J. Pharm. Pharmacol. 1986, 38: 595–599
Received December 30, 1985

Mechanism of the inhibitory effect of thiopentone on stimulus-secretion coupling in chromaffin cells

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This study was undertaken to clarify the mechanism of action of thiopentone on stimulus-secretion coupling in cholinergic postsynaptic cells using cultured bovine adrenal chromaffin cells. Thiopentone (20–100 μM) inhibited carbachol-induced Ca^{2+} uptake into, and catecholamine release from, the cells in a concentration-dependent manner. The inhibition of catecholamine release was almost parallel to the inhibition of Ca^{2+} uptake. The inhibitory effect of thiopentone was not overcome by an increase in the concentration of carbachol, whereas that of alcuronium was. Contrary to the inhibition by diltiazem, which was reversed by increasing the Ca^{2+} concentration, the inhibition by thiopentone was not overcome by a high Ca^{2+} concentration. Compared with the inhibition of carbachol-induced catecholamine release, a higher concentration of thiopentone was required to inhibit high K^+ (56 mM KCl)-induced catecholamine release (200 μM thiopentone exhibited 32% inhibition). The results suggest that thiopentone blocks the stimulus-secretion coupling in the chromaffin cells as a result of inhibiting Ca^{2+} uptake through nicotinic receptor-linked channels. The linkage between receptor stimulation and Ca^{2+} channel activation seems to be the process most susceptible to inhibition by the barbiturate.

It has been proposed that barbiturate anaesthesia is produced by the additive effects of augmented inhibitory and depressed excitatory synaptic transmission, resulting in a diminution of neuronal excitability (Ho & Harris 1981). Depression of excitatory synaptic transmission has been observed at a variety of peripheral and central nervous system synapses. However, because of the intrinsic complexity of the central nervous system, it has seemed appropriate to study peripheral synapses in an attempt to explore where and how a barbiturate anaesthetic acts at specific synapses. For this purpose, sympathetic nerves and the adrenal medulla have been used as a peripheral model of the cholinergic synapse.

Göthert & Rieckesmann (1978) have reported that pentobarbitone inhibits noradrenaline (NA) secretion from sympathetic nerve endings induced by drugs or electrical stimulation. Recently, Marin & Recio (1982) examined the effect of pentobarbitone on NA release from cat arteries stimulated by various drugs and showed that this anaesthetic selectively inhibits Ca^{2+}-dependent secretion of NA, but not Ca^{2+}-independent secretion. Sumikawa et al (1983) have reported that thiopentone at clinical concentrations, as well as the volatile anaesthetic, halothane, selectively inhibit nicotinic receptor-mediated catecholamine (CA) release, but not muscarinic receptor-mediated release. They drew attention to the susceptibility of nicotinic receptor-linked Ca^{2+} channels to the effects of anaesthetics. Interaction of anaesthetics and Ca^{2+} on CA release would be of interest, because Ca^{2+} plays the role of coupler in stimulus-secretion coupling. However, these reports have not analysed this interaction.

The present work was undertaken to examine the effects of thiopentone on stimulus-secretion coupling in adrenal chromaffin cells. Cultured bovine adrenal chromaffin cells were used because they have been shown to be a suitable model for analysing the role of Ca^{2+} and the mechanism of drug action affecting the stimulus-secretion coupling (Kilpatrick et al 1982).

MATERIALS AND METHODS

Cell cultures

Fresh bovine adrenal glands were obtained from a local slaughterhouse. Adrenal chromaffin cells were isolated by collagenase digestion of slices of the adrenal medulla as described previously (Wada et al 1985). Subsequently, the cells were suspended on Eagle's MEM supplemented with 10% heat-inactivated foetal calf serum, aminobenzyl penicillin (60 μg ml^{-1}), streptomycin (100 μg ml^{-1}) and amphotericin B (0·3 μg ml^{-1}), and plated at a density of 4×10^6 cells per dish (Falcon, 35 mm diameter).

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After three days at 37 °C in 5% CO_2–95% air, the chromaffin cells had formed monolayers containing 46·7 ± 2·4 μg (n = 8, mean ± s.e.) of CA per dish. The culture medium was replaced at three-day intervals, and experiments were performed between days 3 and 7. The viability of the cells was more than 95% as judged by the trypan blue exclusion test.

