RAPID COMMUNICATION

Activin A stimulates catecholamine secretion from rat adrenal chromaffin cells: a new physiological mechanism

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Abstract

Activin A is a member of the transforming growth factor- β family and has known roles in the adrenal cortex, from which activin A is secreted. We aimed to find whether activin A induces secretion of catecholamines from chromaffin cells of the adrenal medulla, which neighbours the adrenal cortex *in vivo*. Using carbon fibre amperometry, we were able to measure catecholamine secretion in real-time from single chromaffin cells dissociated from the rat adrenal medulla. Activin A stimulated catecholamine secretion in a rapid and dose-dependent manner from chromaffin cells. This effect was fully reversible upon washout of activin A. The minimum dose at which activin A had a maximal effect was 2 nM, with an EC₅₀ of 1.1 nM. The degree of secretion induced by activin A

(2 nM) was smaller than that due to membrane depolarization caused by an increase in the external K⁺ concentration from 5 to 70 mM. No response to activin A was seen when Ca²⁺ channels were blocked by Cd²⁺ (200 μ M). We conclude from these findings that activin A is capable of stimulating a robust level of catecholamine secretion from adrenal chromaffin cells in a concentration-dependent manner. This occurs via the opening of voltage-gated Ca²⁺ channels, causing Ca²⁺ entry, thereby triggering exocytosis. These findings illustrate a new physiological role of activin A and a new mechanism in the control of catecholamine secretion from the adrenal medulla.

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Introduction

Activins belong to the transforming growth factor- β (TGF- β) superfamily of cytokines. Members of the TGF- β family are important in a variety of biological functions including cell proliferation and death, homeostasis, differentiation, metabolism, immune responses and endocrine function (Harrison *et al.* 2005) and are required for the normal growth and function of a multitude of different tissues (Bilezikjian *et al.* 2004). Alterations or disruptions in the function of members of the TGF- β family result in several types of cancers, fibrotic disorders of the liver, kidney and other organs as well as various inflammatory disorders (Massague 1998).

Activins have roles in regulating pituitary and reproductive function, controlling, for example, the production of pituitary follicle-stimulating hormone (FSH) (Massague 1998). Activin A is a member of the activin family and is a dimer composed of two activin β A-subunits. Apart from the classical role of activins in reproductive control, activin A has also been shown to effect ion channel function in

different endocrine tissues. Activin A is located in insulin-containing human pancreatic β -cells (Wada *et al.* 1996) and increases insulin secretion in the presence of glucose in human pancreatic islets (Florio *et al.* 2000). Activin A closes K_{ATP} channels and increases Ca²⁺ channel amplitude in HIT-T15 insulinoma cells, resulting in membrane depolarization and Ca²⁺ entry (Mogami *et al.* 1995). Activin A also stimulates FSH secretion by causing membrane depolarization and the opening of Ca²⁺ channels in human FSH-secreting tumor cells (Takano *et al.* 1992, Takano *et al.* 1994). Neuronal survival of rat hippocampal cultures is also promoted by activin A and this effect is blocked by Ca²⁺ channel antagonists (Iwahori *et al.* 1997).

Activin signalling components are expressed in the adrenal cortex and activin A reduces secretion of several steroid hormones from this tissue (Vanttinen *et al.* 2003). mRNA for activin A and its receptor has been identified in human fetal and adult adrenal cortical cells and cultured adrenocortical cell lines, and adrenocorticotrophin induces secretion of activin A from these cell types (Vanttinen *et al.* 2002). The adrenal cortex is located within the adrenal

gland, a tissue that also contains the adrenal medulla. The primary function of the adrenal medulla is catecholamine synthesis and secretion, which is carried out by chromaffin cells. Activin A and its receptors are expressed in human pheochromocytomas, a form of adrenal medullary tumor, and production of activin A in cultured pheochromocytoma cells is induced through the protein kinase A pathway (Liu *et al.* 2000).

