Etomidate elevates intracellular calcium levels and promotes catecholamine secretion in bovine chromaffin cells

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Etomidate, an intravenous imidazole general anaesthetic, is thought to produce anaesthesia by modulating or activating ionotropic Cl^--permeable $GABA_A$ receptors. Chromaffin cells are known to express functional $GABA_A$ receptors with properties similar to their neuronal counterparts. We have shown that activation of the $GABA_A$ receptors, with specific $GABA_A$ agonists, leads to cellular excitation. Our goal was to determine whether etomidate mimicked this response and to explore the functional consequences of this activation. Imaging experiments with the Ca^{2+}-indicator dye fura-2 were used to assay $[Ca^{2+}]_i$. Bovine adrenal chromaffin cells were superfused with a variety of $GABA_A$-selective drugs to determine their effects on $[Ca^{2+}]_i$. Amperometric measurements were used to assay catecholamine release in real-time. We show that bovine adrenal chromaffin cells were excited by etomidate at clinically relevant concentrations. Etomidate directly activated $GABA_A$ receptors found in chromaffin cells thereby elevating $[Ca^{2+}]_i$. The effects of etomidate were mimicked by the specific $GABA_A$ agonist muscimol and blocked by the specific antagonist bicuculline. Our data show that low concentrations of etomidate modulated $GABA_A$ receptor activation by muscimol. Blockade of voltage-dependent Ca^{2+} channels prevented the elevation of $[Ca^{2+}]_i$ by GABA. Application of etomidate directly to the chromaffin cells elicited robust catecholamine secretion from these cells. The data indicate that clinically relevant concentrations of etomidate can directly activate $GABA_A$ receptors, which, due to the positive anion equilibrium potential, depolarizes chromaffin cells. This depolarization activates voltage-dependent Ca^{2+} channels thereby stimulating catecholamine release. Our data suggest that circulating catecholamine levels may be elevated after etomidate application.

(Resubmitted 25 June 2004; accepted after revision 23 August 2004; first published online 26 August 2004)

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Historically, anaesthetics were thought to non-specifically modulate membrane lipid fluidity, thereby exerting their anaesthetic actions. More recently this hypothesis has fallen out of favour. Anaesthetics are thought to act by directly interacting with membrane protein targets, generally thought to be neurotransmitter receptors and perhaps ion channels (Franks & Lieb, 1994; Krasowski & Harrison, 1999; Campagna *et al.* 2003). Anaesthetics are known to modulate a variety of targets including gamma-aminobutyric acid type-A ($GABA_A$), glycine, neuronal nicotinic acetylcholine, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and serotonin type-3 ($5-HT_3$) receptors (Krasowski &

Harrison, 1999; Campagna *et al.* 2003). Voltage-dependent channels may be sensitive to anaesthetics but at concentrations higher than used clinically (Franks & Lieb, 1994; Krasowski & Harrison, 1999; Campagna *et al.* 2003). Etomidate is a widely-used intravenous general anaesthetic that is particularly useful in patients who have myocardial dysfunction because it produces minimal haemodynamic change in these patients (Angelini *et al.* 2001; Rothermel, 2003). Etomidate is thought to act on $GABA_A$ receptors at clinically relevant concentrations (Belelli *et al.* 2003; Reynolds *et al.* 2003). At these same concentrations etomidate has no effect on glycine, AMPA, NMDA, $5-HT_3$ or nicotinic receptors (Krasowski & Harrison, 1999; Belelli *et al.* 2003; Reynolds *et al.* 2003)

but there are effects at higher concentrations (Belelli *et al.* 2003). It is interesting that etomidate is thought to potentiate GABA-mediated transmission at clinically relevant concentrations (in the range from 1 to 8 μM; Giese & Stanley, 1983; Yang & Uchida, 1996; Belelli *et al.* 1999) but at higher concentrations is thought to directly activate GABA$_A$ receptors (Belelli *et al.* 1997; Moody *et al.* 1997). Other drugs that also potentiate GABAergic neurotransmission, such as diazepam or phenobarbital, are not efficacious anaesthetics (Schulz & Macdonald, 1981; Sigel *et al.* 1990; Moody *et al.* 1997). Thus, it seems likely that either etomidate acts on other targets, or that it targets different kinds of GABA$_A$ receptors than do diazepam or phenobarbital. Another possibility is that direct activation of GABA$_A$ receptors may mediate part of the anaesthetic response to etomidate (Schulz & Macdonald, 1981; Sigel *et al.* 1990; Belelli *et al.* 1997; Moody *et al.* 1997).

GABA is the primary inhibitory neurotransmitter in the adult brain, with ionotropic Cl^--permeable GABA$_A$ receptors mediating the majority of fast inhibitory synaptic transmission. It has been estimated that approximately one-third of all synapses in the brain are GABAergic (Krasowski & Harrison, 1999). In neonatal animals activation of Cl^--permeable GABA receptors is excitatory and appears to depend on the expression of a $Na^+-K^+-2Cl^-$ cotransporter (NKCC) which driven by Na^+ and K^+ gradients, acts to accumulate intracellular Cl^- (Ben-Ari, 2002), leading to a depolarized Cl^- equilibrium potential (E_{Cl}) (Alvarez-Leefmans *et al.* 1988; Rohrbough & Spitzer, 1996; Sun & Murali, 1999). The change from excitation to inhibition appears to involve the expression of the K^+-Cl^- cotransporter, KCC2 (Payne *et al.* 1996), which lowers intracellular Cl^- levels resulting in a hyperpolarized E_{Cl} (Rivera *et al.* 1999; Ben-Ari, 2002; Owens & Kriegstein, 2002). We previously showed that bovine chromaffin cells from juvenile animals (4–5 months old) are excited by GABA. Activation of GABA$_A$ receptors depolarizes the cells, opens voltage-dependent Ca^{2+} channels, elevates $[Ca^{2+}]_i$ and promotes the release of catecholamines (Xie *et al.* 2003). The extrapolated anion reversal potential in these cells was ~-28 mV indicating a resting intracellular anion concentration of ~50 mM. Expression of KCC2 protein was not detected in the juvenile chromaffin cells. In contrast, clear expression of NKCC1 was observed, which explains the presence of the depolarized anion potential. Depolarizing GABA$_A$ actions have also been observed in some adult neurones (Alvarez-Leefmans *et al.* 1988; Staley *et al.* 1996).

In this manuscript we explore the chromaffin cell response to etomidate. Exposing these cells to etomidate caused a large and rapid elevation in $[Ca^{2+}]_i$, which required Ca^{2+} influx from the extracellular space. The elevation of $[Ca^{2+}]_i$ required the activation of GABA$_A$

receptors; the action was mimicked by the selective GABA$_A$ agonist muscimol and blocked by the selective GABA$_A$ antagonist bicuculline. Exposing chromaffin cells to etomidate elicited significant catecholamine secretion. Although we show that etomidate can potentiate GABA responses, the responses outlined in this manuscript resulted from etomidate directly activating the GABA$_A$ receptors and occurred at clinically relevant concentrations. Responses to etomidate were observed at concentrations as low as 1 μM and the EC$_{50}$ was 5.8 μM. The results suggest that some GABA$_A$ receptors can be directly activated at low concentrations of etomidate and that these effects may mediate some of the anaesthetic responses to the drug. Etomidate is used in surgeries where patients have cardiovascular problems, as it has beneficial haemodynamic properties relative to other anaesthetics. Our data suggest that circulating catecholamine levels may be elevated after etomidate application which may help explain the beneficial haemodynamic properties of this anaesthetic.

Methods

Cell culture

Juvenile bovine adrenal glands, from animals 4–5 months old, were obtained from a local slaughterhouse. Glands were harvested immediately following death. Chromaffin cells were prepared by digestion with collagenase followed by density gradient centrifugation as previously described (Xie *et al.* 2003). The cells were plated onto collagen-coated coverslips (at a density of 0.3–0.4×10^6 cells ml^{-1} for $[Ca^{2+}]_i$ measurements) and maintained in an incubator at 37°C in an atmosphere of 93% air and 7% CO_2 with a relative humidity of 90%. Fibroblasts were effectively suppressed with cytosine-arabinoside (10 μM), leaving relatively pure chromaffin cell cultures. Half of the culture medium was exchanged every day. This medium consisted of Dulbecco's modified Eagle's medium (DMEM)\F12 (1 : 1) (Gibco) supplemented with fetal bovine serum (10%), glutamine (2 mM), penicillin/streptomycin (100 units ml^{-1}/100 μg ml^{-1}), cytosine arabinoside (10 μM) and 5-fluorodeoxyuridine (10 μM).

