Effect of Catecholamines on Na/H Exchange in Vascular Smooth Muscle Cells

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Abstract. Catecholamines were found to activate Na/H exchange in a concentration-dependent manner in primary cultures of vascular smooth muscle cells (VSMC). The potency order was found to be epinephrine > norepinephrine > isoproterenol. The major pathway for catecholamine effects appeared to be via interaction with an alpha$_1$ adrenergic receptor. In addition, it was found that alpha$_1$ receptor-mediated Na/H exchange in VSMC was increased by angiotensin II and inhibited by 12-O-tetradecanoyl phorbol-13-acetate (TPA).

Adrenergic receptors have been shown to be coupled to both adenylate cyclase and to inositol phosphate release (Leeb-Lundberg, L. M. F., S. Cotecchia, J. W. Lomasney, J. F. DeBernadis, R. J. Lefkowitz, and M. G. Caron, 1985, *Proc. Natl. Acad. Sci. USA*, 82:5651–5655.). It was found that catecholamines increased AMP levels in the potency order isoproterenol > norepinephrine > epinephrine and the receptor involved was a beta adrenergic receptor. Since these findings did not parallel the results obtained for catecholamine stimulation of Na/H exchange, an increase in AMP levels was probably not the mechanism by which major pathway for catecholamine-stimulated Na/H exchange in VSMC (via the alpha$_1$ receptor) was activated. When the effects of catecholamines were measured on inositol phosphate release, the potency order for catecholamine stimulation was epinephrine > norepinephrine > isoproterenol, and the receptor involved was an alpha$_1$ adrenergic receptor. In addition, angiotensin II increased and TPA inhibited catecholamine-stimulated inositol phosphate release. Since these findings paralleled the results obtained for catecholamine stimulation of Na/H exchange, inositol phosphate release may be the mechanism by which the major pathway for catecholamine-stimulated Na/H exchange in VSMC (via the alpha$_1$ receptor) was activated.

VASCULAR smooth muscle cell (VSMC)[1] proliferation is thought to be important in physiological processes such as wound healing and in pathological processes such as atheromatous plaque formation (18). Concerning the latter, Ross and colleagues proposed that when the vessel endothelial lining is damaged, circulating blood platelets adhere to the exposed collagen. Upon adherence to collagen, platelets become activated and secrete their granular constituents. Among these granular constituents is the peptide mitogen known as platelet-derived growth factor. Although many laboratories have described the mitogenic potency of platelet-derived growth factor (19), its mechanism of action has not been identified.

Since Na/H exchange has been shown to be stimulated by a wide variety of mitogenic agents in many different cell types (8, 9, 15, 16), we hypothesized that platelet-derived growth factor might stimulate Na/H exchange in VSMC. In this regard, it was found that platelet-derived growth factor activated amiloride-sensitive Na influx in VSMC (14). Another agent shown to be mitogenic for VSMC is angiotensin II (6). In addition, Smith et al. have demonstrated that angiotensin II could activate Na/H exchange in VSMC (20). Recently, my laboratory has shown that DNA synthesis and Na/H exchange in VSMC could be stimulated by biologically active phorbol esters such as 12-O-tetradecanoyl phorbol-13-acetate (TPA) (13). These studies demonstrate that three different VSMC mitogens can also stimulate Na/H exchange in VSMC. Since Na/H exchange has been proposed to be important in control of cell proliferation (8, 9, 15, 16), it is important to understand how this transport system is regulated in VSMC.

To study regulation of Na/H exchange in VSMC, we focused on the effects of catecholamines. Catecholamines were chosen because they too have been shown to stimulate DNA synthesis in VSMC (2) and have been postulated to be linked to atherosclerosis (2). Furthermore, catecholamines (such as epinephrine [Epi], norepinephrine [NE], or isoproterenol [Iso]) are known to exert their biological activities via interaction with two types of membrane receptors (designated alpha and beta). These receptor types have been identified by

1. *Abbreviations used in this paper*: ^{14}C-DMO, [^{14}C]5,5-dimethyl-2-4-oxazolidine-dione; Epi, epinephrine; FBS, fetal bovine serum; Iso, isoproterenol; MEM, minimal essential medium; NE, norepinephrine; TPA, 12-O-tetradecanoyl phorbol-13-acetate; VSMC, vascular smooth muscle cells.

evaluating the potency order of known agonists. Thus, the rank potency order for agonist binding to the alpha receptor is Epi ≥ NE > Iso; and the rank potency order for agonist binding to the beta receptor is Iso > Epi ≥ NE (7). The two basic receptor types have been further subdivided using specific antagonists. In this regard, alpha receptors have been categorized as $alpha_1$, which predominate in smooth muscle and gland cells, and $alpha_2$, which are proposed to exist on nerve terminals. Similarly, beta receptors have been classified as $beta_1$, which are found in cardiac tissue and $beta_2$, which are present in smooth muscle and gland tissue.

Finally, adrenergic receptors have been shown to be linked to two second messenger systems: adenylate cyclase (26) and inositol phosphate release (10). Thus, due to their physiological relevance, the wide variety of available receptor probes, and their linkage to second messengers, we chose to study catecholamine effects on Na/H exchange as a means of understanding the regulation of the system. Two questions were addressed: (*a*) what are the characteristics of catecholamine-stimulated Na/H exchange in VSMC? and (*b*) by what mechanism do catecholamines activate Na/H exchange in VSMC?