Although activin A is known to stimulate hormone secretion from several endocrine tissues, activin signalling components are present in the adrenal gland and activin A is secreted from the adrenal cortex, it remains unknown whether activin A effects catecholamine secretion from neighbouring adrenal medullary chromaffin cells. We present in this report the first evidence that catecholamine secretion from single rat chromaffin cells can be stimulated by activin A. This represents a previously undiscovered physiological pathway involving an important signalling molecule regulating the secretion of a hormone which has widespread homeostatic influences.

Materials and Methods

Adult male Wistar rats were killed by carbon dioxide inhalation as approved by the Monash Medical Centre Animal Care Committee. After they were removed, 4–6 rat adrenal glands had their adrenal capsule and cortex dissected away in cold calcium-free saline (Locke's buffer). Locke's buffer consisting of (mM): NaCl, 154; KCl, 5.6; NaHCO₃, 3.6; glucose, 5.6; HEPES, 5.0; pH 7.4. The remaining adrenal medulla tissue was then incubated with collagenase (Collagenase type A, Roche) in Locke's buffer at a concentration of 3 mg/ml, in a shaking bath at 37 °C for 15, 10, and 5 min. The tissue was triturated between incubations. The collagenase was then diluted further in cold Locke's buffer centrifuged at 2 g for 5 min. After centrifugation, cells were resuspended in Dulbecco's modified Eagle's medium (Thermo Electron, Melbourne, Victoria, Australia) supplemented with 1% penicillin/streptomycin (Invitrogen) and 10% FCS (JRH Biosciences, Lenexa, KS, USA) and the resuspended material was filtered through a nylon mesh. Cells were then centrifuged at 200 g for 5 min, resuspended and plated on 35 mm culture dishes and incubated at 37 °C with 10% CO₂. Cells were maintained in primary culture for 5–7 days prior to experiments to maximise secretory capacity.

Catecholamine release from single chromaffin cells was measured using amperometry (Chow *et al.* 1992). A carbon-fibre electrode (ProCFE, Dagan Corporation, Minneapolis, MN, USA) was carefully placed on an individual chromaffin cell and +800 mV applied to the electrode under voltage clamp conditions. The current due to catecholamine oxidation at the tip of the electrode was recorded using an EPC-9 amplifier and Pulse software (HEKA Electronic, Lambrecht, Germany), sampled at

10 kHz and low-pass filtered at 1 kHz. For quantitative analysis the Pulse files were converted to Axon Binary Files (ABF Utility, version 2.1, Synaptosoft, Decatur, GA, USA) and the secretory spikes analysed (Mini Analysis, version 6.0.1, Synaptosoft, Decatur) for a period of 60 s from the start of exposure to the various treatments. The total charge released over this 60 s period was calculated as the average charge of each secretory event multiplied by the number of events within 60 s. The charge (in picocoulombs, pC) of a single secretory event is simply the area under each spike (charge is the product of current and time), which is automatically calculated by the Mini Analysis software.

The standard saline contained (mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 5 D-glucose, 10 Hepes (pH 7.4). High K⁺-containing solution was the same as control bath solution except that 70 mM K⁺ replaced an equimolar amount of NaCl. All solutions with or without the addition of activin or cadmium (Cd²⁺) were applied to cells using a gravity perfusion system, the outlet of which was placed within 500 µm of the recorded cell. All other reagents were obtained from Sigma-Aldrich. All experiments were carried out at room temperature (22–24 °C).

The equation used for the EC₅₀ calculation and the creation of the sigmoidal dose–response curve was obtained from the graphing and analysis software used (Prism 3.0, GraphPad Software, San Diego, CA, USA) and is as follows:

$$Y = \text{Bottom} + \left(\frac{\text{Top} - \text{Bottom}}{1 + 10^{(\text{LogEC}_{50} - X) * \text{Hill Slope}}} \right)$$

where:

X = the logarithm of concentration

Y = the response

Bottom = Y-axis value at the bottom plateau

Top = Y-axis value at the top plateau

Hill slope was calculated to be 6

All data are displayed as mean ± S.E.M. and data were tested for significant differences using one-way ANOVA for analysis between different groups and Student's paired *t*-test for comparisons within groups. *P* < 0.05 was set as the limit for statistical significance.

