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Amphetamine enhances Ca^{2+} entry and catecholamine release via nicotinic receptor activation in bovine adrenal chromaffin cells

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Abstract

Amphetamine, a psychostimulant, has been shown to act as a channel blocker of muscle nicotinic receptors and to induce a Ca^{2+}-dependent secretion from adrenal chromaffin cells. In this study, the relationship between amphetamine and nicotinic receptors was studied using bovine adrenal chromaffin cells as a model system. Our results show that D-amphetamine sulfate alone induced an increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) and [^{3}H]norepinephrine release in a dose-dependent and extracellular Ca^{2+}-dependent manner. Two common nicotinic receptor antagonists, hexamethonium and mecamylamine, suppressed the D-amphetamine sulfate-induced $[Ca^{2+}]_c$ rise and [^{3}H]norepinephrine release. In addition, D-amphetamine sulfate inhibited the 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP)-induced $[Ca^{2+}]_c$ rise and [^{3}H]norepinephrine release, but not the high K^+- or veratridine-induced $[Ca^{2+}]_c$ increase and [^{3}H]norepinephrine release. Antagonists, including α-bungarotoxin and choline, that are more specific for α7 nicotinic receptors were capable of inhibiting the D-amphetamine sulfate-induced $[Ca^{2+}]_c$ rise, while D-amphetamine sulfate was found to be capable of inhibiting the $[Ca^{2+}]_c$ rise induced by the α7-nicotinic receptor agonists, epibatidine and choline. Moreover, D-amphetamine sulfate dose-dependently suppressed [^{3}H]nicotine binding to chromaffin cells. We, therefore, conclude that D-amphetamine sulfate acts as a nicotinic receptor agonist to induce $[Ca^{2+}]_c$ increase and [^{3}H]norepinephrine release in bovine adrenal chromaffin cells.

Keywords: Amphetamine; Adrenal chromaffin cell, bovine; Nicotinic receptor; Catecholamine secretion; Ca^{2+} concentration, cytosolic; Receptor binding

1. Introduction

Amphetamine is a versatile drug that was used in the clinical treatment of such maladies as obesity, narcolepsy and attention deficit/hyperactivity disorders until its addictive potential was fully acknowledged (Seiden et al., 1993). Because amphetamine is capable of inducing behavioral changes and triggering euphoric reactions, it has become a widely abused drug. Amphetamine induces hyperkinesis and stereotyped behaviors by enhancing the release of dopamine in the striatum and nucleus accumbens (Carboni et al., 1989; Karler et al., 1990; Amalric and Koob, 1993; Giros et al., 1996).

Dopamine is released via one of two mechanisms (Raiteri et al., 1979; Richter et al., 1995): (1) vesicular release, which is Ca^{2+}- and impulse-dependent, and (2) transporter-mediated release, which is much less dependent on Ca^{2+} and is impulse-independent (Hurd and Ungerstedt, 1989; Pierce and Kalivas, 1997). Studies have shown that amphetamine releases dopamine by means of a carrier-mediated process that depends on a plasmalemmal (Burnette et al., 1996; Sitte et al., 1998; Pifl et al., 1999) or vesicular monoamine transporter (VMAT) (Pifl et al., 1995). However, amphetamine-induced dopamine release far exceeds the relatively weak binding capacity of the dopamine transporter (DAT) (Andersen, 1987; Ritz et al., 1987; Wayment et al., 1998; Wu and Gu, 1999). Furthermore, amphetamine-induced dopamine release has been shown to have a weak correlation with the DAT density (Laruelle et al., 2000). It has been shown that amphetamine reduces the accumulation

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of monoamines in synaptic vesicle preparations that lack a plasma membrane transporter (Knepper et al., 1988). In addition to its actions on transporters, Mundorf et al. (1999) reported that amphetamine induces Ca^{2+}-dependent catecholamine release. Thus, we suspect that calcium signaling-related mechanisms might be involved in the actions of amphetamine.

The influence of amphetamine on neuroeffector systems other than the dopaminergic system remains unclear. Amphetamine exerts its psychostimulatory action on central and sympathetic neuronal systems. The postsynaptic sympathetic ganglion cells receive primarily cholinergic stimulation. In adrenal chromaffin cells, which are derived from the same embryonic origin as sympathetic neurons, amphetamine can induce an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) and a Ca^{2+}-dependent secretion (Mundorf et al., 1999). It has been shown that amphetamine acts as a channel blocker of muscle-type nicotinic receptors (Spitzmaul et al., 1999). In this study, the action of amphetamine on nicotinic receptors was investigated by using bovine adrenal chromaffin cells. Bovine chromaffin cells possess two primary nicotinic receptor subtypes: one composed of an α7 subunit that is sensitive to α-bungarotoxin (Garcia-Guzman et al., 1995) and the other composed of an α3 subunit (Criado et al., 1992). Our results suggest that amphetamine acts as both agonist and antagonist at nicotinic receptors.