$[Ca^{2+}]_i$ measurements

Experiments were conducted 1–5 days after chromaffin cells were prepared. Cells were incubated in Hanks' balanced salt solution (HBSS) with 2 μM acetoxymethyl ester form of fura-2 (fura-2 AM) and 1 mg ml^{-1} albumin for 45 min and then washed in a fura-2-free solution for 1 h. Fura-2 loading and wash was carried out at 37°C. The coverslip was transferred to an experimental chamber for recording. Backgrounds at 340 nm and 380 nm were

obtained using an area of the coverslip devoid of cells. Data were continuously collected throughout the experiment using a Coho series 4920 cooled CCD camera. On each coverslip, 10–40 chromaffin cells were selected and individually imaged. Image pairs (one at 340 nm and 380 nm) were obtained every 2 s by averaging 16 frames at each wavelength. Backgrounds were subtracted from the individual wavelengths, and the 340 nm image was divided by the 380 nm image to provide a ratiometric image. Ratios were converted to free $[Ca^{2+}]_i$ by comparing data to fura-2 calibration curves made *in vitro* by adding fura-2 (50 μM free acid) to solutions that contained known concentrations of calcium (0 to 2000 nM). The recording chamber was continuously perfused with fresh solution from gravity-fed reservoirs. All drugs were applied via this perfusion system.

Amperometric measurement of catecholamine release from populations of cells

The recording bath was designed so that it was small (< 1 cm in diameter) and the volume of the bath was kept at around 300–350 μl. Carbon fibre electrodes (Dagan Instruments) were backfilled with 3 M KCl and held at a potential of +700 mV using a modified Warner PC-501 patch clamp amplifier. Electrodes were positioned within the bathing medium. In this configuration the electrode detected increases in the local catecholamine concentration due to release from neighbouring cells. Amperometric data from the patch clamp was recorded continuously using AxoTape (an 8-pole Bessel filter was set at 300–500 Hz and acquired at 1 kHz). The cells were washed with HBSS for several minutes prior to application of etomidate or high K^+-containing solutions. Under control conditions (HBSS), stopping the flow of the solution in the recording bath for 90–120 s did not produce any change in amperometric current, indicating that the cells were not releasing appreciable amounts of catecholamine under basal conditions. Cells were stimulated by stopping the flow of the bath solution for 15–30 s and then directly applying a 50 μl bolus, using a pipette, of one of the following: HBSS (negative control), high K^+ (50 mM) (positive control) or etomidate (100 μM; final bath concentration of ~20 μM). After 30–60 s the flow of HBSS through the bath was resumed to wash away the drug and any released catecholamine.

Amperometric measurement of catecholamine release from individual cells

Carbon fibre electrodes were fabricated with 7-μm diameter carbon fibres (Fortafil Fibres Inc., Knoxville, TN, USA). The carbon fibre was threaded through a glass capillary pipette (Drummond Scientific, Broomall, PA, USA), and pulled with a vertical glass pipette puller (Narashige, Tokyo, Japan). An epoxy seal between

the carbon fibre and glass was created by dipping the glass–fibre tip into a 14% hardener/resin mixture (w/w), pre-heated to ~100°C (hardener, meta-phenylene diamene; resin, 828 Epon Resin; Miller-Stephenson Chemical Co., Morton Grove, IL, USA). Electrodes were baked overnight at 66°C. The glass portion of the tip was painted with Sylgard (Dow Corning, Midland, MI, USA) and then baked at 66°C for at least 1 h. On the day of the experiment the carbon fibre was cut with a scalpel blade. The electrode was backfilled with 3 M KCl. The electrode was held at +700 mV using an EPC-8 amplifier (HEKA Electronics, Lambrecht, Germany) to oxidize catecholamine transmitter. The amperometric signal was low-pass filtered at 1 kHz (8-pole Bessel; Warner Instruments, Hamden, CT, USA). A 16-bit analog-to-digital converter (National Instruments Corp., Austin, TX, USA) was interfaced with in-house written data acquisition software. The amperometric signal was acquired at 5 kHz and stored on a personal computer. Data were analysed with a program kindly supplied by Eugene Mosharov (Columbia University). This program identified amperometric spikes and then integrated the area under each event to provide quantal size. The detection threshold for each event was set at five times the baseline root mean square (RMS) of the noise; spikes were automatically detected. For these experiments, the recording chamber was continuously perfused with fresh solution from gravity-fed reservoirs. All drugs were applied via this perfusion system.

Solutions

Experiments were carried out in Hanks' balanced salt solution (HBSS; Gibco) which contained (mM): NaCl 138, KCl 5, $CaCl_2$ 1.3, KH_2PO_4 0.3, $MgSO_4$ 0.8, Na_2HPO_4 0.3, D-glucose 5.6 and Hepes 20. Nominally Ca^{2+}-free HBSS was prepared by treating Ca^{2+}-free, Mg^{2+}-free, HCO_3-free HBSS with Chelex-100, then adding $MgCl_2$ to a final concentration of 1 mM. The etomidate solution contained polyethylene glycol (PG; 35% v/v). PG was tested to determine whether it could elicit a response. Muscimol hydrobromide (Calbiochem) was prepared as a 25 mM stock in H_2O. Muscimol was diluted to a final concentration with HBSS. (−)-Bicuculline methiodide (Sigma) was prepared as a 100 mM stock in H_2O and then diluted to a final concentration of 100 μM with HBSS. Lanthanum chloride (Sigma) was prepared as a 100 mM stock in H_2O and then diluted to a final concentration of 1 mM with HBSS. The solution containing sodium thiocyanate (Sigma) contained (mM): NaSCN 140, $CaCl_2$ 1.3, KCl 5, $MgCl_2$ 1, glucose 5.6 and Hepes 20; pH 7.35. For the amperometry experiments the bath contained HBSS. All experiments were carried out at room temperature (~23°C).

Results

Intracellular calcium measurements: etomidate elevated intracellular calcium

Chromaffin cells were tested after 1–5 days in primary culture. A coverslip that contained cells was transferred
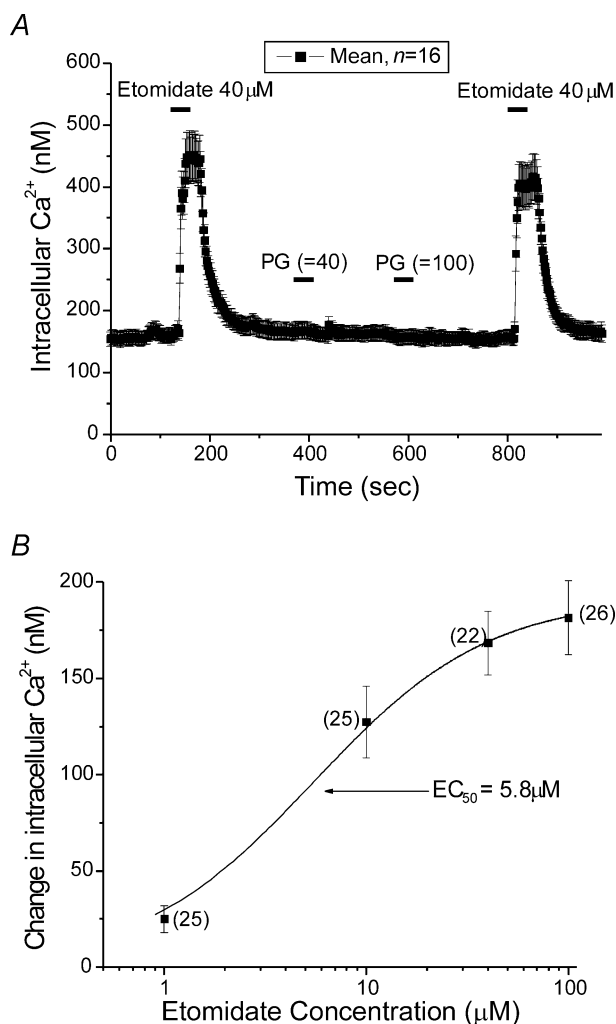

Figure 1. Etomidate elevates $[Ca^{2+}]_i$ in chromaffin cells
A, mean $[Ca^{2+}]_i$ in 16 individual chromaffin cells was determined every 2 s throughout the experiment (see Methods for details). Note that each time point is mean ± S.E.M. Etomidate (40 μM) was applied in a Ca^{2+}-containing HBSS solution during the time indicated by the bars. The data are from a single representative experiment. The etomidate solution contained PG (35% v/v). We tested PG by itself twice; first at the concentration found in the 40 μM etomidate solution (24 mM PG) and then at the concentration found in 100 μM etomidate solution (60 mM PG). Both applications of PG were without effect. *B*, etomidate dose–response relation for $[Ca^{2+}]_i$ elevation. The mean elevation of $[Ca^{2+}]_i$ is plotted as a function of $\log_{10}$ of the concentration of etomidate that elicited the elevation. Data are from cells in Ca^{2+}-based HBSS solution. The number of cells is indicated next to each data point along with standard error bars. The data was fitted by a curve defined by the function $Y = Y_{max} \times 1/1 + (EC_{50}/X)$ in which Y is the $[Ca^{2+}]_i$ elevation and X is the etomidate concentration.