Materials and Methods

Materials

Benzamil was provided by Dr. E. Cragoe of Merck, Sharp & Dohme Div., West Point, PA. Phentolamine was obtained from Ciba-Geigy Corp. (Ardsley, NY). Prazosin was the gift of Pfizer Chemicals Div., Pfizer, Inc. (New York, NY). B-HT 920 was from Boehringer Ingleheim, Elmsford, NY. Rauwolscine was purchased from Atomergic Chemetals Corp., Plainview, NY. Angiotensin II, Epi, Iso, NE, ouabain, phenylephrine, propranalol, and TPA were purchased from Sigma Chemical Co., St. Louis, MO. Dulbecco's modified Eagle's medium (DME), minimal essential medium (MEM), and fetal bovine serum (FBS) were purchased from K.C. Biologicals Inc., Lenexa, KS. [^{3}H]Myoinositol and [^{14}C]5,5-dimethyl-2-4-oxazolidine-dione (^{14}C-DMO) were purchased from Amersham Corp., Arlington Heights, IL.

Cell Culture

VSMC were obtained from rat thoracic aorta by the explant method described by Ross (17). Female Sprague Dawley rats weighing 200–300 g were decapitated and the thoracic aorta removed. All subsequent procedures were performed under sterile conditions. After removal of fat and connective tissue, the aorta was opened, pinned in a wax-coated petri dish, and immersed in phenol red-free Hanks' salts. The intima/media layer was peeled away from the adventitia with forceps, and the pieces of intima media were rinsed several times with Hanks' salts. They were then minced into small pieces (~1 mm square) and placed in 35-ml Falcon "Primaria" flasks (Falcon Labwares, Oxnard, CA). The explants were allowed to partially dry onto the surface of the flask and were then fed with 5 ml DME + 20% FBS, 50 U/ml penicillin and streptomycin, and 500 μg/ml gentamycin sulfate. The flasks were loosely stoppered and placed in a 37°C incubator.

Smooth muscle cells grew out of some of the explants within 7–21 d. When the colonies became confluent the cells were detached by treatment with 0.01% trypsin and resuspended into a T-75 Falcon flask containing 10 ml DME + 10% FBS + antibiotics (penicillin, streptomycin, and gentamycin). The medium was replaced after 24 h, and the cells were passaged when they reached confluence. Smooth muscle cell morphology was verified by light and electron microscopy; cells were used between passages 5–15.

Net Na Influx

Na influx measurements were carried out as described (14). Cells were removed from stock flasks by trypsinization and subcultured onto 60-mm dishes. Cells were used 3–5 d after subculture while they were in the logarithmic phase of growth. Before flux measurements, cells were serum deprived for 4 h at 37°C in an air atmosphere in amino acid–free Hepes-buffered MEM containing 0.1% FBS to reduce basal Na influx to that level observed in quiescent cells. After serum deprivation and with the respective treatments, Na influx was assayed in amino acid–free Hepes-buffered MEM in the presence of 2 mM ouabain, which is added to inhibit the active efflux of Na from the cells by the Na-K ATPase. Amino acid–free medium was used to prevent Na transport via Na/amino acid cotransport systems (i.e., "A system"). At a concentration of 2 mM, ouabain inhibits Na-K ATPase activity immediately (24). Dishes were incubated at 37°C in a gyrotary bath at 100 rpm. Assay times ranged from 1–5 min. Na uptake was terminated by aspiration of the assay medium and washing of the dishes with ice-cold isotonic $MgCl_2$. Cells were extracted with 0.2% SDS for fluorometric protein determination (25) (described below) or with 5% TCA for Na determination. Na concentration was measured by atomic absorption spectrophotometry. Initial net Na influx was taken as the linear phase of a plot of Na content (μmol/g protein) vs. time.

Intracellular pH Measurements

Intracellular pH was determined by measuring weak and distribution with ^{14}C-DMO. Cells were subcultured onto 60-mm dishes as described for Na transport measurements. Cells were serum-deprived for 4 h in amino acid–free Hepes-buffered MEM at 37°C in an air atmosphere. For ^{14}C-DMO distribution the isotope was allowed to equilibrate for 5 min in amino acid–free Hepes-buffered MEM containing 50 μM "cold" DMO. The assay medium was then aspirated and the dishes washed in ice-cold isotonic $MgCl_2$. The medium was then sampled for specific activity determination and the cells extracted with 0.2% SDS. An aliquot of the SDS extract was counted to determine ^{14}C-DMO distribution. Intracellular pH was calculated from the following equation:

$$pH_i = pK_i + \log \frac{(TA)_i}{(TA)_o} (10^{pHo-pKo} + 1) - 1,$$

where pK for DMO is 6.1 (4).

cAMP Assay

For cAMP measurements, cells were subcultured onto six-well cluster plates (35 mm) at a density of 1×10^5 cells/well in DME + 10% FBS. 24 h after subculture each well was washed with Hepes-buffered MEM and the cells treated with appropriate agents. At the end of the specified incubation period the medium was rapidly removed and the cAMP was extracted with 200 μl of 0.1 N HCl. After 20 min the HCl solution was transferred to tubes and appropriate dilutions of the neutralized HCl extract were acetylated according to the method of Harper and Brooker (7), and cAMP was determined in triplicate by radioimmunoassay as described by Steiner et al. (22). Protein content was determined as described below.

Inositol Phosphate Release Measurements

Inositol phosphate release was measured according to a modification of the procedure of Berridge et al. (1). VSMC were subcultured onto 10-cm plates and allowed to recover from subculture for 4 d. Cells were then labeled with 1 μCi/ml [2-^{3}H]myoinositol for 48 h in serum-free vitamin-free medium.