2. Materials and methods

2.1. Chemicals

Collagenase was purchased from Worthington Biochemical. D-Amphetamine sulfate, α-bungarotoxin, carbachol, collagenase, 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP), digitonin, epibatidine, hexamethonium, mecamylamine, nicotine, nifedipine, verapamil and veratridine were all obtained from Sigma. [^{3}H]Norepinephrine and [^{3}H]nicotine were from New England Nuclear. NaCl, KCl, choline chloride and other chemicals were from Merck. Fura-2 acetoxymethyl ester was obtained from Molecular Probes.

2.2. Cell isolation

Bovine adrenal chromaffin cells were isolated with the aid of collagenase as described by Wilson (1987) with slight modifications (Liu et al., 1995). Freshly isolated cells were used for $[Ca^{2+}]_c$ measurements. Cells cultured in 96-well plates (2×10^5 cells/well) were used for measurements of [^{3}H]norepinephrine release and [^{3}H]nicotine binding assay.

2.3. [^{3}H]norepinephrine release

Cultured cells were incubated for 1 h at 37 °C with 0.65 μM [^{3}H]norepinephrine (1.54 Ci/mmol) in loading buffer (150 mM NaCl, 5 mM KCl, 2.2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, pH 7.4) containing 1% ascorbic acid and 0.5% bovine serum albumin (Kilpatrick et al., 1980). [^{3}H]Norepinephrine not taken up by the cells was washed out with loading buffer three times for 15 min each. Chromaffin cells were then stimulated with 10 μM DMPP or high-K^+ buffer in the presence or absence of D-amphetamine sulfate. The high-K^+ buffer was prepared by equimolar substitution of NaCl with 56 mM KCl. After 10 min, the supernatant was removed and a solution of 0.1% Triton X-100 and 2 mM EGTA was added to the pellet. After measuring the radioactivity of the supernatant and pellets, the percentage of total cell secretion was calculated. Results represent the means ± S.E.M. of three to six experiments performed in triplicate using separate cell batches.

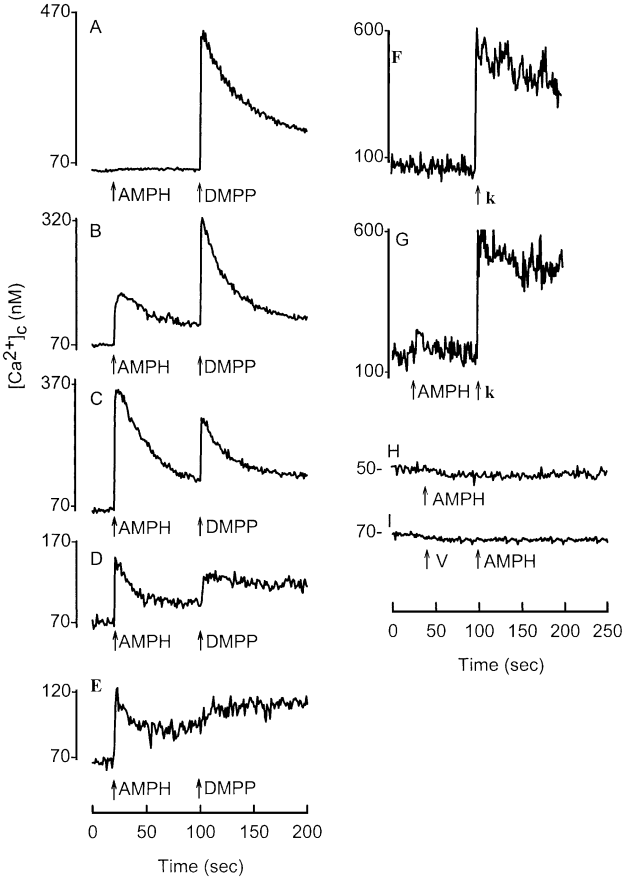

Fig. 1. D-Amphetamine sulfate induced the changes of $[Ca^{2+}]_c$. In panels (A)–(E), cells loaded with fura-2 were treated with various concentrations of D-amphetamine sulfate (↑ AMPH) (A, 0 μM, control; B, 50 μM; C, 100 μM; D, 500 μM; E, 1 mM) prior to the addition of 10 μM DMPP (↑ DMPP). In panels (F) and (G), cells were stimulated by 50 mM KCl (↑ K) either with (G) or without (F) pretreatment with 1 mM D-amphetamine sulfate. In panel (H), 100 μM D-amphetamine sulfate was added to chromaffin cells in a Ca^{2+}-free buffer containing 0.5 mM EGTA. In panel (I), 0.1 mM verapamil (↑ V) was added 50 s prior to the addition of 100 μM D-amphetamine sulfate.

