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Contraction of smooth muscle by activation of endothelin receptors on autonomic neurons

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Endothelin receptors, predominantly of the ET_B type, were localized to cell bodies, processes, and varicosities of cholinergic and adrenergic intramural autonomic neurons that were present in primary cultures of guinea pig tracheal smooth muscle. Stimulation of the neuronal ET_B receptor produced a tetrodotoxin-sensitive increase in the intracellular calcium concentration in neurons which was followed by contraction of the neighboring smooth muscle cells. These observations suggest that endothelins can induce smooth muscle contraction by means of a neuronally mediated mechanism, in addition to their direct actions on the smooth muscle.

Endothelin; Receptor; Autonomic neuron; Smooth muscle; Contraction

1. INTRODUCTION

Endothelin (ET), discovered as a potent vasoactive peptide produced by cultural endothelial cells [1], is a family of peptides, ET-1, ET-2, and ET-3 [2,3]. Sarafotoxins, isolated from the venom of the snake *Atractaspis engaddensis*, possess structural features and pharmacological activities similar to those of ETs [4]. ETs and sarafotoxins exert pharmacological actions such as transient vasodilation and prolonged contraction of bronchial, intestinal, uterine, and other smooth muscle [5,6]. These pharmacological actions are mediated by two types of ET receptor: ET_A receptor (selective for ET-1 and ET-2) [7] and ET_B receptor (nonselective) [8]. In the airway, ETs are implicated in the pathogenesis of asthma because inhalation of ET-1 produced long-lasting bronchoconstriction and because ET-1 production by the airway epithelium was higher in patients with asthma [5,6,9]. The ET-induced bronchoconstriction is often associated with hypersensitivity against other bronchoconstrictors such as acetylcholine and histamine [10,11]. We report here a second pathway of ET-induced smooth muscle contraction.

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Abbreviations: ET, endothelin; DMEM, Dulbecco's modified Eagle's medium; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{125}I]ET-1, [^{125}I]ET-3 and [^{125}I]IRL 1620(~ 74 TBq/mmol) were purchased from DuPont-New England Nuclear (Boston, MA). Authentic ET-1 and ET-3 (Peptide Institute Inc., Osaka, Japan), BQ-123 (Peninsula, CA), and fura-2-acetoxymethylester (Dojin, Kumamoto, Japan) were obtained commercially. IRL 1620 was synthesized as reported previously [12]. All other chemicals used were of reagent grade.

2.2. *Cell preparation*

Guinea pig tracheal smooth muscle was incubated at 37°C for 1 h in oxygenated (95% O_2 and 5% CO_2) Hank's solution containing 200 U/ml collagenase (Worthington type I), 0.5 U/ml elastase (Biozyme type E2) and 100 U/ml DNase (Sigma) with mild shaking (120 strokes/min). The tissue was minced into smaller pieces (~ 1 mm^3) and then incubated for 2 h. The dispersed cells were suspended to a density of 7×10^4 cells/ml in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 50 U/ml penicillin-G, 50 μg/ml streptomycin and 0.5 mM L-glutamine on slides in 95% O_2 and 5% CO_2 at 37°C for 5 to 7 days.

2.3. *Autoradiography and immunocytochemistry*

The primary cultures were incubated at 37°C for 1 h with 30 pM [^{125}I]ETs in the absence or presence of unlabeled ligands in 20 mM HEPES (pH 7.4) containing 140 mM NaCl, 4 mM KCl, 1 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose, and 0.1% bovine serum albumin. The slides were then fixed with 2% paraformaldehyde in 0.1 M sodium phosphate (pH 7.4) for 10 min, air-dried, dipped into Konica NR-M2 emulsion, and stored in the dark. After an exposure for 2 weeks, the slides were developed with Konidol X for 6 min at 20°C. Neurons were identified by staining with antibodies against choline acetyltransferase and tyrosine hydroxylase (Chemicon, CA) and with an avidin–biotin–peroxidase complex staining kit (Vector Labs, CA).