To measure inositol phosphate release, [^{3}H]inositol-labeled cells were washed twice with serum-free medium and then preincubated in serum-free medium in the presence of 10 mM LiCl, which inhibits inositol-1-phosphatase. Inositol phosphate release was then initiated by the addition of serum, catecholamines, angiotensin II, or TPA. The reaction was terminated after 10 min by the addition of ice-cold 10% TCA. The TCA was removed from the extract by washing it five times with an equal volume of diethyl ether. The lower aqueous phase was neutralized by addition of sodium tetraborate (to 5 mM) and then transferred to a Dowex 1 formate column and washed twice with water. The labeled inositol phosphates (inositol, glycerol phosphoinositol, inositol 1-phosphate, inositol 1,4-bisphosphate, and inositol 1,4,5-trisphosphate, respectively) were sequentially eluted with: (*a*) water; (*b*) 5 mM disodium tetraborate/60 mM sodium formate; (*c*) 100 mM formic acid/200 mM ammonium formate; (*d*) 100 mM formic acid/500 mM ammonium formate; and (*e*) 100 mM formic acid/1 M ammonium formate. Aliquots were drawn from each elution tube for the quantitation of ^{3}H content by liquid scintillation counting. Identical dishes were used for fluorometric protein determination as described below.

Fluorometric Protein Assay

Cellular protein was determined using a fluorometric assay developed by Villereal and Cook (25). Cells were extracted with 0.2% SDS, and the fluorescence of the SDS extract was assessed at an excitation wavelength of

280 nm and an emission wavelength of 340 nm. Protein concentration was calculated from a standard curve generated using bovine serum albumin.

Calculation of Data

The concentration of an agent using half-maximal stimulation ($K_{½}$) was calculated from a double reciprocal plot. Variation in results is expressed as standard error of the mean. Tests for significance of differences were made by a two-tailed Students *t*-test (unpaired means solution).

Results

Effect of Catecholamines on Na/H Exchange in VSMC

When the concentration dependence of Epi stimulation of Na/H exchange was measured, it was found that Epi stimulated benzamil-sensitive Na influx in serum-deprived cells in a concentration-dependent manner. The $K_{½}$ was found to be 1 μM (Fig. 1). In addition to Epi, the effects of two other catecholamines were tested, i.e., NE and Iso. NE and Iso were also found to stimulate benzamil-sensitive Na influx in a concentration-dependent manner in serum-deprived VSMC. The $K_{½}$ for NE was found to be 2 μM (Fig. 1), and the $K_{½}$ for Iso was found to be 5 μM (Fig. 1).

The Na/H exchange inhibitor benzamil (100 μM, a maximal concentration) was found to inhibit all of the net Na influx in response to a maximal concentration of Epi (10 μM), NE (10 μM), or Iso (10 μM). As previously reported by Owen (14) using VSMC and by Villereal (24) using human fibroblasts, benzamil had no effect on basal net Na influx. In addition to benzamil, the Na/H exchange inhibitor ethylisopropylamiloride was also found to inhibit all of the catecholamine-stimulated Na influx, without affecting basal Na influx (Owen, N., and E. Cragoe, unpublished observations).

Finally, the three catecholamines were observed to cause a Na-dependent shift in intracellular pH. Intracellular pH was assessed by measuring distribution of the weak acid ^{14}C-DMO. It was found that the basal pH of 7.1 was increased to 7.4 in the presence of 10 μM Epi. When Na was removed from the extracellular milieu (and replaced by choline), the pH did not increase upon treatment with 10 μM Epi. The data reported in Fig. 1 demonstrate that catecholamines stimulate Na/H exchange in VSMC in the potency order Epi > NE > Iso.

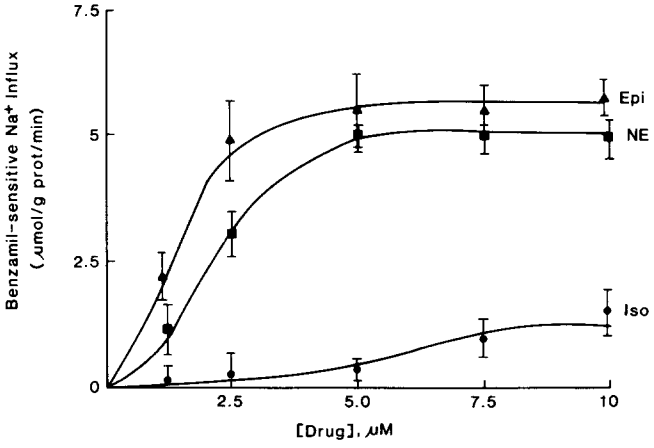

Figure 1. Na/H exchange in VSMC: effect of Epi, NE, or Iso. VSMC were cultured and subcultured as described in Materials and Methods. Cells were serum-deprived for 4 h in amino acid-free Hepes-buffered MEM containing 0.1% FBS. Na influx was assayed in amino acid-free Hepes-buffered MEM that contained varying concentrations of Epi, NE, or Iso in the presence of 1 mM ouabain ± 100 μM benzamil. Data are expressed as benzamil-sensitive Na influx (total Na influx − Na influx in the presence of benzamil). Values represent mean ± SEM from four quadruplicate determinations.