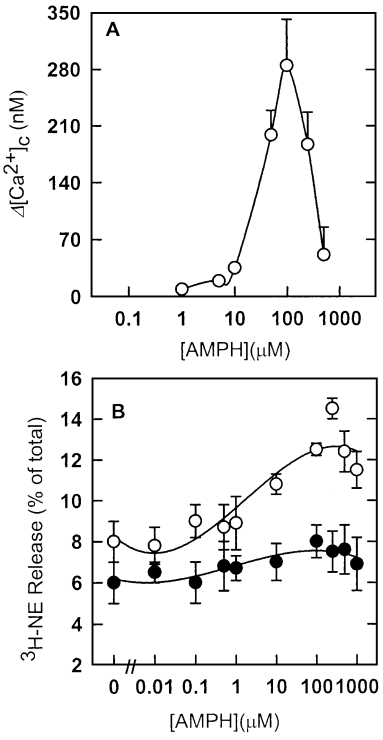

Fig. 2. Concentration-dependent $[Ca^{2+}]_c$ rise and [^{3}H]norepinephrine release induced by D-amphetamine sulfate. Panel (A), $[Ca^{2+}]_c$ rise induced by D-amphetamine sulfate at various concentrations in the presence of extracellular Ca^{2+}. Data were obtained from the cell experiments with similar protocol described in the caption of Fig. 1. Panel (B), [^{3}H]norepinephrine release induced by D-amphetamine sulfate at various concentrations in the absence (●) or presence (○) of extracellular Ca^{2+}. Data are presented as the percentage of total [^{3}H]norepinephrine content in the cells.

2.4. High-performance liquid chromatography (HPLC) analysis of catecholamine

Cultured chromaffin cells were stimulated with secretagogue in the presence or absence of D-amphetamine sulfate for 10 min. The supernatant was then removed and 0.4% perchloric acid was added to the supernatant and the attached cells, respectively, and incubated for 2 h before catecholamine was determined. Measurements of norepinephrine, epinephrine and dopamine were performed by reverse-phase HPLC with electrochemical detection as previously described (Ganhao et al., 1991). The results are presented as means ± S.E.M. of three experiments carried out in triplicate using different batches of cells.

2.5. $[Ca^{2+}]_c$ measurements

Bovine chromaffin cells were loaded with fura-2 acetoxymethyl ester by incubation (5×10^6 cells/ml) with 10 μM fura-2 acetoxymethyl ester at 37 °C for 30 min. Cells were then washed twice with loading buffer. Using a dual-excitation fluorometer (SPEX, CM system), fluorescence was measured at 340 and 380 nm excitation and 505 nm emission. $[Ca^{2+}]_c$ was calculated using a fluorescence ratio at 340 nm to that at 380 nm (Grynkiewicz et al., 1985). R_{max} was achieved by adding 0.01% digitonin to the sample at the end of experiments; excess EGTA was subsequently added to obtain R_{min}. A K_d of 224 nM for Ca^{2+} was used. We performed five experiments in each protocol, using different batches of cells; each experiment was carried out in duplicate.

2.6. [^{3}H]nicotine binding assay

The binding of [^{3}H]nicotine to intact cells was measured using a slightly modified method described by Higgins and Berg (1988). Intact chromaffin cells in 96-well plates (2×10^5 cells/well) were washed twice with loading buffer