to an experimental chamber for each experiment. The microscope was adjusted until a field of view was obtained that had dozens of chromaffin cells visible. $[Ca^{2+}]_i$ measurements from 10 to 40 cells per coverslip were collected every 2 s. Figure 1 shows representative data from such an experiment where $[Ca^{2+}]_i$, assayed with fura-2, was averaged from 16 cells and then plotted. At the bar labelled Etomidate, 40 μM etomidate was perfused into the bath, producing an elevation in $[Ca^{2+}]_i$ of ~300 nM. The etomidate was in a solution that was 35% PG (labelled PG in the figure). Following application of the drug, PEG was applied twice. At the first bar labelled PG, 24 mM PG, a concentration identical to the 40 μM etomidate solution, was applied without effect. At the second bar PG was applied at 60 mM, a concentration similar to that in a 100 μM etomidate solution, again without effect. Etomidate (40 μM) was re-applied at the end of the experiments and produced virtually the same response observed during the first application. The average change in $[Ca^{2+}]_i$ elicited by application of 40 μM etomidate was 220.9 ± 19.3 nM (S.E.M., $n = 40$), while in the same group of cells, application of 60 mM PG was without effect. These data suggest that etomidate elevates $[Ca^{2+}]_i$ and that the carrier, propylene glycol has no effect on resting $[Ca^{2+}]_i$.

Figure 1*B* provides a more detailed analysis of the response of chromaffin cell to etomidate. The average change in $[Ca^{2+}]_i$ observed in response to different etomidate concentrations is plotted. Responses were observed at concentrations as low as 1 μM etomidate, which produced an $[Ca^{2+}]_i$ elevation of 25 ± 7 nM ($n = 25$). Responses at 100 μM were not significantly different from those observed at 40 μM. The data were well fitted with a standard dose–response equation. The EC_{50} provided by the fitting function (see legend) was 5.8 μM.

Multiple responses to higher concentrations of etomidate (> 40 μM) were less reproducible than to lower concentrations (< 40 μM), suggesting desensitization to higher concentrations of the anaesthetic. Figure 2 shows responses from 25 cells to two applications of etomidate (100 μM). The response to the second application was significantly smaller than the first. Application of high concentrations of etomidate always elicited smaller subsequent responses, while application of lower concentrations typically allowed for multiple reproducible responses (see Fig. 1*A*).

Extracellular calcium was required to observe the response to etomidate

Figure 3*A* shows the response of 23 cells to either 10 μM or 40 μM etomidate, in the presence or absence of extracellular Ca^{2+}. Etomidate (10 μM) was perfused into the bath (as indicated), producing a large elevation in $[Ca^{2+}]_i$. After allowing the cell to recover, 40 μM etomidate

was perfused into the bath, producing an even larger change in $[Ca^{2+}]_i$. After recovery, cells were perfused with a Ca^{2+}-free solution and then etomidate was re-applied at $10\,\mu M$ and $40\,\mu M$, without any changes in $[Ca^{2+}]_i$. Subsequently, Ca^{2+} was re-introduced into the bath and then etomidate was re-applied again at $10\,\mu M$ and $40\,\mu M$, this time producing responses not significantly different from the original responses.

Figure 3B shows the average response of chromaffin cells to etomidate. In this experiment etomidate $(40\,\mu M)$ produced a $[Ca^{2+}]_i$ elevation of 152 ± 13.5 nM when applied in the presence of extracellular Ca^{2+} but produced virtually no change of $[Ca^{2+}]_i$ in extracellular Ca^{2+}-free conditions. These data suggest that entry of Ca^{2+} into the chromaffin cells from the extracellular space is required for the response to etomidate. While it is possible that release of Ca^{2+} from intracellular stores plays a role in the responses observed here, perhaps via Ca^{2+}-dependent Ca^{2+} release, such release does not appear to initiate the measured response.

Etomidate elevates $[Ca^{2+}]_i$ via activation of $GABA_A$ receptors

Etomidate is thought to selectively activate $GABA_A$ receptors. In an earlier study we showed that activation of $GABA_A$ receptors produced an increase in $[Ca^{2+}]_i$ (Xie *et al.* 2003). To verify the involvement of $GABA_A$ receptors in the etomidate response, the specific $GABA_A$ receptor antagonist bicuculline was applied to chromaffin cells. Both etomidate $(40\,\mu M)$ and muscimol $(5\,\mu M)$ produced large elevations in $[Ca^{2+}]_i$ when applied to a group of

chromaffin cells (Fig. 4A). In the presence of bicuculline, the elevation of $[Ca^{2+}]_i$ observed with either etomidate or muscimol was almost completely blocked (Fig. 4A). Partial recovery of the response to both etomidate and muscimol was observed upon washout of the bicuculline. Etomidate $(40\,\mu M)$ produced an average elevation in $[Ca^{2+}]_i$ of 284 ± 33.1 nM $(n = 67)$ when applied in the absence of bicuculline but this increase was almost completely blocked, to 29.2 ± 3.5 nM, by bicuculline (Fig. 4B). The data demonstrate that etomidate and muscimol operate via the same mechanism, activation of $GABA_A$ receptors.

Figure 5 shows the supra-additive effects of applying muscimol, a selective $GABA_A$ agonist, and etomidate simultaneously (both drugs were used at non-saturating
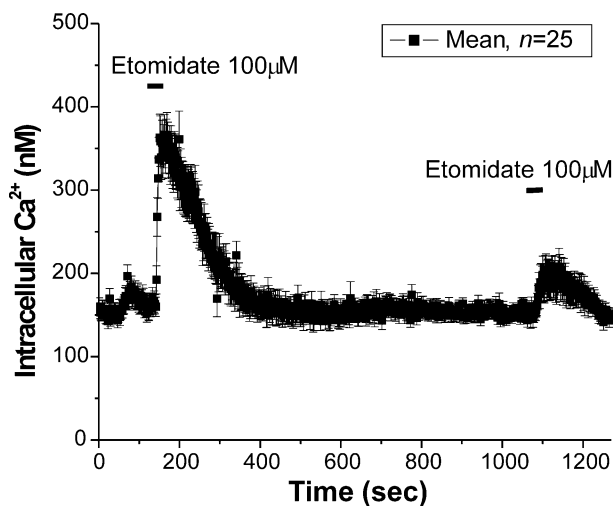
Figure 3. The elevation of $[Ca^{2+}]_i$ produced by etomidate required Ca^{2+} influx into the cells
A, mean $[Ca^{2+}]_i$ in 23 individual chromaffin cells. Etomidate was applied at both $10\,\mu M$ and $40\,\mu M$ in Ca^{2+}-containing HBSS or in a Ca^{2+}-free HBSS solution during the time indicated by the bars. *B*, the mean increase in $[Ca^{2+}]_i$ in 47 cells treated with $40\,\mu M$ etomidate was 152.4 ± 13.5 nM in the Ca^{2+}-containing HBSS, while in the Ca^{2+}-free HBSS the increase was only 12.4 ± 2 nM.