2.4. *Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)*

The cultured cells on quartz slide-glass were incubated at 37°C for 1.5 h with 5 μM fura-2-acetoxymethyl ester in a serum-free DMEM in a CO_2 incubator. The fura-2-loaded cells were transferred to a

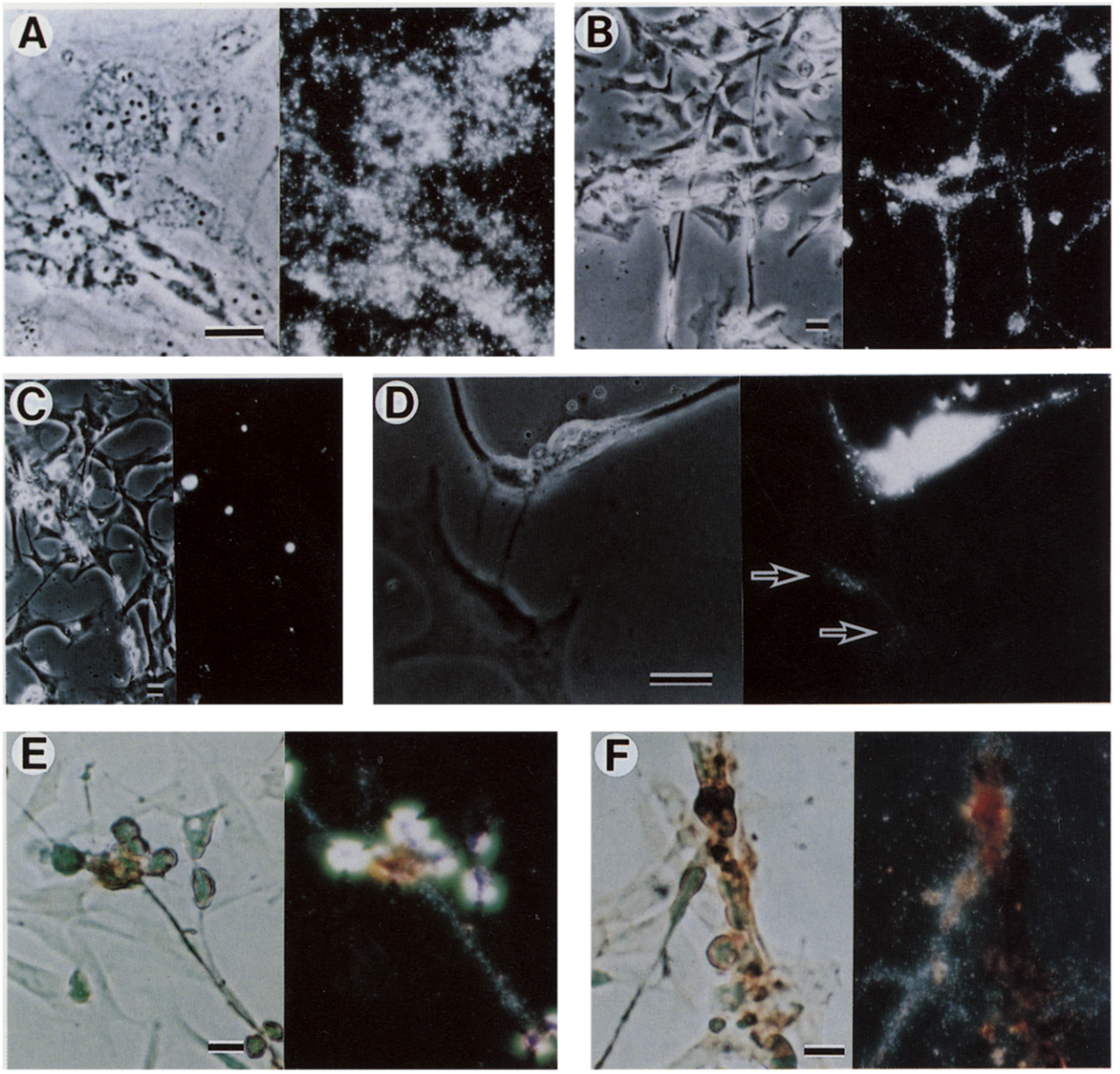


Fig. 1. Autoradiographic detection of ET_A and ET_B receptors in cultured smooth muscle cells accompanied by peripheral neurons. In each pair, the bright-field image is on the left and the dark-field image on the right. (A) [^{125}I]ET-1, (B) [^{125}I]ET-1 in the presence of BQ-123, (C) [^{125}I]ET-1 in the presence of excess unlabeled ET-1, and (D to F) [^{125}I]ET-3. Neurons were labeled with antibodies against choline acetyltransferase (E) and tyrosine hydroxylase (F). Bar = 20 μm.

chamber on the stage of a Mu-1000 Ca^{2+} analyzer (INTER DEC, Osaka, Japan). The $[Ca^{2+}]_i$ change was monitored fluorimetrically with an emission wavelength at 510 nm and excitation wavelengths at 340 and 380 nm, and recorded at 33-ms intervals to video tape for image analyses. Two wavelength ratio images of individual cells were then digitized offline to calculate $[Ca^{2+}]_i$ with a standard curve.