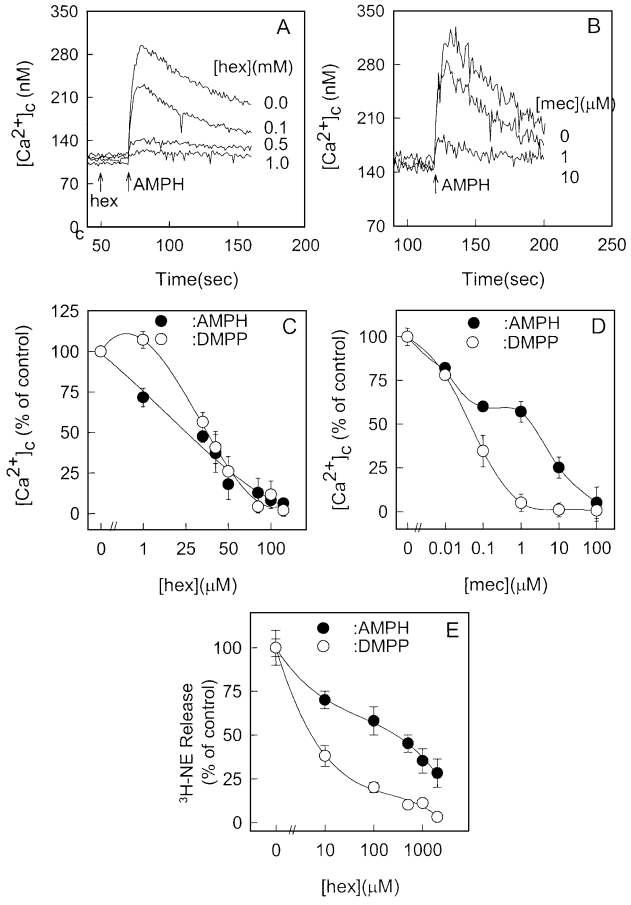

Fig. 3. Dose-dependent hexamethonium- or mecamylamine-based inhibition of D-amphetamine sulfate-induced responses. Cells loaded with fura-2 were stimulated with 200 μM D-amphetamine sulfate (↑ AMPH) in the presence of various concentrations of hexamethonium (hex, panel A) or mecamylamine (mec, panel B). Panel (C) shows the inhibitory potency of hexamethonium (hex) and panel (D) the inhibitory potency of mecamylamine (mec) on $[Ca^{2+}]_c$ changes induced by 10 μM DMPP (○) or 200 μM D-amphetamine sulfate (●). Data are presented as percentages of control responses induced by 10 μM DMPP (○) or 200 μM D-amphetamine sulfate (●). A 100% response represents the difference between basal and peak $[Ca^{2+}]_c$ levels. Panel (E) shows the inhibitory effects of hexamethonium on [^{3}H]norepinephrine induced by 10 μM DMPP (○) or 200 μM D-amphetamine sulfate (●).

and incubated with 40 nM [^{3}H]nicotine in the presence or absence of D-amphetamine sulfate for 90 min. The assay was terminated by aspiration of the incubation medium and cells were washed six times with 0.2 ml ice-cold Ca^{2+}-free loading buffer containing 0.1 mM EGTA. Cells were then treated in 2% Triton X-100 for 2 h before being transferred to vials for scintillation counting. Nonspecific binding (determined by preincubation with 1 mM nicotine for 30 min) was routinely subtracted from the total binding. Data were analyzed and expressed as percent of [^{3}H]nicotine binding in the absence of D-amphetamine sulfate.

3. Results

Fig. 1 shows the effects of D-amphetamine sulfate on $[Ca^{2+}]_c$ in bovine adrenal chromaffin cells. D-Amphetamine sulfate alone induced a transient $[Ca^{2+}]_c$ increase. Its maximal effect—a transient $[Ca^{2+}]_c$ increase to 285 57 nM ($n=30$) followed by a decrease to a sustained level of 124 ± 25 nM within 2 min—was observed at a concentration of 100 μM. Fig. 2A shows that the concentration curve for the D-amphetamine sulfate-induced increase in $[Ca^{2+}]_c$ resembles a bell curve, since the transient rise and decay of $[Ca^{2+}]_c$ both decreased at concentrations above 100 μM. In addition, D-amphetamine sulfate inhibited the $[Ca^{2+}]_c$ rise induced by 10 μM DMPP (a nicotinic receptor agonist) in a concentration-dependent manner (Fig. 1, traces A–E), but did not significantly alter the $[Ca^{2+}]_c$ rise induced by high K^+ (Fig. 1, traces F and G) or veratridine (data not shown). The D-amphetamine sulfate-induced $[Ca^{2+}]_c$ rise was completely suppressed in the absence of extracellular Ca^{2+} (Fig. 1, trace H) and by the presence of the voltage-gated Ca^{2+} channel blockers verapamil (0.1 mM) (Fig. 1, trace I) and nifedipine (10 μM) (data not shown). Thus, it appears that extracellular Ca^{2+} influx was the primary source of the D-amphetamine sulfate-induced Ca^{2+} transient.

Amphetamine is known to induce Ca^{2+}-independent catecholamine release via DAT (Sitte et al., 1998), norepinephrine transporter (Pifl et al., 1999) and VMAT (Pifl et al., 1995). After establishing that D-amphetamine sulfate increased $[Ca^{2+}]_c$, we examined whether D-amphetamine sulfate is capable of inducing catecholamine secretion in the presence or absence of extracellular Ca^{2+}. Our results show that in the absence of extracellular Ca^{2+}, no significant secretion was induced by D-amphetamine sulfate, but in the presence of extracellular Ca^{2+}, D-amphetamine sulfate induced secretion of greater intensity. Fig. 2B shows that [^{3}H]norepinephrine release was almost twice basal release at D-amphetamine sulfate concentrations above 100 μM. These results suggest that D-amphetamine sulfate is capable of inducing Ca^{2+}-dependent catecholamine release.

We used nicotinic receptor antagonists to investigate the process leading to D-amphetamine sulfate-activated $[Ca^{2+}]_c$ rise and to clarify the relationship between nicotinic receptors and D-amphetamine sulfate. We first examined the effects of two common nicotinic receptor antagonists–hexamethonium and mecamylamine. As shown in Fig. 3A, hexamethonium inhibited the $[Ca^{2+}]_c$ rise induced by 200 μM D-amphetamine sulfate in a dose-dependent manner ($IC_{50}=20$ μM, $n=10$); a similar inhibitory effect was noted for the DMPP-induced Ca^{2+} rise ($IC_{50}=30$ μM, $n=10$) (Fig. 3C). Hexamethonium also inhibited the [^{3}H]norepinephrine release induced by either 10 μM DMPP ($IC_{50}=10$ μM, $n=9$) or 200 μM D-amphetamine sulfate ($IC_{50}=100$ μM, $n=9$) (Fig. 3E). Our results also show that mecamylamine was capable of inhibiting the $[Ca^{2+}]_c$ rise induced by D-amphetamine sulfate ($IC_{50}=1$ μM, $n=10$) and DMPP ($IC_{50}=0.3$ μM, $n=10$) (Fig. 3B and D).