$^{45}Ca^{2+}$ uptake and CA release

The incubation medium used was Krebs-Ringer phosphate buffer (mM: NaCl 154, KCl 5·6, $CaCl_2$ 2·2, $MgCl_2$ 1·1, glucose 10, NaH_2PO_4 0·85, Na_2HPO_4 2·15, pH 7·4) containing 0·5% bovine serum albumin or high K^+ medium in which KCl was increased to 56 mM and NaCl reduced to maintain the isotonicity of the medium (other constituents were the same as in the Krebs-Ringer phosphate buffer).

Secretion of CA and influx of $^{45}Ca^{2+}$ were measured simultaneously as described by Wada et al (1985). The cells were washed once with 2 ml of ice-cold Krebs-Ringer phosphate buffer. To obtain equilibrium of thiopentone partition between medium and cells, before starting the reaction, incubation for 1 min was carried out with 2 ml of the medium at 37 °C in the presence or absence of thiopentone. The reaction was started by replacement of the preincubation medium with the reaction medium (2 ml) containing 2 μCi of $^{45}CaCl_2$ (4·4 × 10^6 d min^{-1}) and various concentrations of carbachol or high K^+ in the presence or absence of thiopentone, and was carried out for 1 min at 37 °C. The incubation was terminated by the addition of 0·2 ml of 11 mM diltiazem, after which the medium was transferred to test tubes and the cells were washed four times with 2 ml of ice-cold Ca^{2+}-free Krebs-Ringer phosphate buffer.

The reversibility of the effect of thiopentone was examined as follows. The first incubation was carried out for 1 min in the manner described with various concentrations of thiopentone, except that the medium contained neither $^{45}CaCl_2$ nor carbachol. After incubation the cells were washed twice, and the second incubation was carried out for 1 min to determine CA release followed by the procedure outlined above.

The aliquot in the test tube was acidified with 0·4 M perchloric acid, and the amount of CA secreted into the medium was adsorbed onto aluminium hydroxide and estimated by the trihydroxyindole method (von Euler & Lishajko 1961). This assay method has a limit of sensitivity of 2 ng of each catecholamine. The intra- and interassay variations were less than 5%. In all cases, it was ascertained that the drugs used did not interfere with the assay. Influx of $^{45}Ca^{2+}$ into the cells was measured by detaching and solubilizing the cells in 1 ml of 10% Triton x-100. Radioactivity taken up by the cells was counted in toluene base scintillator using a Beckman LS-7000 liquid scintillation counter. Ca^{2+} uptake was expressed in nmol/10^6 cells, being calculated from the initial activity of $^{45}Ca^{2+}$ in the medium.

The data were expressed as mean ± s.e. The results of repeated measures and multiple groups were analysed by one-way analysis of variance. Comparisons between groups were assessed by Student's *t*-test. A P value $< 0·05$ was considered significant. Drugs used were $^{45}CaCl_2$ (Amersham), collagenase type 1, soyabean trypsin inhibitor, carbachol (Sigma), Eagle's MEM (Nissui), thiopentone, diltiazem (a Ca^{2+} antagonist; Tanabe), and alcuronium (a non-depolarizing neuromuscular blocking agent; Nippon Roche).

RESULTS

Basal release of CA from the cells during a 1 min incubation period with no stimulation was very low, i.e. less than 0·1 μg/10^6 cells (0·8% of the total CA in the cells). Carbachol at 0·3 mM, which was the concentration that caused maximal response of the cells to release CA, increased the release of CA by 1·37 ± 0·12 μg/10^6 cells (n = 6) over the basal release corresponding to 11·8% of the total CA in the cells. Ca^{2+} uptake simultaneously measured was 1·53 ± 0·1 nmol/10^6 cells. Fig. 1 shows carbachol-induced Ca^{2+} uptake and CA release as a function of the extracellular Ca^{2+} concentration. In a range of Ca^{2+} concentrations from 0·5 to 8 mM, Ca^{2+} uptake increased dose-dependently, whereas CA release increased in a range of Ca^{2+} concentrations from 0·5 to 4 mM. As shown in Fig. 2, thiopentone (20–100 μM) inhibited carbachol-induced Ca^{2+} uptake into the cells and CA release from the cells, in a concentration-dependent manner, with 50 μM thiopentone exhibiting 44% inhibition. The inhibition of CA release was almost parallel to that of Ca^{2+} uptake. The inhibitory effect of thiopentone was reversible because, after its removal from the medium, the ability of the cells to release CA was restored to the control level (Table 1).

