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Calcium transients in single adrenal chromaffin cells detected with aequorin

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The effect of 55 mM K^+ and nicotine on intracellular free calcium was monitored in bovine adrenal chromaffin cells microinjected with aequorin. In contrast to results with quin 2, which suggested that stimulation of chromaffin cells resulted in sustained rises in free calcium, aequorin measurements showed that 55 mM K^+ and nicotine resulted in a transient (60–90 s) elevation of free calcium. The peak free calcium and duration of the transient elicited by nicotine were dose-dependent. The concentration of nicotine (10 μM) giving a maximal secretory response gave a peak rise in free calcium of up to 1 μM. 55 mM K^+ which only releases 30% of the catecholamine released by 10 μM nicotine generated a calcium transient indistinguishable from that due to 10 μM nicotine. These results support the idea that nicotinic agonists generate an alternative second messenger in addition to the rise in free calcium.

Ca^{2+}; Chromaffin; Aequorin; Quin 2; Secretion

1. INTRODUCTION

Secretion from bovine adrenal chromaffin cells in response to nicotinic cholinergic agonists requires extracellular calcium and involves calcium influx [1] through voltage-activated calcium channels [2] and a rise [3,4] in the level of intracellular free calcium ($[Ca^{2+}]_i$). In permeabilised chromaffin cells raising free calcium to micromolar levels is sufficient to elicit secretion [5]. These findings appear to indicate that a rise in $[Ca^{2+}]_i$ is necessary and sufficient for secretion. However, there is a discrepancy between the level of $[Ca^{2+}]_i$ required for secretion from permeabilised cells (half-maximal at about 1–2 μM and maximal at 10 μM [5]) and the level of $[Ca^{2+}]_i$ detected with quin 2 in cells stimulated with cholinergic agonists which is usually no more than 300–500 nM [3,4,6–9]. Additionally, we have found that concentrations of calcium ionophores [4] or high K^+ [8,9] that raise $[Ca^{2+}]_i$ to similar or even higher levels than that seen with cholinergic agonists elicit much less secretion than do cholinergic agonists. One possible interpretation of these findings is that a rise in $[Ca^{2+}]_i$ to 300–500 nM is itself not sufficient to elicit a full secretory response and that therefore activation of the nicotinic receptors on bovine chromaffin cells generates an additional second messenger.

The values of $[Ca^{2+}]_i$ obtained thus far [3,4,6–8,10] were derived from experiments in which populations of cells were monitored after loading with the calcium indicator quin 2. An alternative explanation for the discrepancies noted is that the initial rise in $[Ca^{2+}]_i$ and the subsequent time course of the calcium transient in individual cells differs depending on the stimulus presented to

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the cells and that this was obscured in the population response. To examine this possibility we have monitored calcium transients in response to nicotine and high K^+ in single adrenal chromaffin cells microinjected with aequorin.

2. MATERIALS AND METHODS

Chromaffin cells were dissociated from isolated bovine adrenal medulla by enzymatic digestion as described [11] in a Ca^{2+}-free Krebs-Ringer buffer consisting of 145 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose and 20 mM Hepes, pH 7.4 (buffer A). The cells were washed twice by centrifugation in buffer A containing 3 mM $CaCl_2$ and 0.5% bovine serum albumin and preincubated in this buffer for 60 min. All buffers were equilibrated with 95% air/5% CO_2. Determination of catecholamine release and $[Ca^{2+}]_i$ using quin 2 was as described in [8–10].

