Neuroscience Letters, 104 (1989) 83–87
Elsevier Scientific Publishers Ireland Ltd.

NSL 06309

γ-Aminobutyric acid elevates cytosolic Ca in bovine chromaffin cells

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(Received 2 May 1989; Revised version received 12 May 1989; Accepted 12 May 1989)

Key words: Chromaffin cell; γ-Aminobutyric acid; Cytosolic Ca; Fura-2; Calcium channel; Bicuculline; Bovine adrenal medulla

Measurements of the cytosolic Ca concentration ($[Ca]_i$) with the Ca-sensitive dye, fura-2, showed that in intact, but not in voltage-clamped, bovine chromaffin cells γ-aminobutyric acid (GABA, 10 μM) elicited a transient increase in $[Ca]_i$. The Ca transient of intact cells was inhibited by bicuculline (20 μM), by removal of extracellular Ca or by treatment with the Ca channel blocker cobalt (2.5 mM), and enhanced by lowering the extracellular Cl. We conclude, that GABA elevates $[Ca]_i$ by inducing a $GABA_A$-receptor-linked Cl current which depolarizes the cell membrane sufficiently to activate potential-operated Ca channels and cause Ca entry into the cell.

It is established that in chromaffin cells the primary determinant in secretion is an increase in intracellular Ca^{2+} concentration [7, 10]. Recent histochemical [1], biochemical [6, 8] and electrophysiological [3] studies showed that γ-aminobutyric acid (GABA) can modulate the function of the adrenal medulla. The mechanisms by which GABA influences secretion from chromaffin cells have not been established. Of the two known GABA receptor types – $GABA_A$ and $GABA_B$ [2] – only $GABA_A$ receptors have been found in these cells [3, 6, 8]. Binding of GABA to its receptors induces activation of Cl^- channels [2, 3]. The polarity of the membrane potential changes induced by a chloride current and, consequently, their effects on cell function depend critically upon the actual values of the resting membrane potential and the Cl^- equilibrium potential. Because GABA stimulates catecholamine secretion from canine adrenal medulla [6] and cultured bovine chromaffin cells [8] in a Ca-dependent way, it has been deduced that GABA, like ACh, can depolarize the cells and elevate $[Ca]_i$ by opening potential-gated Ca channels.

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The purpose of the present investigation was to evaluate the effects of GABA application on intracellular Ca^{2+} ion concentration. Most experiments were carried out on intact bovine chromaffin cells which preserve normal intracellular ionic milieu and membrane potential. Unlike in the voltage-clamped cells, their membrane potential can vary when ionic currents flow through the membrane. This allows a closer approximation of the physiological conditions.

Bovine chromaffin cells were enzymatically isolated from adrenal medulla as previously described [4], plated on poly-L-lysine-coated cover glasses and cultured for 1–4 days before use. The concentration of intracellular free Ca^{2+} ions was measured by use of the fluorescent indicator dye fura-2. Intact cells were loaded with the dye by incubating (25 min at 37°C) in a control extracellular solution containing 1 μM membrane-permeable fura-2/AM. The method of dual-wavelength measurement of $[Ca]_i$, the instrumentation used and its calibration have been described elsewhere [9]. The patch pipettes, used for intracellular perfusion and voltage-clamping of cells, were filled with a solution containing (in mM) Tris-Cl 140, $MgCl_2$ 1, HEPES-NaOH 10 at pH 7.2, MgATP 0.5 and fura-2 100 μM. Control extracellular solution contained (in mM) $CaCl_2$ 2, $MgCl_2$ 2, KCl 2.8, NaCl 140 and HEPES-NaOH 10 at pH 7.2. In low-Cl solution (10.8 mM Cl^-) NaCl was replaced with an equimolar quantity of sodium-isethionate or Na_2SO_4. Ca-free extracellular solution contained (in mM) NaCl 145, KCl 5.5, EGTA 0.1, HEPES-NaOH 10 at pH 7.2. Applications of drugs were performed locally onto the cell under investigation by pressure ejection from a pipette, whose tip (about 5 μm) was positioned 25–50 μm from the cell. Tetrodotoxin (1 μM) was added to all extracellular solutions. GABA and bicuculline [(−)-bicuculline methiodide] were both from Sigma. They were dissolved in distilled water (10 mM) and added to the test solutions at final concentrations of 10–20 μM.