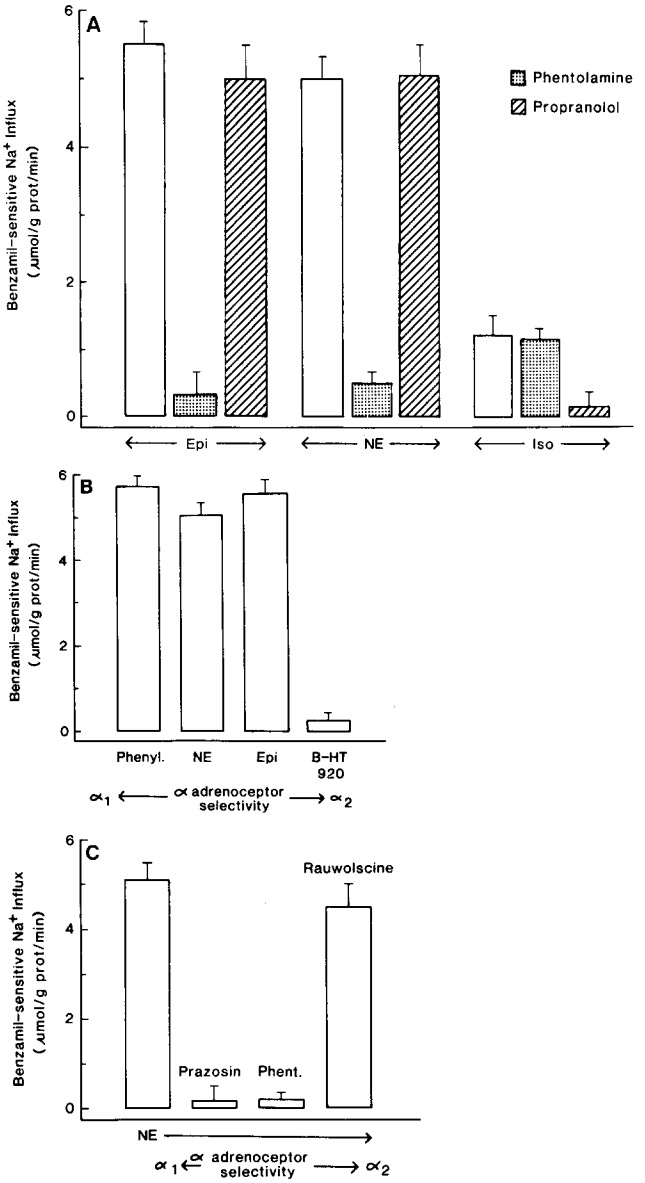

Figure 2. Na/H exchange influx in VSMC: effect of adrenergic agonists and antagonists. VSMC were cultured and subcultured as described in Materials and Methods. Cells were serum-deprived for 4 h in amino acid-free Hepes-buffered MEM containing 0.1% FBS. Na influx was then assayed in amino acid-free Hepes-buffered MEM that contained the indicated additions in the presence of 1 mM ouabain ± 100 μM benzamil. (*A*) Concentrations used were 10 μM Epi, 10 μM NE, 10 μM Iso, 10 μM phentolamine, and 10 μM propranolol. Values represent mean ± SEM from four quadruplicate determinations. (*B*) Concentrations used were 10 μM phenylephrine (*Phenyl.*), 10 μM Epi, 10 μM NE, and 10 μM B-HT 920. Values represent mean ± SEM from five quadruplicate determinations. (*C*) Concentrations used were 10 μM NE, 100 nM prazosin, 10 μM phentolamine (*Phent.*) and 100 nM rauwolscine. Values represent mean ± SEM from five quadruplicate determinations.

Effect of Specific Agonists and Antagonists on Na/H Exchange in VSMC

The data reported in Fig. 1 suggested that the potency order for these three catecholamines was Epi > Ne > Iso. This potency order mimicks that expected for an alpha-type receptor. To support the contention that the catecholamines were interacting with an alpha receptor to stimulate Na/H exchange, the effects of two adrenergic antagonists were measured. As shown in Fig. 2 *A*, both Epi- and NE-stimulated Na/H exchange (each at 10 μM) were inhibited by the alpha receptor antagonist, phentolamine (10 μM), but not by the beta antagonist, propranalol (10 μM). This confirmed that the catecholamines Epi and NE were acting through an alpha receptor to stimulate Na/H exchange. The opposite was observed for Iso-stimulated Na/H exchange. Thus, Iso-stimulated (10 μM) Na/H exchange was not affected by the alpha antagonist, phentolamine (10 μM), but was completely blocked by the beta antagonist, propranolol (10 μM). These results confirmed that Iso was acting through a beta receptor to stimulate Na/H exchange. Given the small effect of Iso (only slightly above basal) and the high concentration required to elicit this effect, it was apparent that the major pathway for catecholamine-stimulated Na/H exchange was via the alpha receptor.

Two types of alpha receptors have been identified; these are designated $alpha_1$ and $alpha_2$. To determine which type of alpha receptor was mediating the Epi or NE effects on Na/H exchange in VSMC, we used specific agonists. Thus, we tested the effects of the $alpha_1$ agonist, phenylephrine (10 μM), and the $alpha_2$ agonist, B-HT 920 (10 μM) on Na/H exchange in VSMC. As shown in Fig. 2 *B*, the $alpha_1$ agonist, phenylephrine, was quite effective in stimulating Na/H exchange, while the $alpha_2$ agonist, B-HT 920, was virtually without effect. These results indicated that the Epi- or NE-stimulated Na/H exchange was mediated through an $alpha_1$ receptor.