For aequorin experiments [12–14] a small number of chromaffin cells were transferred to a 0.1 mm microslide containing a pool of 1.2% agarose (Sigma type VII) in Williams medium E containing 0.2% bovine serum at 37°C (the concentration of bovine serum albumin present was critical for successful microinjection and subsequent survival of cells). The agarose was gelled at 4°C for 3 min. The cells were examined on an inverted microscope (× 600) with Nomarski illumination, and a single cell was selected for its healthy appearance (birefringence, homogeneous appearance without vacuoles or granularity, nucleus not visible) and injected with aequorin solution from a micropipette whose tip had been filled with a 50 μm long column of aequorin solution (80–150 mg/ml). The volume injected into a cell (diameter 12–16 μm) represented approx. 1% of total cell volume. Cells that retained their healthy appearance were maintained at 37°C in a CO_2 incubator for 30 min. The microslides were then transferred to a perfusable cup held at 37°C under a low-noise photomultiplier [14]. The perfusion fluid was buffer A with 0.05% bovine serum albumin, 3 mM $CaCl_2$. Photon counts were sampled every 50 ms by a Sirius microcomputer, and calibrated assuming an intracellular free Mg^{2+} concentration of 1 mM as in [13].

3. RESULTS AND DISCUSSION

To be able to compare results from aequorin with quin 2 measurements and catecholamine release we carried out all three measurements on the same batches of cells at 37°C. We had previously monitored catecholamine release and $[Ca^{2+}]_i$ with quin 2 at room temperature. The resting free calcium level from quin 2 in the present experiments was 110 ± 13 nM (mean ± SE, n = 9). The shape of the rise in $[Ca^{2+}]_i$ in response to 10 μM nicotine or 55 mM K^+ (fig.1) was similar to that reported in [10] with 55 mM K^+ giving a more sustained rise. Stimulation of cells at 37°C for 6 min with 10 μM nicotine resulted in the release of 3-times more catecholamine than resulted from stimulation with 55 mM K^+ (fig.1) despite these being optimal concentrations of the secretagogues. This lower level of release due to high K^+ was found over a wide range of concentrations (1–10 mM) of extracellular calcium.

The averaged resting signal from chromaffin cells microinjected with aequorin indicated a free calcium concentration of 150–200 nM. Nicotine resulted in a single transient rise in $[Ca^{2+}]_i$ the magnitude and duration of which were dose-dependent. The results in fig.2 are taken from a

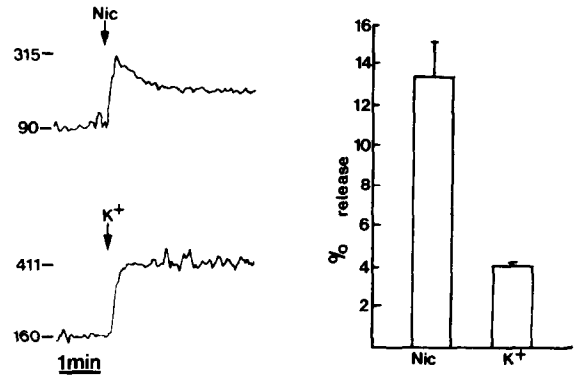

Fig.1. Changes in $[Ca^{2+}]_i$ in chromaffin cells monitored by quin 2 and catecholamine release in response to nicotine and 55 mM K^+. (Left) Upper trace, response to addition of 10 μM nicotine (Nic); lower trace, response to elevation of extracellular K^+ from 5 mM to a final concentration of 55 mM K^+. (Right) Catecholamine release following challenge with 10 μM nicotine or 55 mM K^+ for 6 min at 37°C. Released catecholamine is expressed as a percentage of total cellular catecholamine.