Figure 2. High doses of etomidate produce desensitizing responses
Mean $[Ca^{2+}]_i$ in 25 individual chromaffin cells. The cells were exposed to etomidate $(100\,\mu M)$, during the times indicated. Note that the second response was significantly smaller than the first. Run-down in the response to multiple exposures of etomidate was observed at concentrations greater than $40\,\mu M$.
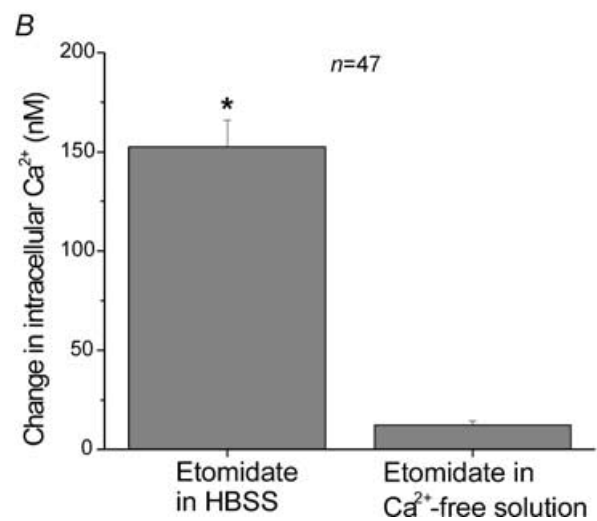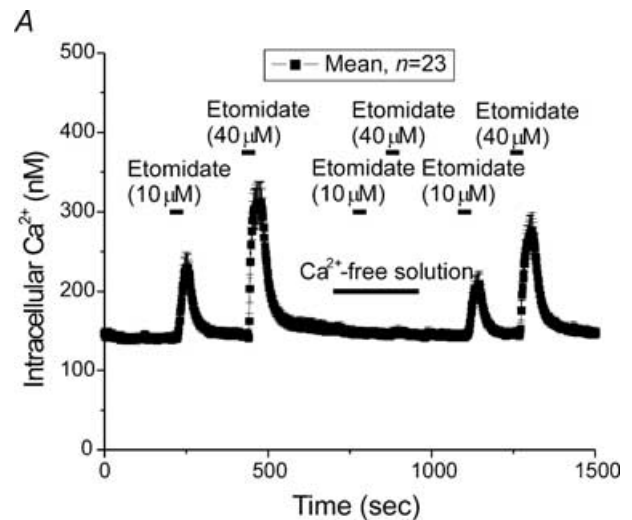
concentrations). At the first bar in Fig. 5, 4 μM etomidate was introduced into the bath. This concentration of etomidate produced a small elevation in $[Ca^{2+}]_i$. Then 1 μM muscimol was introduced into the bath. This concentration of muscimol produced a modest elevation in $[Ca^{2+}]_i$. When both drugs were applied simultaneously, a large increase in $[Ca^{2+}]_i$ was observed suggesting that the drugs were supra-additive, under these conditions. Thus, low concentrations of etomidate can potentiate responses to GABA (or in this case muscimol), as has been described in neurones (Yang & Uchida, 1996; Reynolds *et al.* 2003), in addition to the direct effects on the GABA$_A$ receptors shown above.
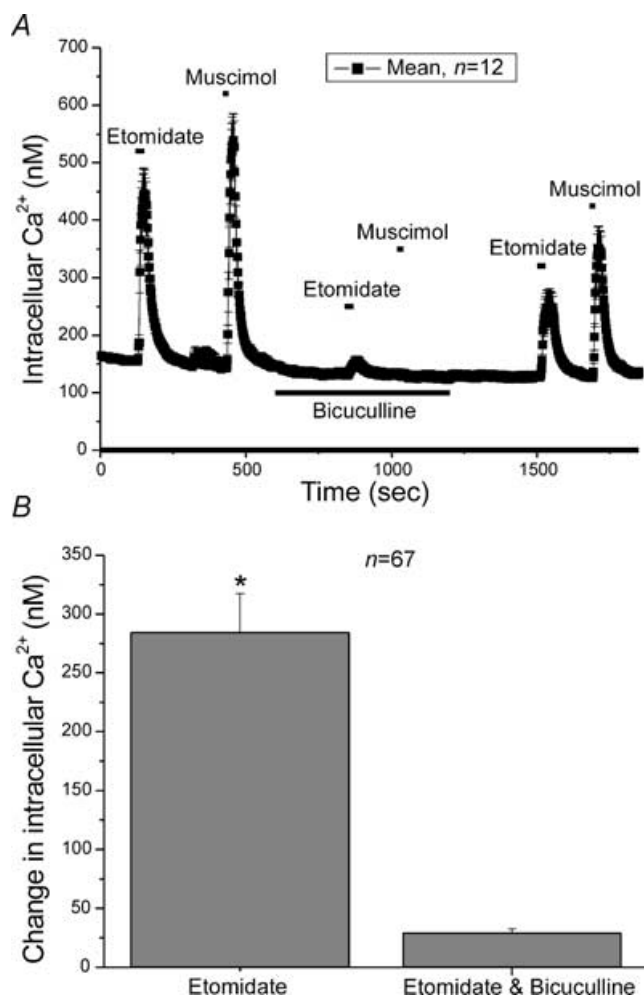
Voltage-dependent Ca^{2+} channels probably mediate Ca^{2+} influx into chromaffin cells

We wanted to verify that etomidate operated through the activation of voltage-dependent Ca^{2+} channels. Another way to activate voltage-dependent Ca^{2+} channels is to depolarize cells; high K$^+$-based solutions depolarize cells and activate voltage-dependent Ca^{2+} channels. In turn, this elevates $[Ca^{2+}]_i$, and elicits robust catecholamine secretion. Ca^{2+} channel blockers such as La^{3+}, block the
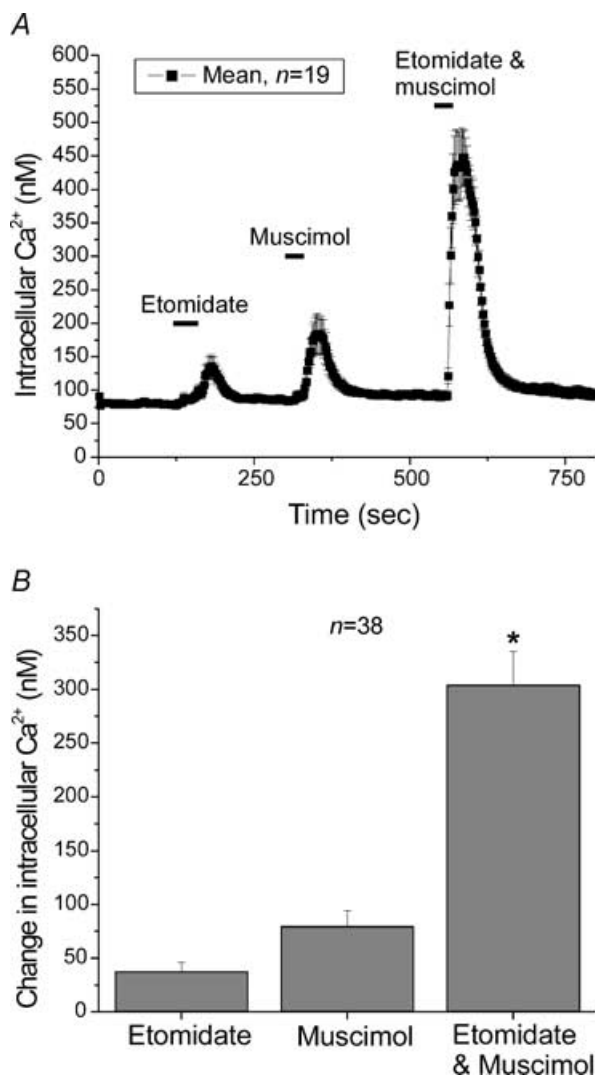

Figure 4. The GABA$_A$ antagonist bicuculline blocked the effects of etomidate on $[Ca^{2+}]_i$

A, mean $[Ca^{2+}]_i$ in 12 individual chromaffin cells was determined every 2 s. The cells were exposed to either etomidate (40 μM) or muscimol (5 μM) during the times indicated. Both drugs were first added to the bath in the absence of bicuculline and then re-applied in the presence of 100 μM bicuculline. The bicuculline was washed out of the bath and etomidate and muscimol were re-applied. *B*, the mean increase in $[Ca^{2+}]_i$ in response to application of 40 μM etomidate in either the presence or absence of bicuculline. Etomidate elevated $[Ca^{2+}]_i$ by 284 ± 33.2 nM (*n* = 67), when applied in the absence of bicuculline. This increase was only 29.2 ± 3.5 nM in the presence of bicuculline.

Figure 5. Submaximal concentrations of etomidate and muscimol produced elevations of $[Ca^{2+}]_i$ that were supra-additive

A, mean $[Ca^{2+}]_i$ in 19 individual chromaffin cells was determined every 2 s. The cells were exposed to either etomidate (4 μM) or muscimol (1 μM) during the times indicated. After allowing the cells to recover, etomidate and muscimol were applied simultaneously. *B*, the mean increase in $[Ca^{2+}]_i$ in response to application of 4 μM etomidate, 1 μM muscimol or the combination of 4 μM etomidate and 1 μM muscimol. Etomidate by itself elevated $[Ca^{2+}]_i$ by 37.6 ± 8.4 nM (*n* = 38), muscimol by itself elevated $[Ca^{2+}]_i$ by 79.6 ± 14.6 nM (*n* = 38) while etomidate and muscimol applied together elevated $[Ca^{2+}]_i$ by 303.7 ± 31.5 (*n* = 38).

Ca^{2+} influx produced by high-K^+ stimulation. Would La^{3+} also block the response to etomidate? La^{3+} (1 mM) blocked the Ca^{2+} influx elicited by either high-K^+ (50 mM K^+) or etomidate (40 μM). In Fig. 6, the bar at the bottom shows when La^{3+} was introduced into the bath. In the presence of La^{3+} neither etomidate nor high K^+-containing solutions elicited significant changes in $[Ca^{2+}]_i$. Washing La^{3+} from the bath and then re-applying both etomidate and high K^+-containing solution, subsequently produced large changes in $[Ca^{2+}]_i$. In a previous study we showed that Cd^{2+} blocked the Ca^{2+} influx elicited by the activation of $GABA_A$ receptors (Xie *et al.* 2003).

Figure 6*B* summarizes average data from 42 cells showing that La^{3+} completely blocks the $[Ca^{2+}]_i$ elevation observed with etomidate. These results suggest that etomidate activates voltage-dependent Ca^{2+} channels, thereby producing the Ca^{2+} influx which elevates $[Ca^{2+}]_i$.

