriuretic peptide failed to identify the receptor accounting for its activity. Most natriuretic peptide responses are thought to be mediated by activation of guanylyl cyclases leading to elevations in cyclic guanosine monophosphate concentrations (Anand-Srivastava and Trachte, 1993). C-type natriuretic peptide interacts with both the natriuretic peptide B and C receptor (Koller *et al.*, 1991) but the PC12 cell line is devoid of the natriuretic peptide B receptor (Suga *et al.*, 1992). Therefore, C-type natriuretic peptide represents a selective ligand for the natriuretic peptide C receptor in this cell line (Trachte *et al.*, 1995). C-type natriuretic peptide exhibited the same neuromodulatory effect as atrial natriuretic peptide in permeablized cells (fig. 8). These data are consistent with natriuretic peptide C receptors mediating natriuretic peptide effects to attenuate catecholamine release from adrenergic tissue by attenuating catecholamine efflux in response to calcium.

In stark contrast to our findings indicating that natriuretic peptides suppress catecholamine efflux by activating the natriuretic peptide C receptor, Rodriguez-Pascual *et al.* (1996) found that natriuretic peptides suppress catecholamine efflux from bovine chromaffin cells by activating guanylyl cyclase and suppressing calcium concentrations. The rationale for the different findings could involve either the different cell types used or the different experimental conditions. For instance our results were obtained after five minute exposures to natriuretic peptides, whereas Rodriguez-Pascual *et al.* (1996) used 45-min experimental periods. Other investigators also have observed stimulatory (Tsutsui *et al.*, 1994) or

inhibitory effects (Soares-da-Silva and Fernandes, 1992) of natriuretic peptides on catecholamine synthesis in bovine adrenal cells and rat kidney, respectively. Thus, the ultimate effects of natriuretic peptides on adrenergic tissue may be quite complex and involve multiple receptors.

In conclusion, our results demonstrate that atrial natriuretic peptide reduces catecholamine release although simultaneously increasing intracellular free calcium concentrations, probably by facilitating calcium entry through the plasma membrane. Atrial natriuretic peptide presumably affects an intracellular calcium-dependent process to suppress dopamine efflux, probably involving activation of the natriuretic peptide receptor C because C-type natriuretic peptide mimicked the action. The novel findings of this study include the potentiative effects of atrial natriuretic peptide on calcium conductance and intracellular calcium concentrations in adrenergic tissue, as well as the inhibition of calcium-induced dopamine exocytosis. The latter observation represents a relatively unique finding, in that atrial natriuretic peptide previously has been reported to alter the sensitivity of a biological process to calcium solely in the adrenal glomerulosa (Lotshaw *et al.*, 1991).

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