High K^+-induced CA release was almost comparable to the carbachol-induced release, i.e. high K^+ increased the release of CA by 1·30 ± 0·12 μg/10^6 cells (n = 4) over the basal release and Ca^{2+} uptake by 1·41 ± 0·15 nmol/10^6 cells. Compared with the inhibition of carbachol-induced CA release, a higher

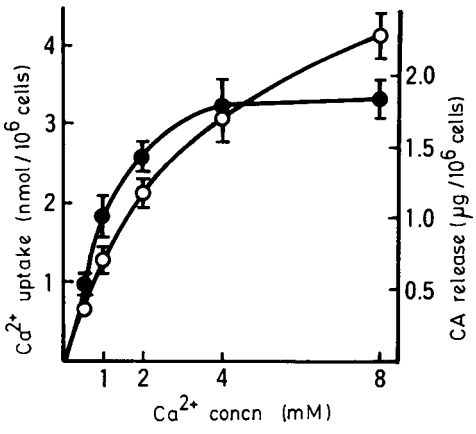

FIG. 1. Effects of varying the Ca^{2+} concentration on carbachol-induced Ca^{2+} uptake (○) and CA release (●) in cultured bovine adrenal chromaffin cells (mean ± s.e., n = 4 for each point). The cells (4×10^6) were incubated in Krebs-Ringer phosphate buffer at 37 °C for 1 min with carbachol (0·3 mM) and $^{45}CaCl_2$ (2 μCi, $4{\cdot}4 \times 10^6$ d min^{-1}). CA released into the medium and $^{45}Ca^{2+}$ taken up by the cells were measured. Ca^{2+} uptake was expressed in nmol/10^6 cells, being calculated from the initial activity of $^{45}Ca^{2+}$ in the medium.

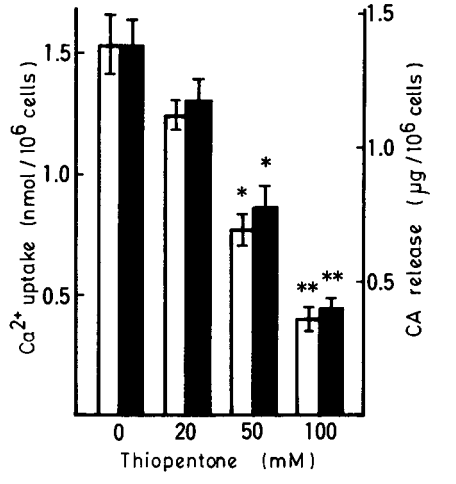

FIG. 2. Effects of thiopentone on carbachol-induced Ca^{2+} uptake (□) and CA release (■) in the chromaffin cells (mean ± s.e., n = 6 for each value). The cells were incubated in Krebs-Ringer phosphate buffer at 37 °C for 1 min with 0·3 mM carbachol and $^{45}CaCl_2$ in the presence of various concentrations of thiopentone.

concentration of thiopentone was required to inhibit high K^+-induced CA release, i.e., 50 μM thiopentone exhibited 46% inhibition of carbachol-induced CA release but no inhibition of high K^+-induced CA release, and 200 μM thiopentone inhibited high K^+-induced CA release by 32% (Table 2).

Table 1. Reversibility of the inhibitory effect of thiopentone on carbachol-induced CA release from chromaffin cells. The cells were first incubated at 37 °C for 1 min in the presence of various concentrations of thiopentone; after washing out the drug, a second incubation was carried out to determine catecholamine release.

Concentration of thiopentone (μM)	Catecholamine release (% of total) Presence of thiopentone	After washing out thiopentone
0	11·8 ± 0·8 (n = 6)	11·4 ± 0·9 (n = 4)
20	8·7 ± 0·6 (n = 6)*	10·7 ± 0·9 (n = 4)
50	4·6 ± 0·3 (n = 6)**	11·1 ± 0·6 (n = 4)
100	2·8 ± 0·2 (n = 6)**	10·8 ± 0·7 (n = 4)

Statistical significance: $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}001$, compared with control.

Table 2. Effects of thiopentone on Ca^{2+} uptake and CA release in chromaffin cells stimulated by carbachol and high K^+. The cells were incubated at 37 °C for 1 min with carbachol or high K^+ in the presence of various concentrations of thiopentone. $^{45}Ca^{2+}$ taken up by the cells and CA released into the medium were measured.