Nicotinic receptors are not involved in the elevated $[Ca^{2+}]_i$ response to etomidate

In chromaffin cells, ACh release from pre-synaptic splanchnic neurones activates neuronal nicotinic receptors (nAChRs) which elicit action potentials in chromaffin cells. Ca^{2+} influx into chromaffin cells is mediated by both voltage-dependent Ca^{2+} channels as well as nicotinic receptors. Recent experiments suggest that transient receptor potential (TRP) channels may also allow Ca^{2+} into cells.

Although etomidate is not thought to act on nAChRs at physiologically relevant concentrations, only a few subunit combinations have been tested (Belelli *et al.* 2003; Reynolds *et al.* 2003). Thus we decided to test the involvement of these receptors in the elevated $[Ca^{2+}]_i$ response to etomidate. Figure 7*A* shows $[Ca^{2+}]_i$ averaged from 35 cells. In the presence of a cocktail of nicotinic antagonists (methyllycaconitine, 1 μM; mecamylamine, 20 μM; dihydro-β-erythroidine, 20 μM; all from Research Biochemicals International, Natick, MA, USA), the response to nicotine (100 μM) was effectively blocked, but etomidate (40 μM) still produced a large elevation in $[Ca^{2+}]_i$ not different from that observed in control conditions. Figure 7*B* summarizes data averaged from 70 cells showing that the nicotinic antagonists effectively blocked the response to nicotine (231.3 $\pm$ 17 nM, in absence of antagonists; 26.9 $\pm$ 6.5 nM, in the presence of antagonists; $n = 70$ for both), but had little effect on responses to etomidate (153.8 $\pm$ 15.5, $n = 35$ in absence of antagonists; 175.1 $\pm$ 17.2, $n = 70$, in the presence of antagonists). Thus, our data suggest that the elevation of $[Ca^{2+}]_i$ observed upon application of etomidate does not involve the activation of nicotinic receptors.

Evidence of a depolarized anion equilibrium potential in chromaffin cells

In order for GABA to depolarize a cell sufficiently to activate voltage-dependent Ca^{2+} channels, the anion equilibrium potential must be quite depolarized. To test
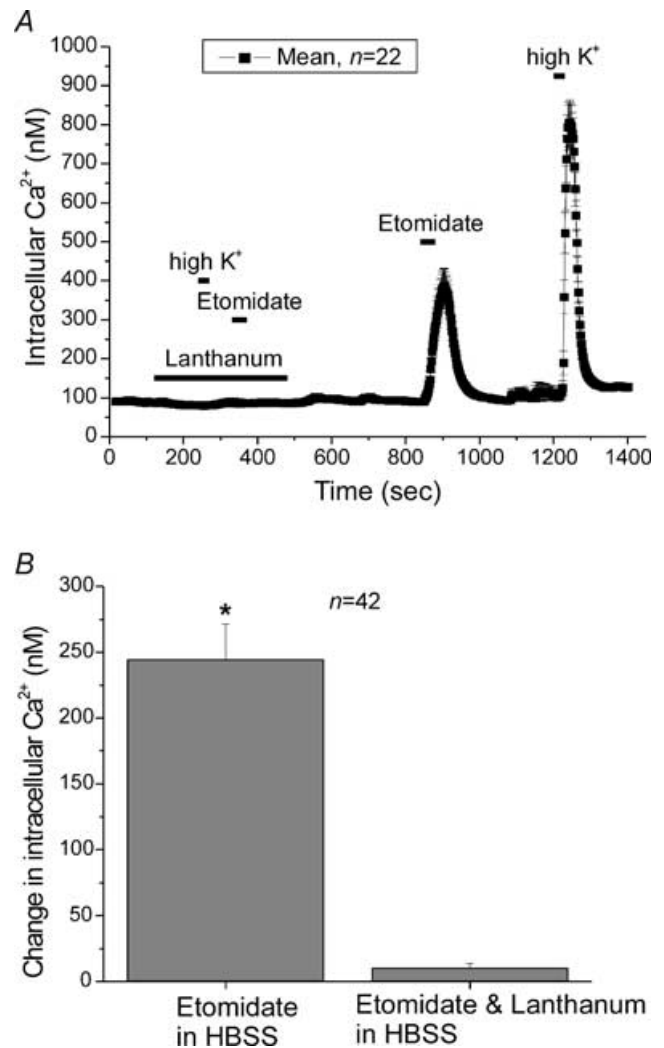

Figure 6. The Ca^{2+} influx observed after activation of $GABA_A$ receptors by etomidate involves voltage-dependent Ca^{2+} channels

A, mean $[Ca^{2+}]_i$ in 22 individual chromaffin cells was determined every 2 s. La^{3+} (1 mM), a non-selective Ca^{2+} channel blocker, was superfused onto the cells during the time indicated by the bar. In the presence of La^{3+} neither depolarization of chromaffin cells with a high K^+-containing solution (see Methods) nor the application of 40 μM etomidate elevated $[Ca^{2+}]_i$. After washing La^{3+} out of the bath, increases in $[Ca^{2+}]_i$ were observed in response to both etomidate and the high K^+-containing solution. Note the high K^+-containing solution contained 2 mM Ca^{2+}, not the 1.3 mM Ca^{2+} found in the usual HBSS. The data are from a single representative experiment. *B*, the mean increase in $[Ca^{2+}]_i$ in 42 cells treated with etomidate in the absence of La^{3+} was 244.2 $\pm$ 27 nM, while in the presence of La^{3+} the increase was only 10.3 $\pm$ 3.4 nM. Note that the response to etomidate was obtained after exposing cells to La^{3+} and may have been somewhat diminished.

this hypothesis we altered the Cl^- reversal potential by replacing most of the extracellular Cl^- by SCN^-, an anion that is more permeant than Cl^- itself in $GABA_A$ receptors and other chloride channels. Replacing Cl^- by SCN^- typically results in a negative shift in the anion equilibrium potential. If the shift in the anion reversal potential is large enough it would preclude activation of voltage-dependent Ca^{2+} channels in response to etomidate or muscimol. Indeed when cells were exposed to either 40 μM etomidate or 5 μM muscimol in the presence of SCN^- (140 mM) only a small elevation of $[Ca^{2+}]_i$ was

observed (Fig. 8*A*). The mean response of chromaffin cells to etomidate in a solution containing SCN^- was an elevation in $[Ca^{2+}]_i$ of 12.4 ± 2 nM, but an elevation in $[Ca^{2+}]_i$ of 222.7 ± 18.4 nM ($n = 51$) was observed when applied in HBSS (Fig. 8*B*). The data support the idea that the anion equilibrium potential of the cells is such that application of muscimol produces a depolarization that is strong enough to activate voltage-dependent Ca^{2+} channels.
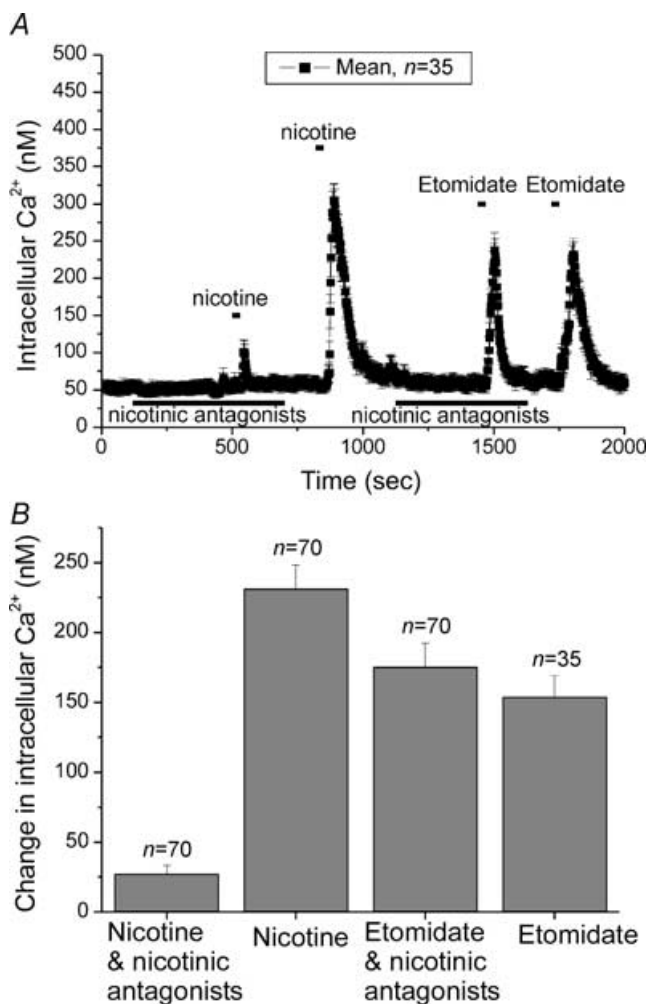