Further support that the effects of Epi or NE were mediated through an $alpha_1$ receptor was provided through the use of specific $alpha_1$ and $alpha_2$ antagonists. Thus, the $alpha_1$ antagonist, prazosin (100 nM), totally abolished NE-stimulated (10 μM) Na/H exchange, while the $alpha_2$ antagonist, rauwolscine (100 nM), was without effect (Fig. 2 *C*). Similar effects were obtained using Epi. These studies demonstrated that the major pathway for catecholamine-stimulated Na/H exchange in VSMC is mediated by an $alpha_1$ adrenergic receptor.

Effect of Angiotensin II or TPA on Catecholamine-stimulated Na/H Exchange in VSMC

To further characterize the effects of catecholamines on Na/H exchange in VSMC, we sought to determine the effect of Epi or NE on Na/H exchange when added in combination with other known activators of this transport system (i.e., growth factors such as angiotensin II [20] or phorbol esters such as TPA [13]). We focused on $alpha_1$ receptor-mediated effects because this was clearly the major pathway for catecholamine-stimulated Na/H exchange in VSMC. When serum-deprived VSMC were challenged with maximal concentrations of angiotensin II (100 nM) in the presence of either Epi (10 μM) or NE (10 μM), Na/H exchange was markedly increased over the levels seen with the catecholamines alone (Fig. 3 *A*). The next step was to determine the

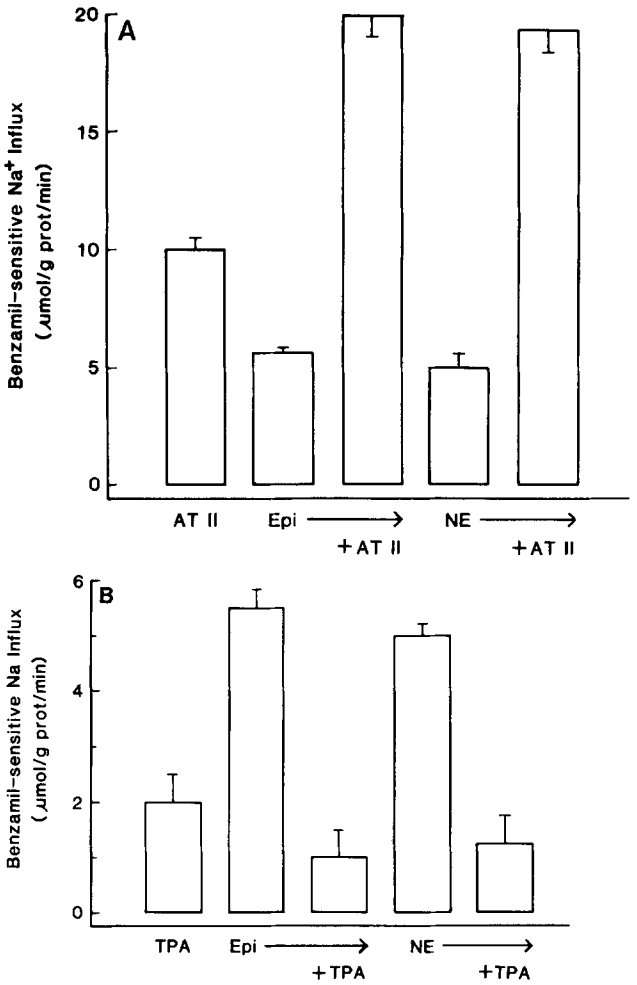

Figure 3. Na/H exchange in VSMC: effect of catecholamines and angiotensin II and TPA. VSMC were cultured and subcultured as described in Materials and Methods. Cells were serum-deprived for 4 h in amino acid-free Hepes-buffered MEM containing 0.1% FBS. (*A*) Na influx was assayed in amino acid-free Hepes-buffered MEM in the presence of 1 mM ouabain ± 100 μM benzamil and the indicated additions. Concentrations used were 10% FBS, 10 μM Epi, 10 μM NE, and 100 nM angiotensin II (*AT II*). Values represent mean ± SEM from six quadruplicate determinations. (*B*) Cells were then preincubated for 15 min in amino acid-free Hepes-buffered MEM that contained 0.01% drug vehicle (control, *FBS, Epi, NE*) or 0.16 μM TPA. Na influx was assayed in identical medium in the presence of 1 mM ouabain ± 100 μM benzamil with the indicated additions. Concentrations used were 10% FBS, 10 μM Epi, 10 μM NE, and 0.16 μM TPA. Values represent mean ± SEM from four quadruplicate determinations.

effect of TPA on $alpha_1$ receptor-mediated Na/H exchange. As shown in Fig. 3 *B*, TPA (0.16 μM) inhibited both Epi-stimulated (10 μM) and NE-stimulated (10 μM) Na/H exchange. These findings demonstrated that angiotensin II increases $alpha_1$ receptor-mediated Na/H exchange in VSMC. They also show that TPA inhibits $alpha_1$ receptor-mediated Na/H exchange in VSMC.

Effect of Catecholamines on cAMP Levels in VSMC

After characterizing catecholamine effects on Na/H exchange in VSMC with respect to potency order, receptor type, and effect of angiotensin II and TPA, the second major