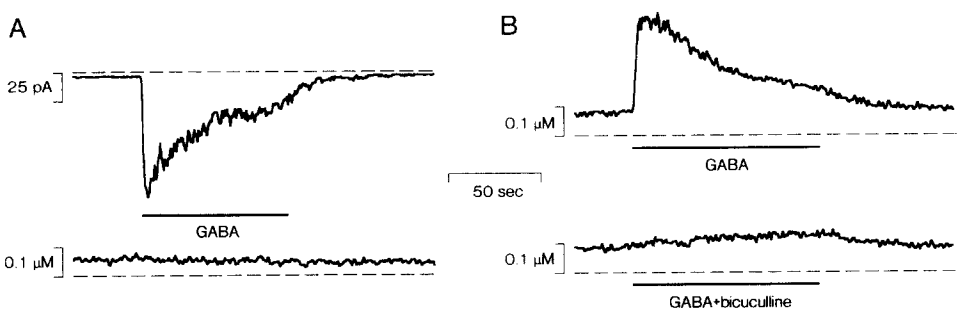

Fig. 1. The effects of GABA application on voltage-clamped and intact bovine chromaffin cells. A: upper trace shows the transmembrane current activated during application of 10 μM GABA; lower trace shows the simultaneously measured intracellular $[Ca]_i$ in the intracellularly perfused voltage-clamped cell. The application is indicated by a horizontal bar (63 s). The dashed lines give zero-value levels. The bath and the application pipette contained the control extracellular solution. Holding potential −60 mV. B: the traces here and in the next Figure show successive parts (from above to bottom) of a continuous recording of $[Ca]_i$ during a typical experiment on an intact cell. Horizontal bars (100 s) indicate applications. During the first application the pipette contained the control extracellular solution with 10 μM GABA, to which 20 μM bicuculline was added during the second application (380 s after the first one, lower trace). The dashed lines show zero $[Ca]_i$ level.

Fura-2, pentapotassium salt, and fura-2/AM, acetoxymethyl ester, were from Molecular Probes. All experimental data were sampled at 2 Hz with a laboratory computer (PDP 11/73).

Under whole-cell voltage clamp conditions, application of 10 μM GABA produced an inward current (upper trace, Fig. 1A) whose time course, reversal potential (close to 0 mV in the given experimental conditions) and single-channel conductance (about 20 pS as estimated from the current variance) closely corresponded to those of $GABA_A$-receptor-activated chloride current described by Bormann and Clapham [3]. Simultaneous measurements of intracellular Ca^{2+} ion concentration in voltage-clamped cells revealed no detectable changes in $[Ca]_i$ during GABA application (lower trace in Fig. 1A). These results show that GABA does not directly increase the Ca permeability of the cell membrane or release Ca^{2+} ions from intracellular stores.

However, intact (non-perfused and not-voltage-clamped) chromaffin cells loaded with fura-2/AM often responded to GABA with an increase in $[Ca]_i$. The mean concentration of free Ca^{2+} ions in these cells at rest was 0.08 ± 0.02 μM (range 0.05–0.14 μM, $n = 25$). In about a quarter of the 70 chromaffin cells studied, application of GABA produced a transient increase in $[Ca]_i$ (upper trace, Fig. 1B). During GABA application $[Ca]_i$ increased rapidly to a peak and then declined to a plateau level. The peak value produced by the application of 10 μM GABA averaged 0.34 ± 0.13 μM in the cells which responded to GABA ($n = 16$). During 100-s applications the plateau $[Ca]_i$ reached a mean value of 0.20 ± 0.10 μM ($n = 12$). Afterwards $[Ca]_i$ returned to the resting level during the next 50–100 s. This GABA-induced increase in intracellular Ca^{2+} level could be effectively and reversibly blocked by 20 μM bicuculline (lower trace, Fig. 1B), which suggests that the GABA response is mediated by $GABA_A$ receptors.

Taken together, these experiments performed on intact and voltage-clamped cells suggest that it is the activation of chloride channels via $GABA_A$ receptors that leads to an influx of extracellular calcium into intact cells. Further, they show that Ca influx does not occur when the membrane potential is clamped near its resting level. These findings are consistent with the idea that GABA promotes Ca influx into intact cells by depolarizing the membrane and consequent opening of voltage-activated Ca channels [4]. Two types of experiments were performed to explore this possibility. First, when the control extracellular solution (both in the bath and in the application pipette) was replaced with Ca-free solution, application of GABA (10 μM) did not lead to an increase in $[Ca]_i$ (Fig. 2A). Also, the GABA-induced $[Ca]_i$ transients were completely inhibited when the Ca channel blocker, cobalt (2.5 mM) was added to the test solution in the application pipette (data not shown). This concentration of Co^{2+} ions was sufficient to completely block the $[Ca]_i$ increase in fura-2/AM-loaded cells caused by opening of voltage-gated Ca channels with a high-K (50 mM) extracellular solution. Both these effects of Co^{2+} ions were readily reversible.