Figure 7. Nicotinic ACh receptor antagonists did not block the response to etomidate
A, mean $[Ca^{2+}]_i$ in 35 individual chromaffin cells was determined every 2 s. A cocktail of nicotinic antagonists (methyllycaconitine, 1 μM; mecamylamine, 20 μM; and dihydro-β-erythroidine, 20 μM) was added, as indicated. This cocktail suppressed the response to nicotine but not etomidate. *B*, the mean elevation of $[Ca^{2+}]_i$ in chromaffin cells exposed to nicotine (100 μM) in HBSS solution was 231.3 ± 17 nM ($n = 70$) in absence of nicotinic antagonists and 26.9 ± 6.5 nM ($n = 70$) in the presence of antagonists. The mean elevation of $[Ca^{2+}]_i$ exposed to etomidate (40 μM) in HBSS solution was 153.8 ± 15.5 nM ($n = 35$) in absence of nicotinic antagonists 175.1 ± 17.2 nM ($n = 70$) in the presence of antagonists.
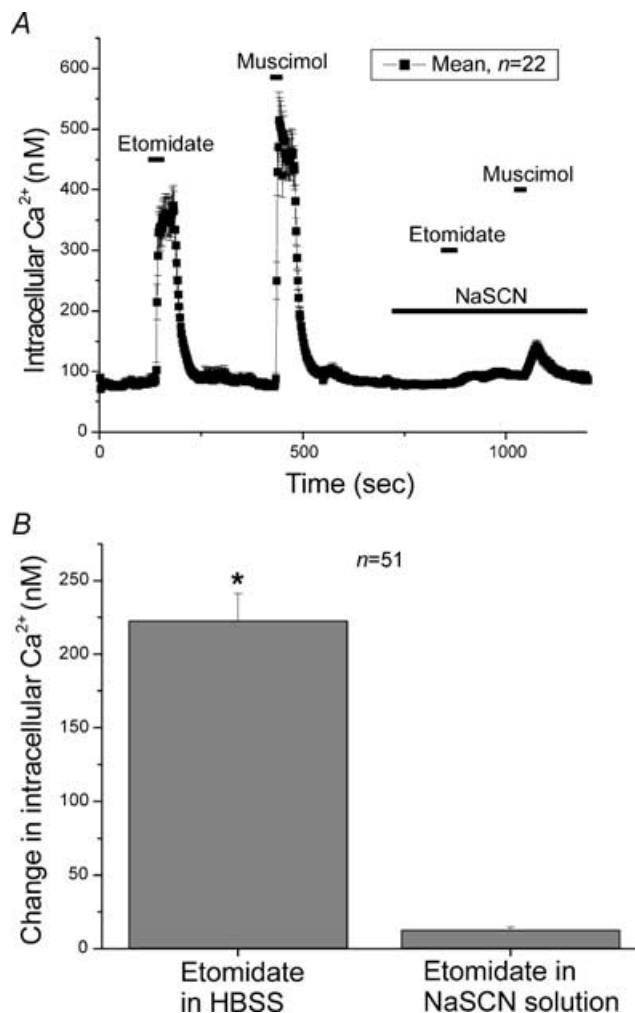
Figure 8. Evidence for a depolarized E_{Cl} in chromaffin cells
A, mean $[Ca^{2+}]_i$ in 22 individual chromaffin cells was determined every 2 s. After the first applications of 40 μM etomidate or 5 μM muscimol, the anion equilibrium potential of the chromaffin cells was altered by exposing them to 140 mM SCN^-, an anion which is more permeant in $GABA_A$ receptors than Cl^-. Replacing most of the Cl^- by SCN^- should result in a negative shift in the Cl^- equilibrium potential. When cells were re-exposed to either 40 μM etomidate or 5 μM muscimol in the presence of SCN^- only a small elevation of $[Ca^{2+}]_i$ was observed. *B*, the mean elevation of $[Ca^{2+}]_i$ in chromaffin cells exposed to 40 μM etomidate in HBSS solution was 222.7 ± 18.4 nM ($n = 51$). When etomidate was applied during superfusion with 140 mM SCN^- the increase was only 12.4 ± 2 nM.

Etomidate-induced catecholamine release

The robust elevations of $[Ca^{2+}]_i$ observed upon exposing cells to etomidate suggest that the anaesthetic may be able to elicit catecholamine secretion in chromaffin cells. Figure 9 shows that in fact, etomidate does promote secretion. An amperometric electrode was positioned inside a small bath which contained chromaffin cells. The electrode sampled the bath concentration of catecholamine. At the time indicated by the arrow, a solution containing control HBSS solution (Fig. 9, grey trace) or etomidate (40 μM; Fig. 9, black trace) was introduced into the bath. Prior to introduction of each of the solutions the bath flow was stopped. This allowed any catecholamine released by the cells to accumulate in the bath. The etomidate-containing solution elicited secretion. However, application of control solution (HBSS) caused no increase in bath catecholamine concentration.

Figure 9B shows data averaged from five experiments. This figure shows that etomidate elicited slightly more than one-quarter the secretion of that produced by a solution containing 50 mM K$^+$ (labelled KCl).
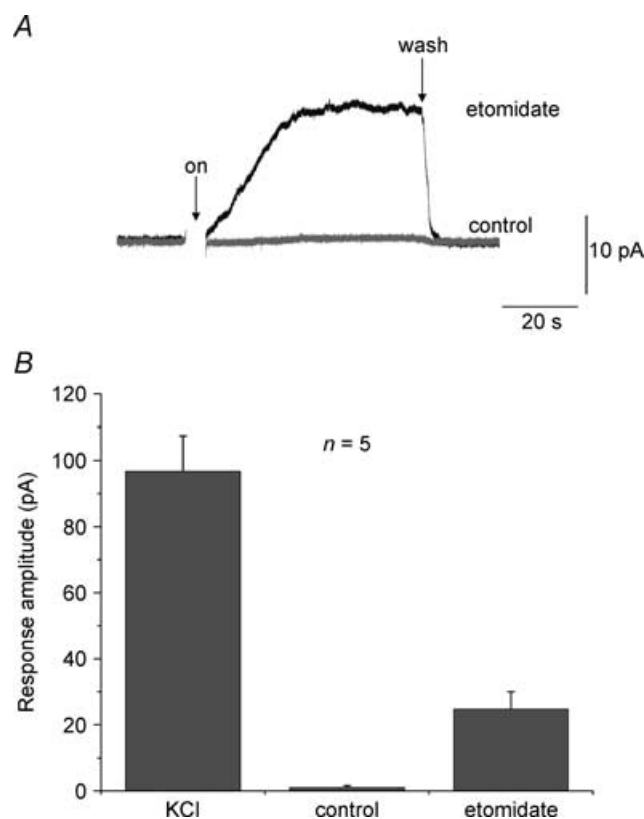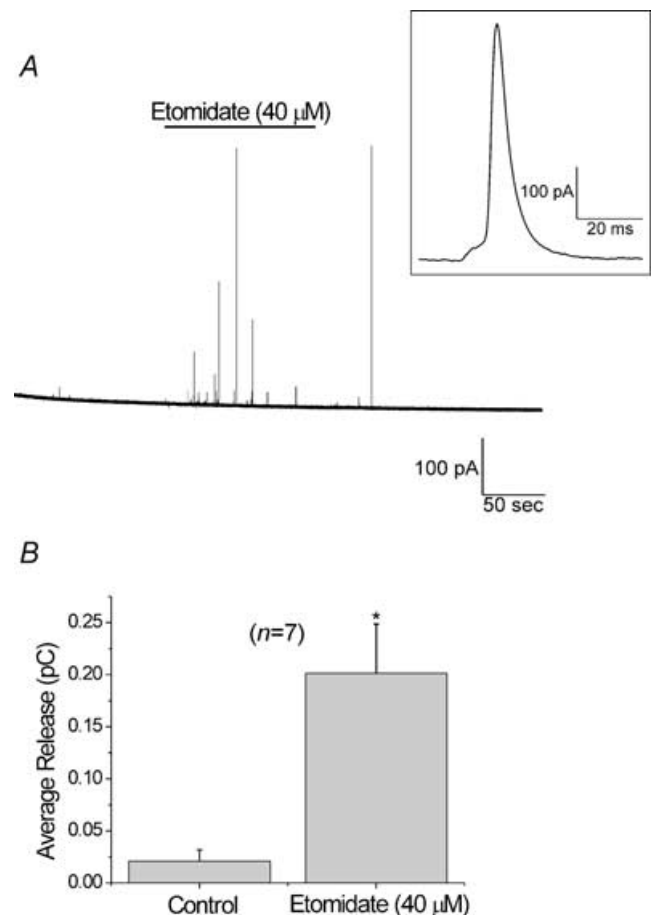

Figure 9. Activation of GABA$_A$ receptors by etomidate elicits catecholamine secretion in bovine chromaffin cells
An amperometric electrode, used to sample the catecholamine concentration, was positioned inside a small bath that contained chromaffin cells. A, amperometric traces recorded from cells sequentially exposed to two different solutions. At the arrow a solution containing control HBSS solution (grey trace) or etomidate (40 μM, black trace) was introduced into the bath. Prior to introduction of each of the solutions the bath flow was stopped. This allowed any catecholamine released by the cells to accumulate in the bath. Stopping the bath flow, without etomidate, caused no increase in bath catecholamine concentration. The etomidate-containing solution elicited secretion. B, mean catecholamine release as measured by the peak amperometric response was averaged from five experiments. Although not as effective as KCl, etomidate application clearly resulted in significant release.