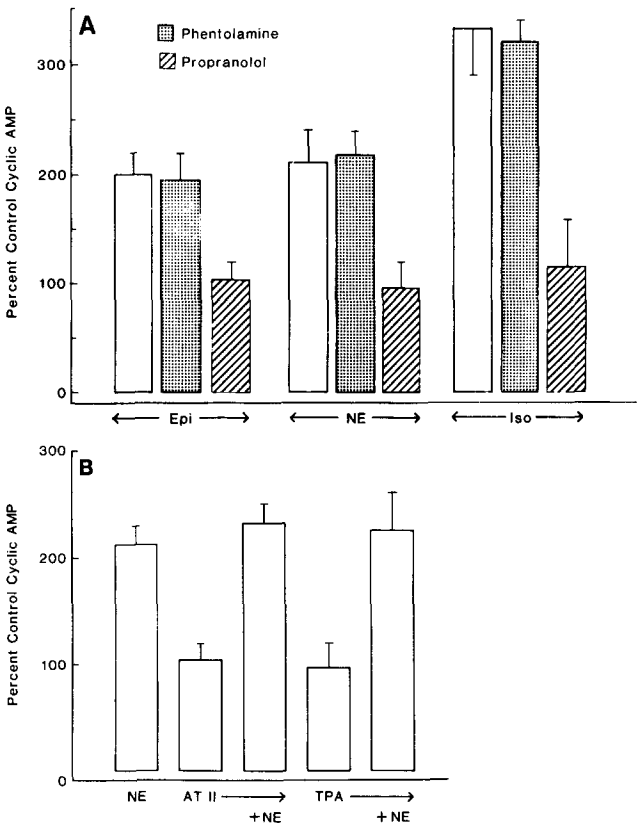

Figure 4. AMP levels in VSMC: effect of catecholamines. VSMC were cultured and subcultured as described in Materials and Methods. Cells were then treated with indicated agents and cAMP measured as described in Materials and Methods. (*A*) The following concentrations were used: 10 μM Epi, 10 μM NE, 10 μM Iso, 10 μM phentolamine, and 10 μM propranalol. Data are expressed as percent control where control is basal cAMP level. Values represent mean ± SEM from four triplicate determinations. (*B*) The following concentrations were used: 10 μM NE, 100 nM angiotensin II (*AT II*), and 0.16 μM TPA. Data are expressed as percent control where control is basal cAMP level. Values represent mean ± SEM from six triplicate determinations.

goal of our studies was to identify the mechanism by which catecholamines activate Na/H exchange. To identify the mechanism by which catecholamines activate Na/H exchange, we measured catecholamine effects on the two second messenger systems known to be linked to adrenergic receptors, that is cAMP (26) and inositol phosphate release (10).

cAMP was measured by radioimmunoassay (22) with the acetylation modification (7). As shown in Fig. 4 *A*, each of the catecholamines (10 μM) stimulated cAMP levels significantly above control, and the potency order was found to be Iso > NE > Epi. This order is clearly different from that observed for stimulation of Na/H exchange (i.e., Epi > NE > Iso).

To assess the receptor type involved, cells were treated with the alpha antagonist, phentolamine (10 μM) and with the beta antagonist, propranolol (10 μM). As shown in Fig. 4 *A*, phentolamine had no effect on catecholamine-stimulated (at 10 μM) cAMP levels, while propranolol completely inhibited the cAMP increase. This suggests that the receptor involved in catecholamine-stimulated cAMP increases is the beta adrenergic receptor. This is clearly different from the alpha receptor involvement proposed for effects on Na/H exchange.

To further rule out a role for cAMP in $alpha_1$ receptor-mediated Na/H exchange, we tested the effect of angiotensin II or TPA on NE-stimulated cAMP levels. As shown in Fig. 4 *B*, neither angiotensin II (100 nM) nor TPA (0.16 μM) had any effect on NE-stimulated (10 μM) cAMP levels. The finding that NE-stimulated Na/H exchange could be significantly increased by angiotensin II or markedly inhibited by TPA, while NE-stimulated cAMP levels were unchanged (in addition to the potency order and the involvement of the beta receptor described above, argued strongly against cAMP playing a role in $alpha_1$ mediated Na/H exchange. However, while cAMP clearly does not play a role in Epi- or NE-stimulated Na/H exchange, it may still be involved in the slight stimulation of Na/H exchange measured in response to the beta agonist Iso.

Effect of Catecholamines on Inositol Phosphate Release in VSMC

Another second messenger system recently shown to be linked to adrenergic receptors is inositol phosphate release (10). Concerning this finding, we questioned whether catecholamine-stimulated Na/H exchange in VSMC was mediated through inositol phosphate release. Inositol phosphate release was measured in the presence of LiCl according to the method of Berridge (1) and is expressed as the sum of inositol phosphate, inositol biphosphate, and inositol triphosphate. As shown in Fig. 5 *A*, both Epi (10 μM) and NE (10 μM) stimulated inositol phosphate release significantly above control levels, whereas Iso did not. This potency order Epi > NE > Iso parallels that observed for stimulation of Na/H exchange.

The receptor involvement was evaluated by measuring the effects of the alpha antagonist, phentolamine, and the beta antagonist, propranolol. As shown in Fig. 5 *A*, the effects of Epi (10 μM) and NE (10 μM) were inhibited by the alpha blocker, phentolamine (10 μM), but not by the beta blocker, propranolol (10 μM). This suggests that the receptor type involved in mediating inositol phosphate release is the alpha receptor. This is identical to the findings for Epi and NE effects on Na/H exchange. These observations led to the suggestion that catecholamine stimulation of inositol phosphate release was mediated through an alpha adrenergic receptor.

To clarify the type of alpha receptor involved in stimulation of inositol phosphate release, we again used the specific $alpha_1$ agonist, phenylephrine, and the specific $alpha_2$ agonist, B-HT 920. As shown in Fig. 5 *B*, phenylephrine (10 μM) significantly stimulated inositol phosphate release above basal, while B-HT 920 (10 μM) did not. This observation suggested that catecholamine-stimulated inositol phosphate release was mediated through an $alpha_1$ receptor.