3. RESULTS AND DISCUSSION

In primary cultures of guinea pig tracheal smooth muscle, intramural autonomic neurons coexisted with smooth muscle cells innervated by these neurons. Most neurons grew over smooth muscle cells and reformed a network of neuronal processes that often were seen in the periphery of smooth muscle cell colonies. As assessed immunocytochemically, about 20% of the neurons reacted with anti-choline acetyltransferase antibody and about 60% with anti-tyrosine hydroxylase antibody; the remaining neurons were neither cholinergic nor adrenergic.

Both smooth muscle cells and neurons were labeled with silver grains by autoradiography after incubation with 30 pM [^{125}I]ET-1, a ligand that binds to ET_A and ET_B receptors (Fig. 1A). In the presence of 10 μM BQ-123, an ET_A-antagonist [13], neurons were densely la-

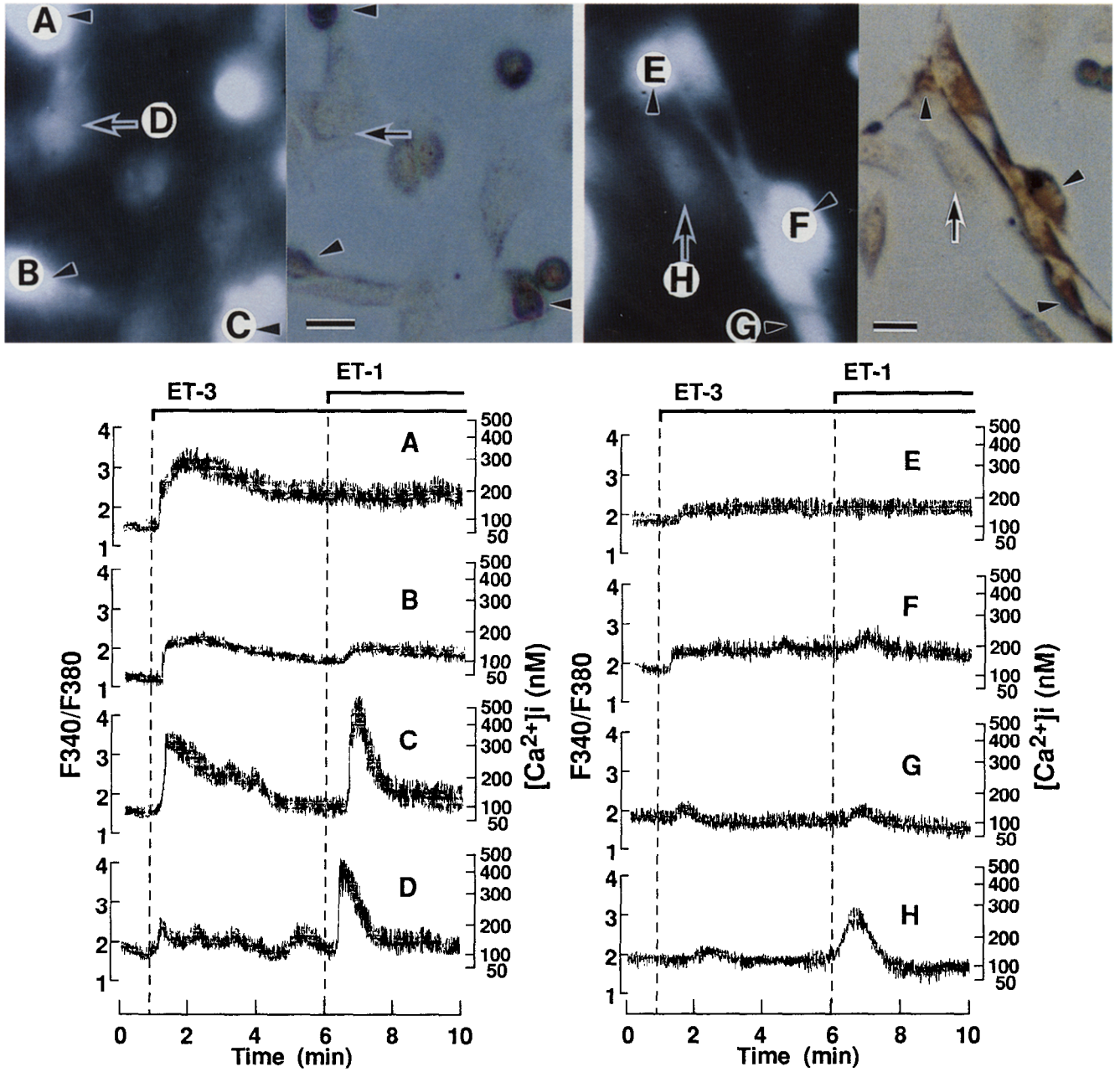
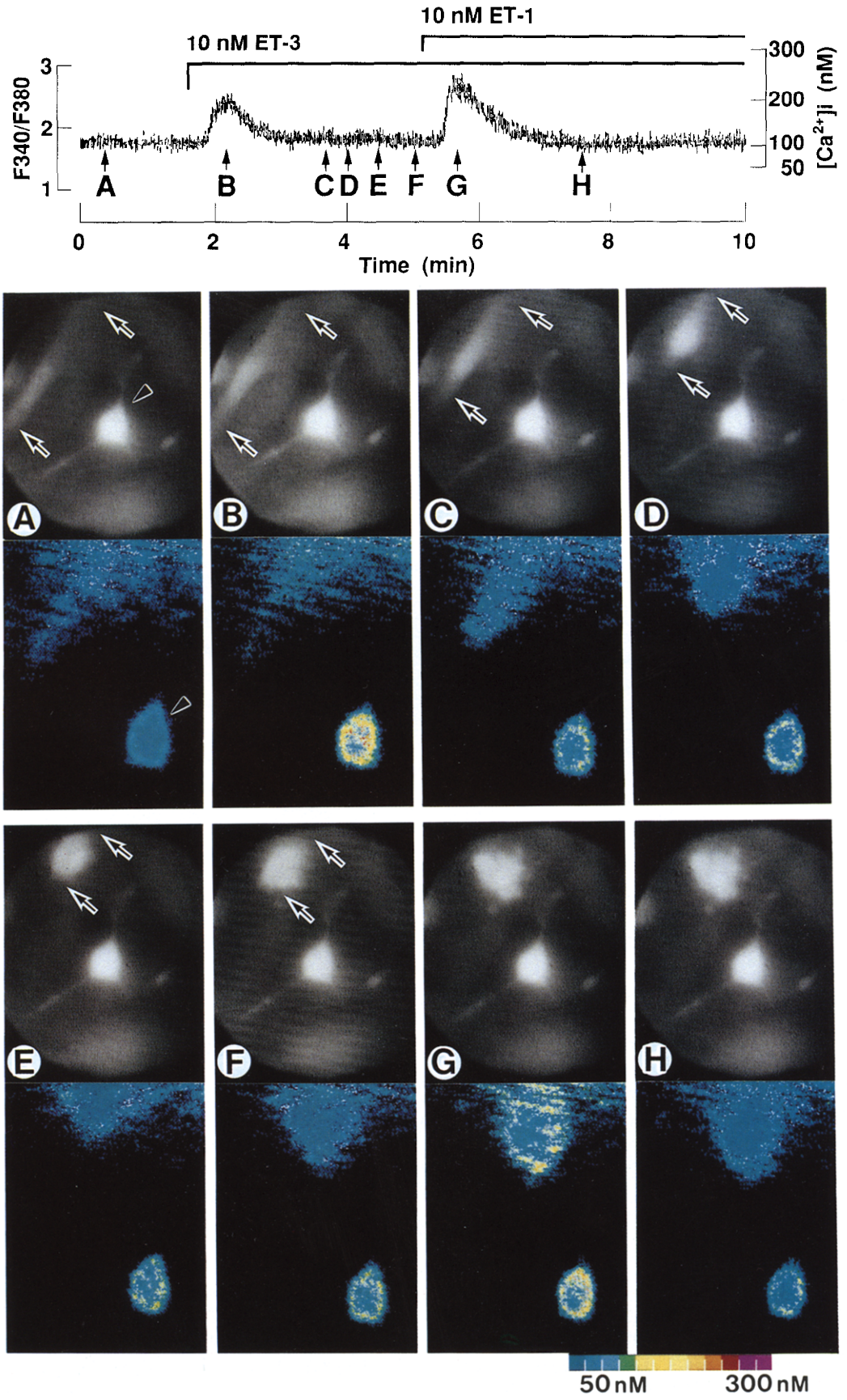