Stimulant	Thiopentone (μM)	Ca^{2+} uptake (nmol/10^6 cells)	CA release (μg/10^6 cells)
Carbachol 0·3 mM	0	1·53 ± 0·12 (n = 6)	1·37 ± 0·10 (n = 6)
	50	0·92 ± 0·08 (n = 6)*	0·76 ± 0·08 (n = 6)**
High K^+ 56 mM	0	1·58 ± 0·13 (n = 4)	1·32 ± 0·12 (n = 4)
	50	1·47 ± 0·13 (n = 4)	1·28 ± 0·10 (n = 4)
	200	1·04 ± 0·10 (n = 4)*	0·90 ± 0·08 (n = 4)*

Statistical significance: $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, compared with control.

Inhibition by thiopentone was examined as a function of the carbachol concentration in comparison with alcuronium. As shown in Fig. 3, the inhibitory effect of thiopentone was not overcome by an increase in the concentration of carbachol, whereas that of alcuronium was overcome to a considerable extent.

Similar results were obtained for the inhibition by thiopentone and diltiazem of carbachol-induced CA release, which was examined as a function of the extracellular Ca^{2+} concentration (Fig. 4). Contrary to the inhibition by diltiazem which was reversed by increasing the Ca^{2+} concentration, the inhibition by thiopentone was not overcome by a high Ca^{2+} concentration.

DISCUSSION

The results have shown that thiopentone inhibited CA release from cultured chromaffin cells stimulated by carbachol. Thiopentone at 50 μM exhibited 44% inhibition, which is similar to the values of IC50 for inhibiting the nicotinic receptor-mediated response of the dog perfused adrenal medulla (Sumikawa et al

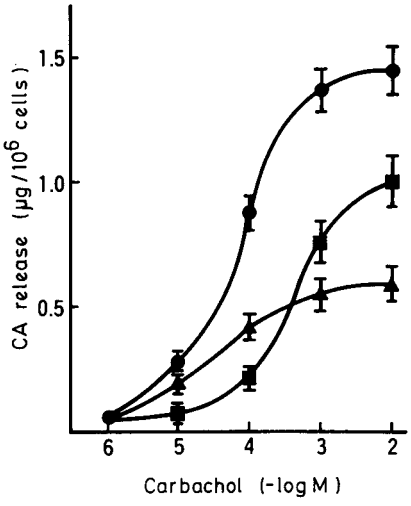

FIG. 3. Effect of varying the carbachol concentration on the inhibition by thiopentone and alcuronium of carbachol-induced CA release (mean ± s.e., n = 4 for each point). The cells were incubated in Krebs-Ringer phosphate buffer at 37 °C for 1 min with various concentrations of carbachol in the absence (control, ●) and presence of (▲) thiopentone, 100 μM, and (■) alcuronium, 1 μM.

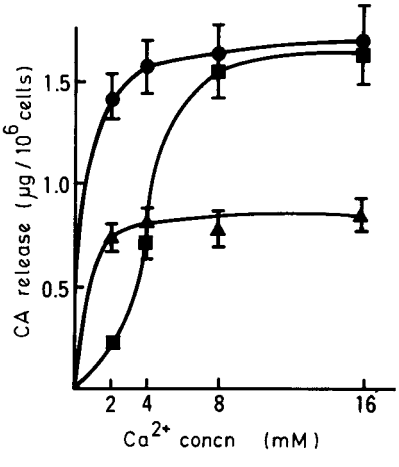

FIG. 4. Effect of varying the Ca^{2+} concentration on the inhibition by thiopentone and diltiazem of carbachol-induced CA release (mean ± s.e., n = 4 for each point). The cells were incubated in Krebs-Ringer phosphate buffer at 37 °C for 1 min with 0·3 mM carbachol and various concentrations of Ca^{2+} in the absence (control, ●) and presence of (▲) thiopentone, 100 μM, and (■) diltiazem, 3 μM.

1983) and does not deviate from the clinical concentration (Becker 1978). This effect of thiopentone was reversible, since the chromaffin cells regained their ability to release CA after washing out the barbiturate. Contrary to the dog adrenal medulla, in which both nicotinic and muscarinic receptors play a role in inducing CA release (Tsujimoto & Nishikawa 1975), acetylcholine (ACh) receptors in the bovine adrenal medulla are entirely nicotinic (Sumikawa et al 1979). Accordingly, in this experiment, carbachol induced CA release as a result of a Ca^{2+} influx through nicotinic receptor-linked Ca^{2+} channels (Sumikawa et al 1983). The mechanism involved in the inhibition of carbachol-induced CA release by thiopentone would be diminution of the Ca^{2+} influx into the cells, because parallelism was obvious between the diminution in Ca^{2+} influx and inhibition of CA release; there was also a close relation between the amount of Ca^{2+} taken up and the amount of CA released.