Further proof that catecholamine-stimulated inositol phosphate release was mediated through an $alpha_1$ receptor was provided through the use of specific antagonists. As shown in Fig. 5 *C*, the specific $alpha_1$ antagonist, prazosin (100 nM), reduced NE-stimulated (10 μM) inositol phosphate release to basal levels, while the specific $alpha_2$ antagonist, rauwolscine (100 nM), had no effect on NE-stimulated (10 μM) inositol phosphate release.

Finally, we looked at the effect of angiotensin II and TPA on catecholamine-stimulated inositol phosphate release. As

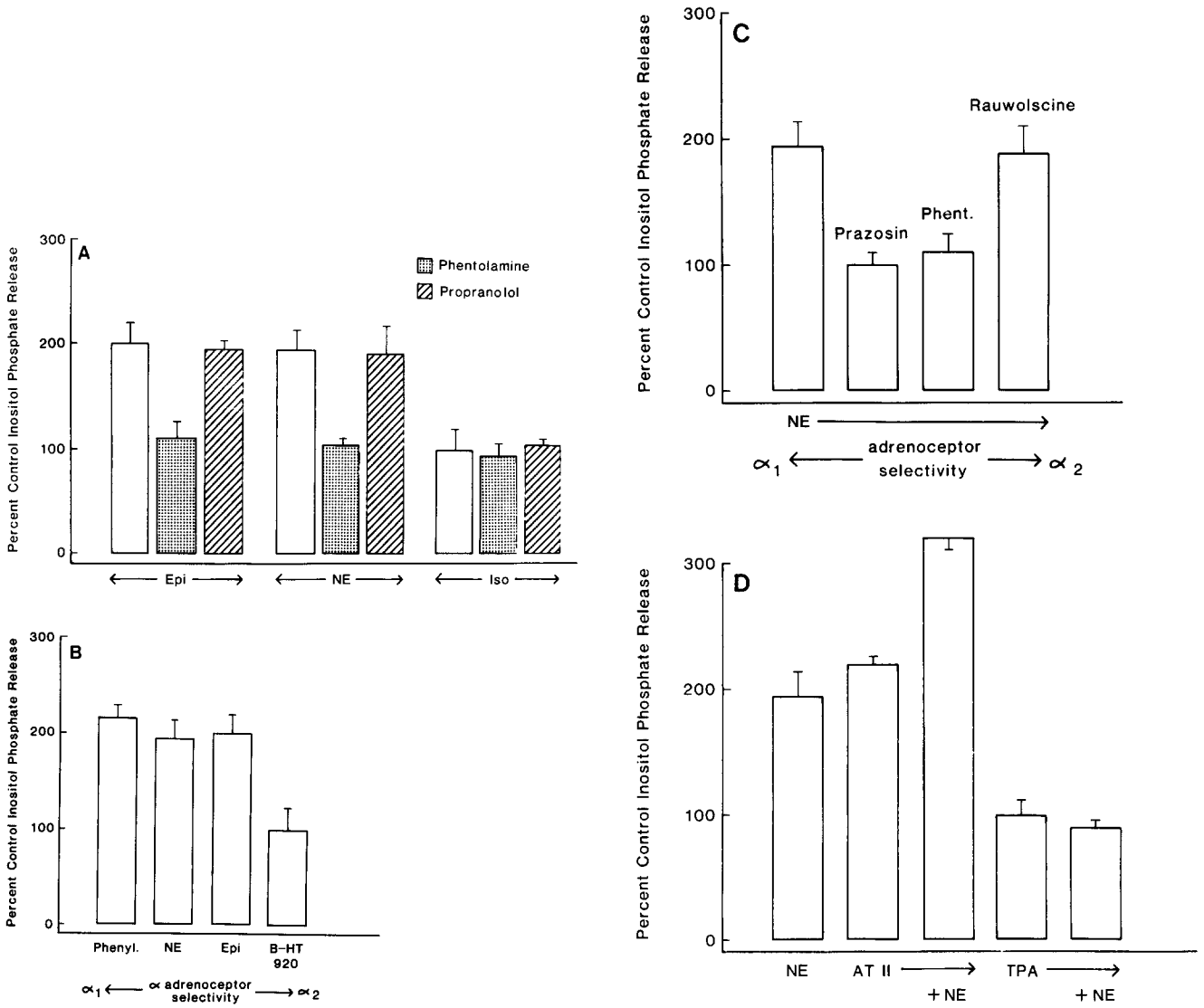

Figure 5. Inositol phosphate release in VSMC: effect of catecholamines. VSMC were cultured and subcultured as described in Materials and Methods. Cells were labeled with [^{3}H]inositol for 48 h in vitamin-free MEM containing 0.1% FBS. Inositol phosphate release was stimulated with the indicated additions in the presence of 10 mM LiCl, and terminated after 10 min by the addition of 10% TCA. (*A*) Concentrations used were 10 μM Epi, 10 μM NE, 10 μM Iso, 10 μM phentolamine, and 10 μM propranolol. Data are expressed as percent of control, where control is basal inositol phosphate release. Values represent mean ± SEM from three determinations. (*B*) Concentrations used were 10 μM phenylephrine (*phenyl.*), 10 μM NE, 10 μM Epi, and 10 μM B-HT 920. Data are expressed as percent of control, where control is basal inositol phosphate release. Values represent mean ± SEM from five determinations. (*C*) Concentrations used were 10 μM NE, 100 nM prazosin, 10 μM phentolamine (*Phent.*), and 100 nM rauwolscine. Data are expressed as percent control where control is basal inositol phosphate release. Values represent mean ± SEM from four determinations. (*D*) Concentrations used were 10 μM NE, 100 nM angiotensin II (*AT II*), and 0.16 μM TPA. Data are expressed as percent control where control is basal inositol phosphate release. Values represent mean ± SEM from six determinations.

shown in Fig. 5 *D*, angiotensin II (100 nM) also increased NE-stimulated (10 μM) inositol phosphate release, and TPA (0.16 μM) inhibited NE-stimulated (10 μM) inositol phosphate release. The finding that the potency order, receptor type, and effects of angiotensin II and TPA were identical for the major pathway of catecholamine stimulation of Na/H exchange and inositol phosphate release led us to conclude that the major pathway of catecholamine-stimulated Na/H exchange in VSMC may be mediated through inositol phosphate release.