Results

A variety of concentrations of activin A were applied acutely to cultured chromaffin cells (Figure 1A). Activin A was found to be without significant effect on secretion, recorded using amperometry, at very low levels (0.1 and 0.5 nM) but secretion reached maximal levels at a concentration of 2 nM. This concentration-dependency followed a sigmoidal relationship, enabling an EC₅₀ value of

Figure 1 Dose–response effect of activin on catecholamine secretion. Chromaffin cells were exposed to various concentrations of activin for 60 s. Total charge released over this period was evaluated as stated in the Materials and Methods section. The lowest concentration with a maximal effect was 2 nM. All data from these figures are from 5–7 cells.

1.1 nM to be calculated (Figure 1B). Given that the minimal concentration of activin that gave maximal response was 2 nM, this concentration was used in all subsequent experiments.

All chromaffin cells were tested for their initial secretory capacity by 60 s exposure to high K^+ (70 mM) solution in order to be sure that any cell tested with activin could secrete in response to a known strong stimulus. This induced an immediate and large level of secretion from single cells (Figure 2A), which was completely reversible upon washout with control bath solution ($n=11$). The application of activin (2 nM) also caused a rapid and substantial response that was reversible upon washout (Figure 2A). Approximately 50% of cells tested were found to respond to activin A. To test whether the stimulation caused by activin A induced Ca^{2+} entry through voltage-gated Ca^{2+} channels, we applied Cd^{2+} (200 μ M) to the same cells and then stimulated the cells with activin A in the continued presence of Cd^{2+} . This resulted in a significant reduction in both spike frequency ($P<0.05$) and total charge released ($P<0.01$) when the effect of activin is compared with the same cells in which Cd^{2+} was present or absent. This loss of secretion in response to activin A in the presence of Cd^{2+} indicates that Ca^{2+} entry through voltage-gated Ca^{2+} channels is required for activin-induced secretion to occur. In some experiments, activin A was applied again after exposure to Cd^{2+} , once Cd^{2+} had been washed off the cell, and secretion was found to still occur (results not shown). High K^+ -induced secretion was also blocked by the application of Cd^{2+} to the bath solution (results not shown).

The total amount of secretion, displayed as charge (a representation of the area under each secretory spike), as well as the frequency of secretory spikes during a 60 s exposure under all the above experimental conditions were analysed (Figure 2B and C). These results illustrate that activin A (2 nM) causes a robust level of secretion, which appeared smaller in frequency and magnitude to that caused by 70 mM K^+ . However, due to the relative variability of these results, there was no significant difference in total charge released or spike frequency between the two groups. These results also show that the presence of Cd^{2+} significantly reduces activin-induced secretion ($n=4$; $P<0.01$). We also analysed a variety of spike parameters to investigate whether the kinetics of release of secretory vesicles stimulated by activin A alters from that caused by membrane depolarization (i.e. by 70 mM K^+ solution). The results of this analysis (Table 1) indicate that none of the spike parameters are different between those caused by stimulation with activin A (254 events from 6 cells) and those induced by 70 mM K^+ (878 events from 11 cells).