It has been shown that both ACh binding to nicotinic receptors and high K^+ medium produce a Ca^{2+} influx from the extracellular space into the adrenal chromaffin cells, thereby increasing the amount of Ca^{2+} free in the cytoplasm and causing CA secretion by exocytosis (Holz et al 1982). The present results show that, as with the effects of halothane (Sumikawa et al 1982), the process of exocytosis would not be susceptible to this concentration of thiopentone, because high K^+-induced CA release, in which the common process of exocytosis would be involved, was not inhibited by the barbiturate, and furthermore, the inhibition of carbachol-induced CA release could be explained by the inhibition of the Ca^{2+} uptake.

A specific interaction between barbiturates and ACh receptors has been demonstrated, i.e. there might be a specific barbiturate binding site on the ACh receptor (Miller et al 1982). This barbiturate binding site could decrease ACh binding to the receptor (Dodson & Miller 1983). In the present experiment, we examined the interaction between thiopentone and carbachol. The results show that the inhibition of CA release by thiopentone was not overcome by an increase in the carbachol concentration, whereas that by alcuronium was to a considerable extent. Thus, it seems that the mode of action would not be competitive inhibition of carbachol binding to the nicotinic receptor. The possibility that the barbiturate binding site on the nicotinic receptor might allosterically regulate carbachol binding in a non-competitive manner is not denied by the present results.

The effect of thiopentone on Ca^{2+} influx was compared with a Ca^{2+} antagonist, diltiazem. The inhibitory effect of diltiazem on the Ca^{2+} influx was completely reversed by an increase in the Ca^{2+} concentration, whereas that of thiopentone was not. This result suggests that the mechanism of inhibition of CA release by thiopentone is different from that

by diltiazem, i.e. the Ca^{2+} channel itself would not be the target of this barbiturate. It is likely that the linkage between ACh binding to the nicotinic receptor and activation of the receptor-linked Ca^{2+} channel might be the susceptible process in stimulus-secretion coupling in the chromaffin cells.

The inhibitory effects of barbiturates on Ca^{2+} influx into neuronal cells have been demonstrated using synaptosomes from the rat brain (Blaustein & Ector 1975), leech Retzius cells (Kleinhaus & Prichard 1977), and mouse spinal cord neurons (Heyer & Macdonald 1982). All of these previous reports have examined the effects of barbiturates on Ca^{2+} channels in presynaptic nerve terminals, which would be gated by depolarization upon conduction of an impulse and would, therefore, be voltage-dependent. In the present experiment, we examined the effect of thiopentone on both receptor-linked and voltage-dependent Ca^{2+} channels in the postsynaptic membrane (Holz et al 1982). The concentration required to block the voltage-dependent Ca^{2+} channel by 50% is more than 200 μM, which is almost compatible with the previous reports, whereas 50% inhibition of receptor-linked Ca^{2+} channels was achieved at about 50 μM, indicating that receptor-linked Ca^{2+} channels would be more susceptible to the barbiturate than voltage-dependent channels. During clinical anaesthesia with thiopentone, the free fraction of this anaesthetic in the plasma was reported to be about 24 μM (Becker 1978). Accordingly, it is likely that nicotinic receptor-linked Ca^{2+} channels would be selectively inhibited in a clinical situation, whereas voltage-dependent Ca^{2+} channels might be inhibited only when a high dose of thiopentone is administered.

In addition to the cholinergic synapses, barbiturates would affect other synapses. It has been demonstrated that barbiturates increase γ-aminobutyric acid (GABA)-mediated inhibition (Barker & Ransom 1980) and reduce glutamate-mediated excitation (Richards & Smaje 1976) in the central nervous system. These effects seem to form the basis for barbiturate anticonvulsant action (Schulz & Macdonald 1981). At present, there is no agreement about the pathways in the brain that are responsible for the maintenance of consciousness (Richards 1978).

In conclusion, thiopentone inhibits the stimulus-secretion coupling in cholinergic postsynaptic cells of the adrenal medulla. A contributing mechanism would be inhibition of Ca^{2+} uptake through nicotinic receptor-linked Ca^{2+} channels. The linkage between the receptor and Ca^{2+} channels seems to be the process most susceptible to barbiturate inhibition.

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