Fig. 2. Time courses of $[Ca^{2+}]_i$ changes in cholinergic (A, B and C) and adrenergic (E, F and G) neurons (arrowheads) and in smooth muscle cells (arrows) (D and H) in response to ET-3 and ET-1. Fluorescence images (left in each pair) and the corresponding bright-field images (right in each pair) stained for choline acetyltransferase and tyrosine hydroxylase are shown in the top. Numbers of cholinergic and adrenergic neurons showing $[Ca^{2+}]_i$ changes similar to those of (A and E) were 7 and 41; (B and F), 8 and 21; and (C and G), 14 and 26; respectively. No changes were seen in 4 cholinergic and 10 adrenergic neurons. Neurons were loaded with more fura-2 than smooth muscle cells producing the brighter images, however, the calculated $[Ca^{2+}]_i$ did not differ significantly between the two cell types. Bar = 20 μm.

beled with [^{125}I]ET-1 whereas the labeling of smooth muscle cells was reduced greatly (Fig. 1B). A similar labeling pattern to that with [^{125}I]ET-1 plus BQ-123, was obtained with 30 pM [^{125}I]ET-3 (Fig. 1D–F) or 30 pM [^{125}I]IRL 1620, a potent and highly selective ET_B-agonist [12,14]. Labeling was eliminated by an excess (200 nM) of unlabeled ET-1 (Fig. 1C). These results indicate that the peripheral neurons predominantly possess ET_B receptors whereas the smooth muscle cells have ET_A receptors.

The ET_B receptor was distributed evenly over neuronal cell bodies and their processes with varicosities that often spread over the muscle cells (Fig. 1D). Both cholinergic (79 of the 152 cells) and adrenergic (288 of


10 nM ET-3
10 nM ET-1
F340/F380
3
2
1
300
200
100
50
[Ca2+]i (nM)
A
B
C
D
E
F
G
H
0
2
4
6
8
10
Time (min)
50 nM
300 nM

the 446 cells) neurons expressed the ET_B receptor (Fig. 1E and F).

We measured changes in $[Ca^{2+}]_i$ of individual cells after stimulation of ET_B receptors with 10 nM ET-3 or 500 nM IRL 1620. About 90% of the 131 neurons examined responded with $[Ca^{2+}]_i$ increases. Cholinergic neurons (29 of the 33 cells) displayed large increases of $[Ca^{2+}]_i$ (> 100 nM from basal concentrations of 30 to 100 nM) within 1 min of stimulation. Adrenergic neurons (88 of the 98 cells), in contrast, showed smaller $[Ca^{2+}]_i$ increases (~ 50 nM from basal). These increases were followed by decline to a sustained concentration still elevated over basal (Fig. 2A,B,E,F), or by a return to the basal concentration (Fig. 2C and G). Stimulation of ET_A receptors with 10 nM ET-1 after decline to the plateau produced no further increase in 7 cholinergic (Fig. 2A) and 41 adrenergic neurons (Fig. 2E) but a secondary $[Ca^{2+}]_i$ increase in 22 cholinergic (Fig. 2B and C) and 47 adrenergic neurons (Fig. 2F and G), suggesting that the latter neurons possess both ET_A and ET_B receptors. On the other hand, a second stimulation with ET-3, instead of ET-1, resulted in no further increase in $[Ca^{2+}]_i$. In the absence of extracellular calcium or after incubation with 300 nM tetrodotoxin for 2 min, the neuronal $[Ca^{2+}]_i$ responses were attenuated to a slight increase (< 30 nM from basal). These results indicate that stimulation of ET receptors leads to activation of neuronal signal transduction [15,16] which would be followed by neuronal responses including transmitter release and elongation or degeneration of neuronal processes.