Discussion

Previous studies have shown that DNA synthesis in VSMC can be stimulated by peptide growth factors (14, 19, 20), phorbol esters (13), and catecholamines (2). In addition, both growth factors (14, 19) and phorbol esters (13) have been shown to also stimulate Na/H exchange. Concerning these results, it has been proposed that VSMC in common with a wide variety of other cells (8, 9, 15, 16) have activation of Na/H exchange as a step in the stimulus-transfer pathway for DNA synthesis. In the present study, regulation of Na/H exchange was investigated by studying the effect of catechol-

amines on Na/H exchange in VSMC. As described in the results, three catecholamines, Epi, NE, and Iso, were found to stimulate Na/H exchange (benzamil-sensitive net Na influx) in a concentration-dependent manner. The $K_{½}$ values were 1 μM (Epi), 2 μM (NE), and 5 μM (Iso) which were in the appropriate range for binding to a specific membrane receptor. These results are the first evidence to suggest that catecholamines can stimulate Na/H exchange in VSMC.

The major pathway for catecholamine-stimulated Na/H exchange in VSMC is via an $alpha_1$ adrenergic receptor. Evidence to support this contention is provided by the observed potency order for the three agonists on Na/H exchange, i.e., Epi > NE > Iso. Additional support is provided by the finding that catecholamine-stimulated Na/H exchange was significantly blocked by the alpha antagonist phentolamine but not by the beta antagonist propranalol. Finally, only $alpha_1$ agonists were found to stimulate Na/H exchange ($alpha_2$ agonists were ineffective) and only $alpha_1$ antagonists were found to block NE-stimulated Na/H exchange.

Previous studies by Owen (14) and by Whiteley et al. (27) have examined the relationship between growth factors and the phorbol ester, TPA, in activating Na/H exchange. These experiments suggested that while TPA alone stimulated Na/H exchange, when TPA was added together with epidermal growth factor it caused inhibition of epidermal growth factor–stimulated Na/H exchange. The present study probed the effect of both the growth factor, angiotensin II, and the phorbol ester, TPA, on catecholamine-stimulated Na/H exchange. VSMC provide a unique model system in which to evaluate these interactions. In this regard, many cultured cells appear to respond to growth factors and phorbol esters but few cultured cells have adrenergic receptors.

Angiotensin II was found to increase $alpha_1$ receptor mediated Na/H exchange. These results provide the first evidence that angiotensin II and catecholamines can have additive effects on Na/H exchange in VSMC. Furthermore, TPA was found to inhibit $alpha_1$ receptor-mediated Na/H exchange in VSMC. These results provide the first evidence that TPA can inhibit catecholamine-stimulated Na/H exchange in VSMC.

When the effects of catecholamines on cAMP levels were measured, it was found that the potency order, receptor involvement, and effects of angiotensin II and TPA were not in agreement with the results reported for catecholamine-stimulated Na/H exchange. This result was not unexpected since catecholamine-stimulated effects on cAMP are usually mediated through a beta receptor (26). The lack of agreement between catecholamine effects on cAMP and on Na/H exchange were in marked contrast to the effects of catecholamines on inositol phosphate release. In the latter case, the potency order, receptor involvement, and effects of angiotensin II and TPA were identical to the results reported for catecholamine-stimulated Na/H exchange. This is the first report of catecholamine stimulation of inositol phosphate release in VSMC, although others have made a similar observation using vas deferens smooth muscle cells (10). In addition, these data are the first to demonstrate that NE can enhance angiotensin II–stimulated inositol phosphate release in VSMC (although others have measured the effect of angiotensin II [5, 11, 21]). Finally our work confirms and extends reports which have suggested that TPA may modulate the activity of other agents (e.g., NE [8], angiotensin II [5], or carbachol [12, 23]) by inhibiting their ability to stimulate inositol phosphate release.

On the basis of the studies measuring cAMP and inositol phosphate release, we propose that NE or Epi interact with an $alpha_1$ receptor to cause inositol phosphate release which somehow, possibly via inositol triphosphate-mediated Ca mobilization (11, 21), activates Na/H exchange. Both Smith et al. (21) and Nabika et al. (11) have provided evidence to suggest that inositol triphosphate can mobilize intracellular Ca in VSMC. It is important to note that Iso was also found to slightly stimulate Na/H exchange. Based on the present data, it seems that Iso interacts with a beta receptor to cause an increase in cAMP levels which somehow stimulates Na/H exchange. Evidence that cAMP may be able to stimulate Na/H exchange in VSMC is provided by our previously reported finding that the permeable cAMP analogue 8-Br-cAMP can mimick the effects of Iso in stimulating Na/H exchange (3). However, it should be noted that given the magnitude of the effects, it appears that the putative cAMP-mediated pathway is a minor pathway and may not be physiologically important.

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