We then examined the effects of two α7 nicotinic receptor-specific antagonists: α-bungarotoxin and choline. Our results show that 0.5 μM freshly prepared α-bungarotoxin suppressed the D-amphetamine sulfate-induced $[Ca^{2+}]_c$ rise by $52 \pm 10\%$ ($n=8$) (Fig. 4A). In contrast, the peak carbachol-induced transient $[Ca^{2+}]_c$ rise was not

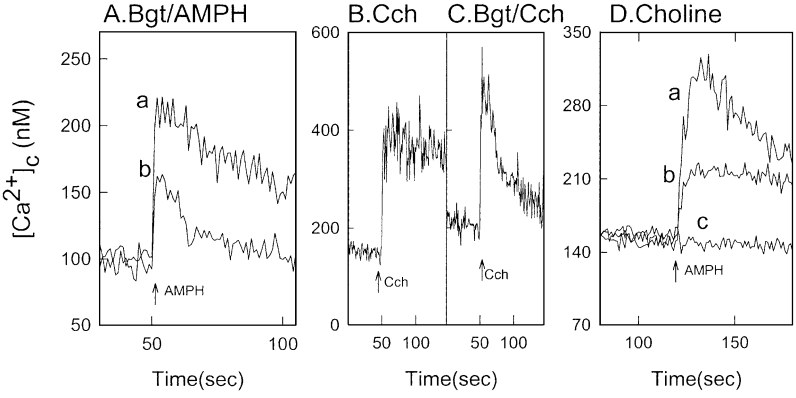

Fig. 4. Suppression of D-amphetamine sulfate-induced $[Ca^{2+}]_c$ rise by choline and α-bungarotoxin (Bgt). Panel (A), cells loaded with fura-2 were stimulated with 200 μM D-amphetamine sulfate (↑ AMPH) in the absence (curve a) or presence (curve b) of 0.5 μM α-bungarotoxin. Panel (B), cells stimulated with 0.3 mM carbachol (↑ Cch) in the absence of α-bungarotoxin. Panel (C), cells stimulated with 0.3 mM carbachol in the presence of 2 μM α-bungarotoxin. Panel (D), cells stimulated with 200 μM D-amphetamine sulfate (↑ AMPH) in the presence of choline (curve a, 0 μM; curve b, 100 μM; curve c, 500 μM).

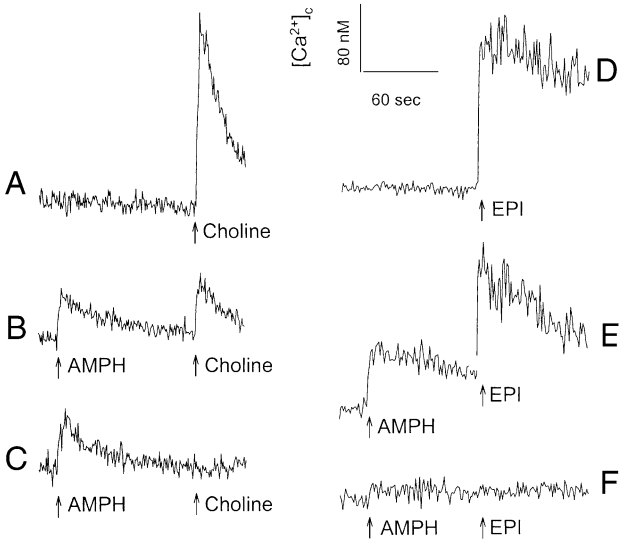

Fig. 5. Inhibitory effects of D-amphetamine sulfate on choline- and epibatidine (EPI)-induced $[Ca^{2+}]_c$ rise. Cells loaded with fura-2 were stimulated with 3 mM choline (↑ choline; traces A, B, C) and 0.5 μM epibatidine (↑ EPI; traces D, E, F). Cells were treated with D-amphetamine sulfate (↑ AMPH) 100 and 80 s prior to the addition of choline or epibatidine (EPI), respectively. D-Amphetamine sulfate concentrations in traces A, B, C were 0, 100, 300 μM, and traces D, E and F were 0 μM, 100 μM, and 1 mM, respectively.

affected by treatment with 2 μM α-bungarotoxin—however, the rate at which the $[Ca^{2+}]_c$ rise returned to a resting level was increased (Fig. 4B and C). As shown in Fig. 4D, choline inhibited the D-amphetamine sulfate-induced $[Ca^{2+}]_c$ rise in a concentration-dependent manner. It is known that choline acts as both a nicotinic receptor agonist and antagonist, and is more potent when interacting with α7 nicotinic receptors than with other subtypes (Alkondon et al., 1997). We then examined the effects of D-amphetamine sulfate on the α7 nicotinic receptor agonist-induced $[Ca^{2+}]_c$ rise. Epibatidine is recognized as a potent α7 nicotinic receptor agonist (Alkondon et al., 1997; Decker and Meyer, 1999; Gerzanich et al., 1995). D-Amphetamine sulfate inhibited the $[Ca^{2+}]_c$ rise induced by either 3 mM choline or 0.5 μM epibatidine (Fig. 5).