Second, as changes in the membrane potential due to chloride current depend on the Cl^- ion equilibrium potential, its shift to more positive values should result in a larger depolarization of the membrane. This should cause more effective activation

of the Ca channels and, consequently, larger Ca influx. We could achieve this by lowering the Cl^- ion concentration in the extracellular medium (low-Cl extracellular solution with 11 mM Cl^- ions). Indeed, when the application pipette contained low-Cl solution with 10 μM GABA, the induced Ca transients were observed in a larger proportion of the cells, had larger amplitudes and more steeply rising phases compared to the control (Fig. 2B).

In the mammalian central nervous system GABA is an inhibitory transmitter and this has often been suggested for the adrenal medulla as well (e.g. ref. 3). Whether activation of the chloride current would lead to depolarization or hyperpolarization of the cell membrane depends on the relation of the chloride equilibrium potential to the actual membrane potential of the cell. Existing data on the resting membrane potential of bovine chromaffin cells in culture range from -40 to -80 mV [4, 5]. The data on intracellular Cl^- ion concentration in these cells are not available. The results we obtained with non-perfused, intact chromaffin cells give new information on this question. The observed increase in the $[Ca]_i$ of some cells during GABA application means that these cells had a Cl^- equilibrium potential above -40 mV, which is the threshold for activation of their Ca channels [4, 5]. With an extracellular Cl^- ion concentration of 150 mM, the intracellular concentration of Cl^- ions in these cells should be about 35 mM.

The fact that not every cell examined responded to GABA application with an increase in $[Ca]_i$ can be explained, in part, by possible differences in the intracellular Cl^- ion concentration in different cells. Taken together with the recent demonstrations of a rich innervation of GABAergic neurons in the adrenal medulla of mam-

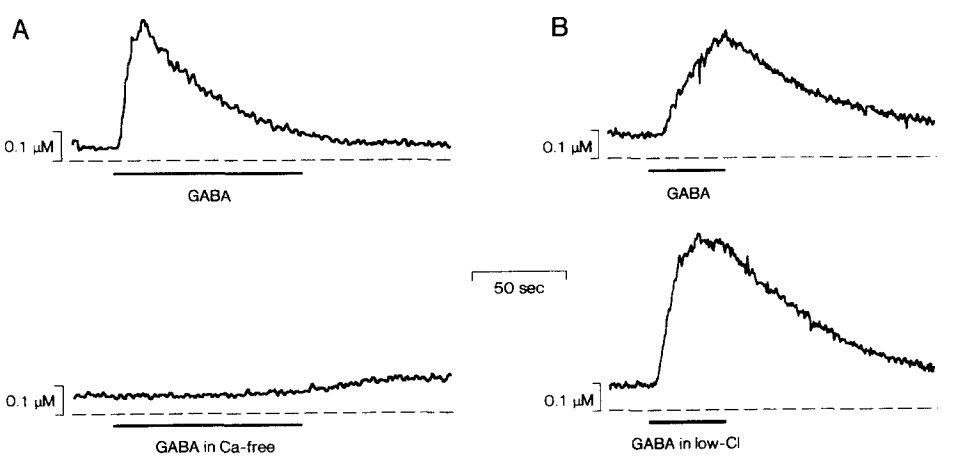

Fig. 2. Dependence of the GABA-induced $[Ca]_i$ transient on extracellular calcium (A) and chloride (B). A: the $[Ca]_i$ recordings from an intact cell during 10 μM GABA applications (horizontal bars, 100 s) with the control solution (upper trace) and with the Ca-free solution (lower trace) in the bath and in the pipette. Before the second application solution in the bath was changed several fold to Ca-free. B: shown are $[Ca]_i$ transients in an intact cell bathed in the control solution during 10 μM GABA applications (horizontal bars, 40 s) with the control (upper trace) and the low-Cl, isethionate-substituted, (lower trace) solutions in the application pipette. Time interval between the two applications was 320 s.

mals [1, 6], this could permit certain subpopulations of chromaffin cells to respond to GABA with either an increased or a decreased rate of secretion.

In conclusion, our results show that GABA can produce a significant increase in intracellular Ca^{2+} ion concentration in intact bovine chromaffin cells, bathed in a solution with physiological ionic composition, thereby confirming the ability of this neurotransmitter to modulate secretion from these cells. The proposed mechanism of GABA action involves development of a $GABA_A$ receptor-linked Cl^- current with, under the appropriate ionic conditions, subsequent depolarization of the cell membrane above the threshold for activation of potential-operated Ca channels.

I thank Professor E. Neher for providing facilities and advice, Professor Neher, Dr. R. Penner and Professor G. Augustine for commenting on this paper, and Mr. M. Pilot for excellent technical assistance. Financial support was provided by the Alexander von Humboldt Foundation.

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