Discussion

In this report we provide evidence of a new physiological mechanism in which activin A stimulates catecholamine secretion from rat chromaffin cells of the adrenal medulla. This is a physiologically relevant finding given the well established role and presence of the activin A signalling system in the adrenal cortex (Vantinen *et al.* 2002,

Table 1 Average spike parameters from all cells exposed to high K^+ (70 mM; $n=11$) and activin A (2 nM; $n=6$)

	Frequency (Hz)	Amplitude (pA)	Area (fC)	Half-width (ms)	Rise time (ms)	Decay time (ms)
High K^+	1.3 $\pm$ 0.3	63.2 $\pm$ 9.2	214 $\pm$ 23.2	4.3 $\pm$ 0.7	3.7 $\pm$ 0.5	7.0 $\pm$ 1.2
Activin	0.7 $\pm$ 0.2	72.4 $\pm$ 6.6	236 $\pm$ 22.3	3.9 $\pm$ 0.8	3.2 $\pm$ 0.5	6.3 $\pm$ 1.1

Figure 2 Amperometric detection of catecholamine secretion from a single rat chromaffin cell. (A) Example trace of an amperometry experiment in which a single cell was exposed to 70 mM K⁺ solution, activin A (2 nM) and activin A in the presence of Cd²⁺ (200 μ M). This cell was selected as it displayed a very high response to activin. Spikes indicate release of a single secretory vesicle. Mean ($\pm$ S.E.M.) results of total charge (B) and frequency of release events (C) when single cells were exposed to various treatments over 60 seconds. * indicates $P < 0.05$ compared with activin group.

Vanttinen *et al.* 2003). The secretion of activin A from the adrenal cortex suggests that paracrine signalling within the adrenal gland may be adequate to cause activin-induced catecholamine release. In the adrenal gland, most of the cortical blood flows through the medulla (Kikuta & Murakami 1982), meaning that the chromaffin cells will be exposed to much higher concentrations of activin A than those seen in the whole body circulation. As activin A is also known to be secreted from human pheochromocytomas (Liu *et al.* 2000), it is plausible that any activin A which might be secreted from chromaffin cells would act in an autocrine manner to induce further catecholamine secretion.

Our results indicate that an activin A concentration of 2 nM is the lowest concentration that gives maximal effects in stimulating catecholamine secretion. This is in close agreement with a previous result showing that the same activin A concentration has significant effects on

insulin secretion and ion channel modulation and maximal effects on stimulating Ca²⁺ influx in dose-response analysis (Mogami *et al.* 1995, Fukuhara *et al.* 1997, Florio *et al.* 2000).

Previous work has illustrated that the effects of activin A can occur through the manipulation of ion channel function in endocrine cells such as insulin-secreting β -cells (Mogami *et al.* 1995) and FSH-secreting tumor cells (Takano *et al.* 1992, Takano *et al.* 1994). This new finding that activin A stimulates catecholamine release from chromaffin cells is physiologically important given the many important roles that catecholamines play in homeostatic regulation. These roles include the triggering of the classical 'flight or fight' response, regulation of heart rate and blood pressure as well as fetal survival during episodes of intrauterine stress (Phillippe 1983).

Our results illustrate that the level of catecholamine secretion stimulated by activin A is similar in magnitude to

that caused by membrane depolarization induced by an increased external K^+ concentration. This is further reinforced by the similarity in all spike parameters analysed from our experiments. As well as this, Ca^{2+} entry through voltage-gated Ca^{2+} channels is required for activin-induced secretion based on the fact that the Ca^{2+} channel antagonist, Cd^{2+} , blocks activin-induced secretion. Thus, activin A seems to cause membrane depolarization as this is required for the opening of these voltage-gated Ca^{2+} channels. Given that activin A induces catecholamine secretion within approximately 10–30 seconds of being applied, we hypothesize that this secretion is caused either by direct effects of activin A on certain ion channels (such as through the reduced opening of K^+ channels, for example) or by indirect effects on such channels through a more complex signalling cascade.

This report examines only the end point of the signalling cascade, secretory vesicle exocytosis, within chromaffin cells. Further investigations will need to be carried out to find whether this effect of activin A is caused by ion channel modulation, what intracellular mechanisms are involved in this response and whether other members of the TGF- β family cause similar effects to those reported here by activin A.

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