In the case of DMPP, a common nicotinic receptor agonist, D-amphetamine sulfate suppressed both the DMPP-induced $[Ca^{2+}]_c$ rise (IC_{50} = 30 μM) (Fig. 6A) and catecholamine secretion (IC_{50} = 200 μM) (Fig. 6B). To further clarify the effects of D-amphetamine sulfate on agonist-induced secretion, we depolarized the plasma membrane of bovine adrenal chromaffin cells with 56 mM K^+ solution or 0.3 mM veratridine. As shown in Fig. 6C, D-amphetamine sulfate had no significant effect on high K^+ and veratridine-induced [^{3}H]norepinephrine release. According

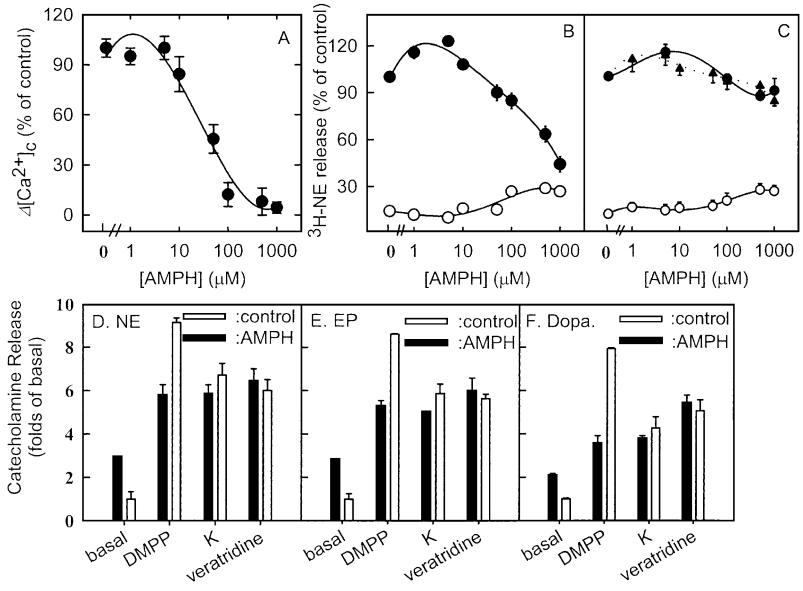

Fig. 6. Effects of D-amphetamine sulfate on $[Ca^{2+}]_c$ and on catecholamine release induced by DMPP, high K^+ or veratridine. Panel (A), inhibitory effect of D-amphetamine sulfate on the $[Ca^{2+}]_c$ changes induced by 10 μM DMPP, calculated by subtracting basal $[Ca^{2+}]_c$ from peak $[Ca^{2+}]_c$. Data are presented as percentage change of DMPP-induced $[Ca^{2+}]_c$. Panel (B), chromaffin cells were stimulated (●) or not (○) with 10 μM DMPP in the presence of various concentrations of D-amphetamine sulfate. DMPP alone induced a 25.3 ± 1.4% secretion of total ^{3}H-norepinephrine from bovine chromaffin cells. This was used to represent 100% secretion. Panel (C), chromaffin cells were treated with vehicle (○, control) or stimulated with 56 mM K^+ (●) or 0.1 mM veratridine (▲) in the presence of D-amphetamine sulfate at various concentrations. Individually, high K^+ or veratridine induced the secretion of 12.5 ± 1.7% and 14.7 ± 3.1% total ^{3}H-norepinephrine from bovine chromaffin cells, respectively. Both were used to represent 100% secretion. Panels (D)–(F), norepinephrine, epinephrine and dopamine-release stimulated by 10 μM DMPP, 56 mM K^+ or 0.1 mM veratridine in the presence (solid bars) or absence (open bars) of 500 μM D-amphetamine sulfate. The analysis of norepinephrine, epinephrine and dopamine was by HPLC. Data are presented as folds of basal catecholamine release (2.2 ± 0.9%, 1.3 ± 0.3% and 4.8 ± 0.2% for norepinephrine, epinephrine and dopamine, respectively). *$P < 0.01$, paired Student's t-test, comparing the data in the presence and absence of D-amphetamine sulfate.