Figure 10. Activation of GABA$_A$ receptors by etomidate elicits catecholamine secretion from individual chromaffin cells
An amperometric electrode was positioned so that it gently touched a cell. Each event corresponds to the release of an individual vesicle. A, a representative 7-min amperometric trace with multiple amperometric events is shown. This cell was exposed to etomidate (40 μM) for 2 min, as indicated. The inset shows an individual amperometric event on an expanded time scale (corresponding to the largest event observed in the presence of etomidate). B, the area under each of the amperometric events, (as shown in the inset), was integrated in order to obtain the quantal size. Control data were obtained in the 2-min period prior to etomidate application, while the data obtained for etomidate correspond to the 2-min exposure to etomidate. Summing the integrals of individual spikes from seven cells provided a measurement of the total catecholamine released during the periods that cells were in either control conditions or in etomidate.

In order to rule out catecholamine release via plasma membrane transporters working in reverse, amperometric measurements were made from individual cells. In these experiments a carbon fibre electrode was gently pushed against a cell. Data were sampled continuously. Figure 10*A* shows the amperometric current observed from a cell

perfused for 2 min with HBSS prior to exposing it to etomidate (40 μM) for 2 min. This was followed by $\sim$3 min where the cell was allowed to recover in HBSS. The current spikes correspond to amperometric events. The inset shows a single event on an expanded time scale. Integrating the area under each amperometric event provides a measure of the catecholamine content of individual vesicles. The sum all of the current integrals is proportional to the total catecholamine released. Figure 10*B* compares the sum of the amperometric current integrals in 2-min periods in the absence and presence of etomidate (40 μM), in seven cells. Both the total number of events (39 events in control conditions, *versus* 219 events in the presence of etomidate), as well as the sum of the current integrals, were significantly larger in the presence of etomidate. Taken together our data indicate that in addition to elevating $[Ca^{2+}]_i$, etomidate also promotes the vesicular release of catecholamines.
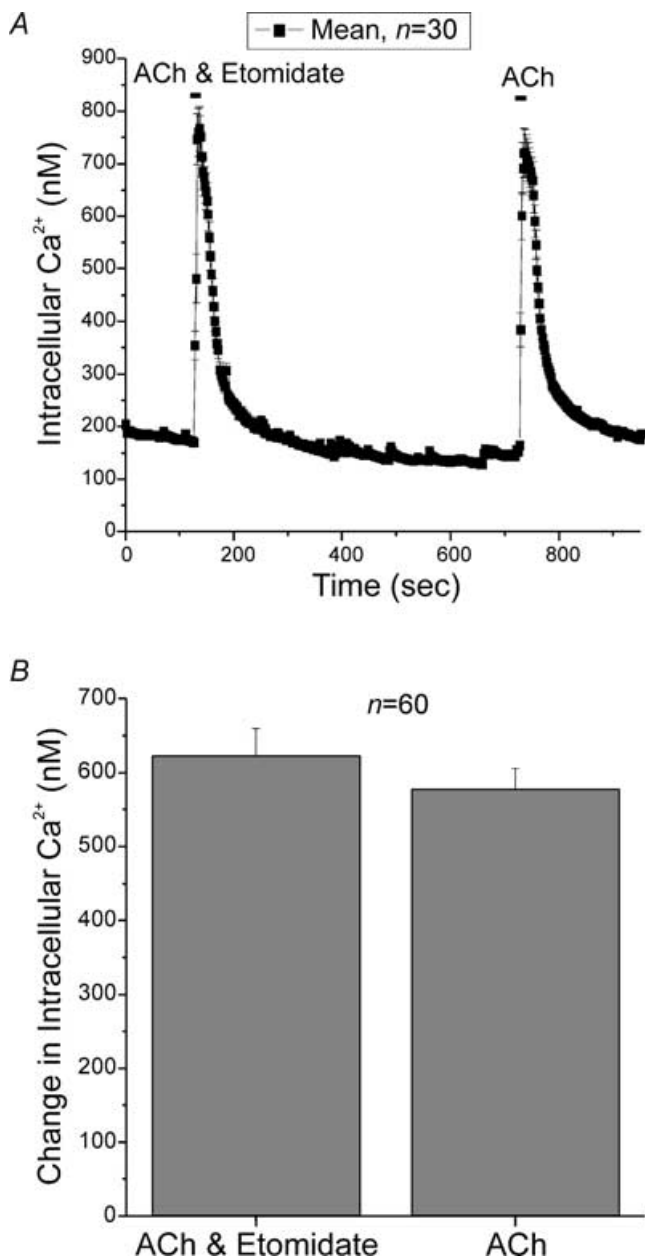
Co-application of ACh and etomidate

ACh released by pre-synaptic splanchnic nerve terminals is thought to be the physiological activator of adrenal chromaffin cells. In some cells activation of $GABA_A$ receptors is depolarizing but is still inhibitory (Zhang & Jackson, 1993). In chromaffin cells, there is considerable controversy regarding whether GABA can inhibit responses to high-K^+ or ACh, even though GABA itself is excitatory (Kataoka *et al.* 1986; Fujimoto *et al.* 1987; Kitayama *et al.* 1990, 1991). We had previously shown that muscimol did not diminish ACh responses. Figure 11 shows that etomidate did not suppress responses to ACh either. Figure 11*A* shows that responses to ACh (100 μM) were virtually identical in the presence or absence of etomidate. The order of presentation of ACh or ACh plus etomidate did not affect the responses (not shown). Figure 11*B* shows mean data from 60 cells; responses to ACh were unaffected by etomidate. In this set of experiments ACh elevated $[Ca^{2+}]_i$ by 622 ± 36.8 nM in the presence of etomidate and by 578 ± 28.2 nM in the absence of etomidate.

Discussion

In this manuscript we describe the response of chromaffin cells to the intravenous anaesthetic etomidate. We show that clinically relevant concentrations of etomidate directly activate $GABA_A$ receptors which then depolarize the cells sufficiently to activate voltage-gated Ca^{2+} channels. The resulting Ca^{2+} influx elicits catecholamine release. While it is possible that release of Ca^{2+} from intracellular stores plays a role in the responses observed, perhaps via Ca^{2+}-dependent Ca^{2+} release, such release does not appear to initiate the measured response. Our results suggest that application of etomidate under clinical conditions will result in elevated levels of circulating catecholamines.

Figure 11. Co-application of ACh and etomidate demonstrates that etomidate did not interfere with the activation of nicotinic ACh receptors
A, elevations in $[Ca^{2+}]_i$ in response to ACh (100 μM), in the presence and absence of etomidate (40 μM). The order of presentation of ACh or ACh plus etomidate did not affect the responses (not shown). *B*, the mean increase in $[Ca^{2+}]_i$ in 60 cells treated with ACh or ACh plus etomidate. ACh elevated $[Ca^{2+}]_i$ by 622 ± 36.8 nM in the presence of etomidate and by 578 ± 28.2 nM in the absence of etomidate.

Whether the release of catecholamines *in vitro* can explain the haemodynamical stability of etomidate *in vivo* remains to be determined.

Etomidate is a popular general anaesthetic. It is rapidly distributed and rapidly cleared which explains its rapid onset and short duration of action. Etomidate is thought to directly activate $GABA_A$ receptors, but only at high concentrations (Yang & Uchida, 1996; Belelli *et al.* 1997; Moody *et al.* 1997). At clinically relevant concentrations it is thought to potentiate GABA-mediated synaptic transmission (Ashton & Wauquier, 1985; Proctor *et al.* 1986; Yang & Uchida, 1996; Belelli *et al.* 1997). In our study we found that the EC_{50} for the elevation of $[Ca^{2+}]_i$ in chromaffin cells was ~5.8 μM. Clear elevations in $[Ca^{2+}]_i$ were observed in response to application of etomidate as low as 1 μM. Chromaffin cells can release GABA upon stimulation (Kataoka *et al.* 1984; Oset Gasque *et al.* 1985, 1990) which raises the possibility that etomidate potentiated the response to endogenously released GABA. We believe this possibility to be unlikely, but not impossible, as the cells were continuously perfused and there were small numbers of cells in a relatively large bath. Any released GABA should be rapidly washed away. More likely, it appears that clinically relevant concentrations of etomidate, defined as concentrations in the range of 1–8 μM (Giese & Stanley, 1983; Yang & Uchida, 1996; Belelli *et al.* 1999), directly activated the $GABA_A$ receptors found in chromaffin cells. If similar channels are found in the CNS, then they too will respond to low concentrations of etomidate. Other drugs that potentiate GABAergic neurotransmission, such as diazepam or phenobarbital, are not effective anaesthetics (Schulz & Macdonald, 1981; Sigel *et al.* 1990; Moody *et al.* 1997). One possible explanation for the difference between etomidate and diazepam/phenobarbital is that there is a subpopulation of $GABA_A$ receptors, like those found in chromaffin cells, that can be directly activated by clinical concentrations of etomidate. For these types of $GABA_A$ receptors, etomidate would both potentiate GABAergic transmission as well as activate the receptors. Part of the anaesthetic response to etomidate would be due to direct receptor activation (Belelli *et al.* 1997; Moody *et al.* 1997). Of course there are other possible explanations for the differences between etomidate and diazepam/phenobarbital. The drugs may target different types of $GABA_A$ receptors preferentially, or they may act on different targets altogether.