Smooth muscle cells (280 cells) showed only small $[Ca^{2+}]_i$ increases (< 50 nM from basal concentrations of 80 to 140 nM) by ET-3 or IRL 1620 treatment, but ET-1 produced much larger increases (> 150 nM from resting) (Fig. 2D and H), indicating that these cells predominantly possess ET_A receptors, in agreement with the results of autoradiography. Tetrodotoxin did not alter ET-induced $[Ca^{2+}]_i$ changes in the smooth muscle cells.

While monitoring $[Ca^{2+}]_i$, we also observed the delayed contraction of smooth muscle cells near neurons (5 of the 74 cells) after stimulation of neuronal ET_B receptors (Fig. 3). In the neuron, ET-3 evoked a transient $[Ca^{2+}]_i$ increase, peaking at 200 nM after 45 s (Fig. 3B). The $[Ca^{2+}]_i$ then declined to a sustained plateau of 120 nM, a response typical of cholinergic neurons (Fig. 2). The smooth muscle cell showed no increase of $[Ca^{2+}]_i$ in response to ET-3 (Fig. 3B–F), yet it began to contract 130 s after ET-3 addition (Fig. 3C). Stimulation with ET-1 produced increases in $[Ca^{2+}]_i$ in both the smooth muscle cell and the neuron after 40 s with a peak of 240 nM for both (Fig. 3G) with recovery within 120 s (Fig. 3H). The ET-3-induced contraction was not observed for smooth muscle cells far from the neuron (206 cells), or in the presence of tetrodotoxin (the 30 monitored cells near neurons). These results suggest that the contraction of the smooth muscle cell was not due to nonspecific cell damage and leakage of Ca^{2+} but was mediated by ET-3-induced activation of neurons followed by a release(s) of diffusible transmitter(s) from its varicosities to the effector smooth muscle cell.

Furthermore, in 4 out of 33 recording sessions, we observed delayed changes in the positions of neuronal cell bodies and varicosities after stimulation of ET_B receptors. In these cases, although we could not identify contracting muscle cells within the monitored area, the position changes are considered to be due to the contraction of smooth muscle cells attached to the neuronal elements, which occurred outside but near the monitored area. Because autonomic neurons connect with smooth muscle cells in vivo more efficiently than in vitro, the neuronally mediated contraction is expected to exist in vivo.

ET-induced contraction has been studied in terms of direct actions of ETs on smooth muscle [5,6]. However, our observations suggest an alternative pathway of ET actions. Similar indirect and possibly neuronally mediated actions of ETs have been observed for vasoconstriction in vivo after stimulation of ET_B receptors [17] and ET-induced increases in colonic ion transport [18]. On the other hand, there are a few reports demonstrating binding sites for [^{125}I]ETs by autoradiography in several types of ganglia [19,20]. However, previous researchers did not precisely distinguish between neurons and non-neuronal (glial) cells in the ganglion. ET receptors predominantly of the ET_B subtype are enriched in the central nervous system, in which the receptors are mainly expressed in glial cells, but not in neurons [21]. In this study, we simultaneously used autoradiography for detection of ET receptors, immunohistochemistry for the identification of neurons, and Ca^{2+} monitoring to demonstrate the functional coupling of neuronal ET receptors. Therefore, this is the first report to demonstrate the existence of ET receptors in autonomic cholinergic and adrenergic neurons. Our conclusion is also supported by several recent reports that ETs modulate neurotransmitter release [22–25].

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Fig. 3. Contraction of a smooth muscle cell after ET-3-induced neuronal activation. Upper panel shows the time course of $[Ca^{2+}]_i$ changes in the selected area directly measured with photomultiplier. Arrows (A to H) correspond to time for fluorescence images (lower panel, each upper photo) and computer-processed $[Ca^{2+}]_i$ images (lower panel, each lower photo) of the cultured cells. A neuron (arrowhead in A) and its processes with varicosities lies in the center with a contracting smooth muscle cell on the left upper side (empty arrows in A to F). The pseudocolor scale used for the $[Ca^{2+}]_i$ images is shown at the bottom.

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