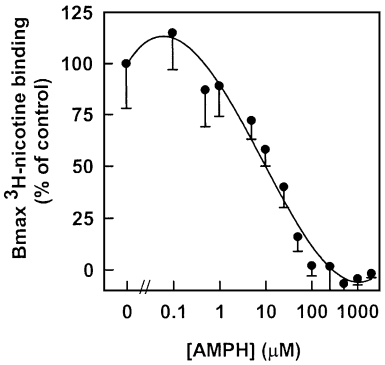

Fig. 7. Inhibitory effects of D-amphetamine sulfate on specific [^{3}H]nicotine binding in bovine adrenal chromaffin cells. Chromaffin cells were incubated with [^{3}H]nicotine (40 nM) in the presence of D-amphetamine sulfate as indicated. Specific [^{3}H]nicotine binding was calculated by subtracting nonspecific binding from total binding (see Materials and methods).

to the results obtained from HPLC analysis, D-amphetamine sulfate (500 μM) induced a statistically significant release of norepinephrine, epinephrine and dopamine (Fig. 6D, E and F; $P < 0.01$). D-Amphetamine sulfate (500 μM) also inhibited the DMPP-induced release of the three catecholamines, but did not significantly suppress high K^+- or veratridine-induced catecholamine release (Fig. 6D).

Based on these results, it is very likely that D-amphetamine sulfate acts as a nicotinic receptor agonist in the process of increasing $[Ca^{2+}]_c$ and inducing catecholamine secretion, while at the same time inhibiting DMPP-stimulated responses. To further support this idea, we conducted a radioligand-binding assay. As shown in Fig. 7, [^{3}H]nicotine binding to intact chromaffin cells was suppressed by D-amphetamine sulfate ($IC_{50} = 50$ μM). These data illustrate that D-amphetamine sulfate was able to replace [^{3}H]nicotine and bind with nicotinic receptors.

4. Discussion

We found three pieces of evidence showing that D-amphetamine sulfate acts as a nicotinic receptor agonist in bovine adrenal chromaffin cells: (a) D-amphetamine sulfate-induced $[Ca^{2+}]_c$ rise and extracellular Ca^{2+}-dependent catecholamine secretion-cellular events that are similar to those associated with nicotinic receptor stimulation; (b) the D-amphetamine sulfate-induced $[Ca^{2+}]_c$ rise and catecholamine secretion were inhibited by various nicotinic receptor antagonists and voltage-gated ion channel blockers, and the $[Ca^{2+}]_c$ rise induced by other nicotinic receptor agonists was inhibited by D-amphetamine sulfate; and (c) D-amphetamine sulfate suppressed [^{3}H]nicotine binding to bovine adrenal chromaffin cells.

Spitzmaul et al. (1999) reported that the influx current of muscle nicotinic receptors is blocked by D-amphetamine sulfate and, therefore, suggested that D-amphetamine sulfate acts as a nicotinic receptor channel blocker. Our $[Ca^{2+}]_c$ and [^{3}H]norepinephrine measurements in the present study show that D-amphetamine sulfate not only blocked nicotinic receptor responses induced by other agonists, but also induced nicotinic receptor responses. These results, while supporting the suggestion of Spitzmaul that amphetamine acts as a nicotinic receptor blocker, also support the idea that D-amphetamine sulfate acts as a nicotinic receptor agonist. The differences may be due to the nicotinic receptor subtypes, tissue cells and amphetamine derivatives that were used: Spitzmaul et al. used the muscle nicotinic receptor expressed in human embryonic kidney cells, while we used bovine chromaffin cells that do not contain any subtype of muscle nicotinic receptors. In addition, different forms of amphetamine were used: we used D-amphetamine sulfate, while Spitzmaul et al. (1999) used an L enantiomer of amphetamine.

It has been shown that amphetamine releases catecholamine by means of an exchange-diffusion process depending on a norepinephrine transporter (Burnette et al., 1996), DAT (Sitte et al., 1998; Pifl et al., 1999) or VMAT (Pifl et al., 1995). The catecholamine release facilitated by VMAT inhibitors is known to be Ca^{2+}-independent (Carboni et al., 1989). However, our data show that in chromaffin cells, D-amphetamine sulfate can induce a transient $[Ca^{2+}]_c$ rise and that the amphetamine-induced catecholamine release is largely, if not totally, Ca^{2+}-dependent. A similar phenomenon was reported by Mundorf et al. (1999), namely, that amphetamine, at 10 μM, triggers a transient increase of $[Ca^{2+}]_c$ (23% with respect to the response to high K^+) and that the secretion elicited by amphetamine in Ca^{2+}-free medium was significantly less than that in Ca^{2+}-containing medium in bovine adrenal chromaffin cells. The absence of Ca^{2+}-independent secretion may be because only a minor fraction of norepinephrine transporter is present at the cell surface of chromaffin cells (Schroeter et al., 2000) or even functionally absent (Wakade et al., 1986), thus, we suggest that in chromaffin cells the $[Ca^{2+}]_c$ transient rise and catecholamine secretion induced by D-amphetamine sulfate, and blocked by hexamethonium and mecamylamine, are mainly associated with nicotinic receptors.