Etomidate is used in surgeries where patients have cardiovascular problems, as it has beneficial haemodynamic properties relative to other anaesthetics. For instance, propofol can cause hypotension during surgery (Minami *et al.* 1996), even though, like etomidate, it acts at $GABA_A$ receptors. It is interesting that propofol inhibits catecholamine release from the adrenal medulla by interfering with Na^+ influx through voltage-dependent Na^+ channels as well as nAChRs (Minami *et al.* 1996). Our data suggest a different mechanism of action for etomidate. Figures 9 and 10 show that, unlike propofol, etomidate directly elicits catecholamine release from chromaffin cells. Also unlike propofol, etomidate does not interfere with the actions of ACh on nicotinic receptors (Fig. 11). These mechanistic differences may give rise to the clinical differences observed.

Although we show that etomidate stimulates catecholamine release from chromaffin cells, elevation of circulating catecholamine levels *in vivo* has not been observed (Mazerolles *et al.* 1996). If the increase in catecholamine levels is small, the level of change may not have been detected. Interestingly, these authors found that pentobarbital decreased catecholamine level while etomidate caused no change. Our data show that etomidate stimulates catecholamine release in chromaffin cells even though the elevations in $[Ca^{2+}]_i$ described in this study are relatively modest. Submicromolar levels of intracellular Ca^{2+} can stimulate exocytosis from chromaffin cells (TerBush & Holz, 1992). Alternatively, it is possible that the depolarization induced by etomidate causes chromaffin cells to fire action potentials, which are not resolved by our imaging system. It is also worth noting that we measured the average response of the entire cell and that Ca^{2+} is substantially higher in microdomains near the release sites.

GABA plays an important role in the physiology of the adrenal medulla: (1) chromaffin cells possess the enzymes that synthesize and degrade GABA (Fernandez-Ramil *et al.* 1983; Kataoka *et al.* 1984); (2) GABA is taken up by chromaffin cells and released in response to depolarizing stimuli (Kataoka *et al.* 1984; Oset Gasque *et al.* 1985, 1990); (3) GABA-immunoreactive nerve fibres are found throughout the adrenal medulla (Kataoka *et al.* 1986; Oomori *et al.* 1993) and bundles of GABA immunoreactive nerve fibres run into the medulla with varicosities often in close contact with chromaffin cells (Kataoka *et al.* 1986; Oomori *et al.* 1993); and (4) chromaffin cells express $GABA_A$ receptors (Bormann & Clapham, 1985; Amenta *et al.* 1988; Peters *et al.* 1989). Functionally it has been shown that GABA can elicit catecholamine release from isolated perfused adrenal glands (Sangiah *et al.* 1974; Kataoka *et al.* 1984; Kitayama *et al.* 1984; Fujimoto *et al.* 1987; Gonzalez *et al.* 1992). Thus agents, like anaesthetics, that either potentiate or directly activate $GABA_A$ receptors are expected to play an important role in normal physiological functioning of chromaffin cells. The responses that we report are similar to those we observed using muscimol to activate $GABA_A$ receptors (Xie *et al.* 2003).

Ionotropic $GABA_A$ receptors are usually associated with inhibition, but during development, $GABA_A$-mediated depolarization has been observed in many regions of the CNS (Mueller *et al.* 1984; Luhmann & Prince, 1991; Yuste & Katz, 1991; Chen *et al.* 1996; Owens *et al.* 1996; Eilers *et al.* 2001; Ben-Ari, 2002). The action

of GABA$_A$ receptors in neurones is determined by the transmembrane Cl$^-$ gradient and the resultant E_{Cl} (Staley *et al.* 1996). The depolarized E_{Cl} observed is due, at least in some cases, to a member of the NKCC family (Alvarez-Leefmans *et al.* 1988; Rohrbough & Spitzer, 1996; Sun & Murali, 1999), which driven by Na$^+$ and K$^+$ gradients, acts to accumulate intracellular Cl$^-$ (Ben-Ari, 2002). Expression of another cotransporter that normally lowers intracellular Cl$^-$, KCC2 (Payne *et al.* 1996), appears to mediate the developmental shift towards inhibition (Rivera *et al.* 1999; Ben-Ari, 2002; Owens & Kriegstein, 2002). We had previously shown that chromaffin cells from animals 4–5 months old did not express KCC2, as expected from animals of this age (Xie *et al.* 2003). Rather these cells keep expressing the NKCC1 cotransporter typically found in embryonic cells. This result provides a molecular explanation for the depolarization produced by activation of GABA$_A$ receptors by etomidate. Data from our current study are consistent with a depolarized anion equilibrium potential. In cells where etomidate has inhibitory effects on release, as in SH-SY5Y neuroblastoma cells (Sikand *et al.* 1997), it is likely that either cells have a hyperpolarized anion equilibrium potential or that etomidate acts on targets other than GABA$_A$ receptors.

GABA responses are more complex than simply altering membrane potential. Activation of GABA$_A$ receptors induced c-Fos immunoreactivity and increased brain-derived neurotrophic factor (BDNF) mRNA expression in embryonic hippocampal neurones, but failed to have any effect in older neurones (Berninger *et al.* 1995). GABA stimulates DNA synthesis (LoTurco *et al.* 1995; Haydar *et al.* 2000) and appears to regulate KCC2 expression (Ganguly *et al.* 2001). It will be of interest to determine whether the GABA-mimetic actions of anaesthetics, such as etomidate, can also mediate some of these other responses.

The subunit composition of the GABA$_A$ receptors in chromaffin cells that responded to etomidate remains unknown. Immunoblots indicate the presence of α_1, α_4, β_{1-3} and γ_2 subunits in chromaffin cells (Parramon *et al.* 1995). Several studies have examined GABA$_A$ receptor subunit composition and responsiveness to etomidate. Uchida *et al.* (1995) showed that responses to etomidate were prolonged in γ_2-containing receptors, but that equivalent potentiation of GABA responses was observed in receptors with or without γ_2 subunits. The ability of etomidate to modulate and activate receptors is dependent on the β subunit (Belelli *et al.* 1997; Sanna *et al.* 1997). β subunits can directly bind etomidate (Belelli *et al.* 1999). Receptors with β_2 or β_3 but not β_1 are highly sensitive to etomidate (Belelli *et al.* 1997; Sanna *et al.* 1997). Single point mutations in β subunits could strongly suppress GABA-modulatory and GABA-mimetic actions of etomidate (Belelli *et al.* 1997). Furthermore, $\alpha_1\gamma_2$-containing receptors were not activated by etomidate

but homomeric β_1 receptors or $\beta_1\gamma_2$ receptors produced large responses to etomidate (Sanna *et al.* 1997). There may be two separate loci where etomidate acts: one for potentiation and another for direct activation of receptors. Single point mutations could reduce the ability of etomidate to activate the GABA$_A$ receptors but still retain ability to potentiate GABA responses (Moody *et al.* 1997) (but see (Belelli *et al.* 1997; Cestari *et al.* 2000) who argue that a single site on GABA$_A$ receptors mediates the actions of etomidate). Sedation and anaesthesia may be mediated by distinct GABA$_A$ receptors with different subunit composition (Reynolds *et al.* 2003). A genetically modified mouse with an etomidate-insensitive β_2 subunit showed that loss of consciousness was mediated through β_3-containing receptors while β_2 subunits mediated the sedative properties (Reynolds *et al.* 2003). Of interest, it was recently shown that the tuberomammillary nucleus plays a key role in the sedative properties of anaesthetics (Nelson *et al.* 2002). Jurd *et al.* (2003) showed that a single point mutation in the β_3 subunit of a genetically modified mouse did not affect responses to volatile anaesthetics but did eliminate responses to etomidate. It would be of interest to see whether the etomidate-induced catecholamine secretion (or elevation in $[Ca^{2+}]_i$) are modified in chromaffin cells from the β_2 or β_3 mutant animals described above.

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