There are various nicotinic receptor subtypes composed of unique combinations of homologous subunits encoded by at least 16 distinct genes ($\alpha_1-\alpha_9$, $\beta_1-\beta_4$, γ, δ and ε) (Sargent, 1993). Bovine adrenal chromaffin cells possess both α7 (Garcia-Guzman et al., 1995) and α3 nicotinic receptors (Criado et al., 1992). It is known that the α7 nicotinic receptor possesses Ca^{2+} permeability and exhibits rapid desensitization, and it is, therefore, believed to generate fast but transient secretion responses in chromaffin cells (Lopez et al., 1998). In contrast, the α3 nicotinic receptor is a slow desensitizing receptor and, therefore, may be responsible for generating more sustained catecholamine release responses (Lopez et al., 1998). We suggest that D-amphetamine sulfate-induced Ca^{2+}-dependent secretion is primarily associated with the α7 nicotinic receptor in

bovine adrenal chromaffin cells, making the secreted level much smaller than that induced by DMPP. This suggestion is supported by our data. First, α-bungarotoxin in particular exerts a potent effect on muscle α1 and neuronal α7–α9 nicotinic receptors (Sargent, 1993), and our data show that α-bungarotoxin could suppress the D-amphetamine sulfate-induced $[Ca^{2+}]_c$ rise, but did not affect the DMPP-induced $[Ca^{2+}]_c$ transient rise. Second, we noted that D-amphetamine sulfate inhibited the $[Ca^{2+}]_c$ transient induced by other potent α7 nicotinic receptor agonists-choline and epibatidine (Fig. 5). Choline, which has been described as a prototypical α7 nicotinic receptor agonist (Alkondon et al., 1997), exerts its effect at a concentration range measured in mM. Epibatidine is also known as an α7 nicotinic receptor agonist (Gotti et al., 2000). It appears that D-amphetamine sulfate acts as both an agonist and antagonist at α7 nicotinic receptors and, thus, induces Ca^{2+}-dependent secretion but in smaller amounts. The possibility D-amphetamine sulfate acting on other nicotinic receptor subtypes remains to be investigated.

D-Amphetamine has been shown to inhibit high K^+-stimulated dopamine release from rat synaptosomes (Bowyer et al., 1987) as well as action potential discharges in rat brains (Mercuri et al., 1989). Mahata et al. (1996) found that some VMAT inhibitors (e.g., reserpine) suppress nicotine- and membrane depolarization-induced secretion by inhibiting voltage-gated Ca^{2+} channels. In our study, we found that D-amphetamine sulfate suppressed nicotinic receptor-associated secretion, but did not significantly affect either high K^+- or veratridine-induced secretion. It is, therefore, unlikely that the inhibitory effect of D-amphetamine sulfate on the nicotinic receptor response involves voltage-gated ion channels. In contrast, we found that the L enantiomer of amphetamine was capable of blocking the high K^+-induced $[Ca^{2+}]_c$ rise by 40% (data not shown), although D-amphetamine sulfate blocked the high K^+-induced $[Ca^{2+}]_c$ rise in bovine adrenal chromaffin cells by less than 10% (Fig. 1). We also found that D-amphetamine sulfate blocked the high K^+-induced $[Ca^{2+}]_c$ rise in rat PC12 cells by approximately 35% (data not shown). The voltage-gated Ca^{2+} channels found in various species have different amino acid sequences and different manners of modulation (Catterall, 2000). In chromaffin cells, studies have shown that voltage-gated Ca^{2+} channel activity differs according to species (Hernandez-Guijo et al., 1997; Gandia et al., 1995). Thus, our conflicting results may be the result of differences in tissue type, the amphetamine derivative used, and/or type of voltage-gated ion channel.

Previous studies on the relationship between amphetamine and nicotinic receptors support our view that D-amphetamine sulfate has a novel role as a nicotinic receptor agonist. Skau and Gerald (1977) found that amphetamine inhibits α-bungarotoxin binding at the neuromuscular junction in mice, and Sulzer et al. (1995) reported that amphetamine suppresses nicotine-induced dopamine release in PC12 cells. That nicotine and nicotinic antagonists counteract the effects of amphetamine finds support from Stevens et al. (1995), who noted that nicotine caused a transient normalization of amphetamine-induced loss in auditory gating. In addition, Karler et al. (1996) claimed that nicotinic antagonists blocked the induction and expression of amphetamine sensitization. The idea of similarities in the actions of amphetamine and nicotine finds support from Fung and Lau (1992), who reported increased activity at nicotinic binding sites and in amphetamine action following long periods of nicotinic treatment, and from a study reporting a partial nicotine discriminative stimulus of amphetamine with unclear involvement of dopamine receptors (Mansbach et al., 1998). Chen et al. (1995) also showed that treatment with either nicotine or amphetamine suppresses asphyxia-induced changes, and that amphetamine counteracts the inhibitory effects of nicotine on these changes.

In conclusion, D-amphetamine sulfate plays a novel role as an agonist at nicotinic receptors. Multiple nicotinic receptor subtypes are present in the central and peripheral nervous systems associated with memory, emotion, neuronal survival and pain perception. By exerting its influence as a psychostimulant on the nervous system, amphetamine can interfere with hearing, emotion and pain perception. The molecular mechanisms of amphetamine have attracted a great deal of research interest. Our results encourage the consideration of a new direction for inquires into this area.

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