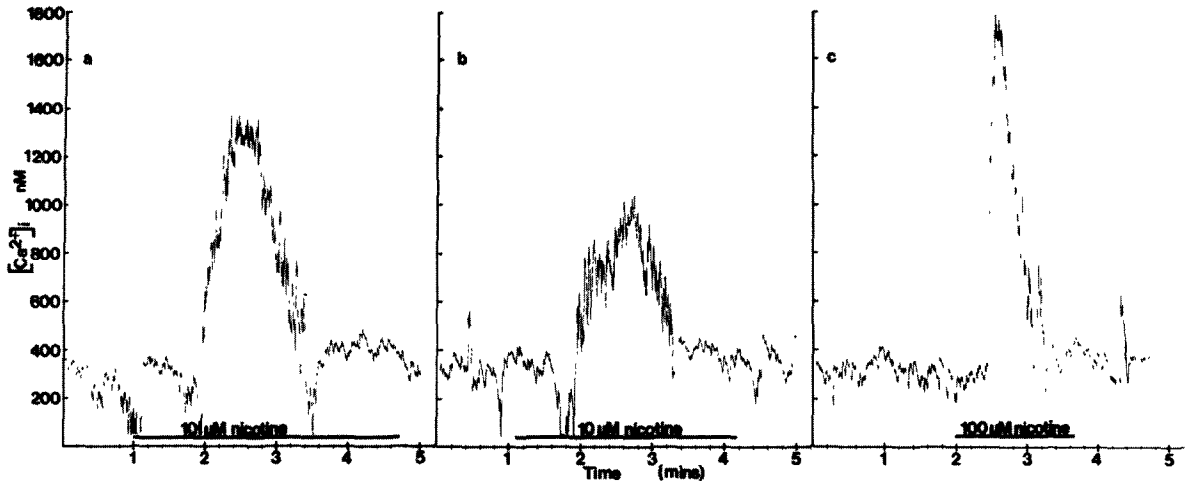

Fig.2. Changes in $[Ca^{2+}]_i$ in response to nicotine in a single chromaffin cell microinjected with aequorin. The three computer plots are excerpts from a recording of the same cell. The cell was challenged with 10 μM nicotine. (a) Subsequent challenge 20 and 48 min later with 0.1 and 1 μM nicotine elicited no response (not shown). The response to a second challenge 67 min after (a), with 10 μM nicotine is shown in (b). The same cell was then challenged 20 min later with 100 μM nicotine. (c) Time constants. 10 s for resting level, 1 s for transient. The apparent lag in the response is probably not real (see text).

single cell which responded to 10 μM nicotine (a dose just giving a maximal secretory response [10]). Peak free calcium was 1350 nM and duration of transient 90 s. This cell did not respond to subsequent challenges with 0.1 and 1.0 μM nicotine (not shown) but did respond 1 h later to a second application of 10 μM nicotine generating a calcium transient somewhat smaller than that elicited by the first 10 μM nicotine challenge. After a 20 min recovery period this cell was challenged with 100 μm nicotine which elicited a calcium transient with a larger peak of $[Ca^{2+}]_i$ (1800 nM) but with a shorter duration (40 s) than that seen following 10 μM nicotine. Calcium transients very similar to that generated by the 100 μM nicotine challenge were seen in other cells that had not previously been exposed to agonist (fig.3). The apparent lag between onset of perfusion with nicotine and the rise in $[Ca^{2+}]_i$ would be an overestimate of the true lag time since it includes time for mixing of solutions in the perfusion cup and diffusion of nicotine through 50–100 μm of agarose within which the cells were embedded.

The rise in free calcium in a single chromaffin cell in response to nicotine is clearly transient in contrast to results from quin 2 on a cell population which appear to show a prolonged elevation of $[Ca^{2+}]_i$ (fig.1). This could be due either to quin 2 prolonging the calcium transient in some fashion or to heterogeneity of response within the population.

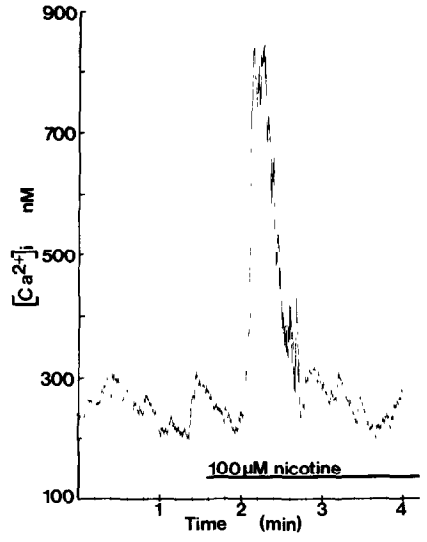

Fig.3. Change in $[Ca^{2+}]$ in response to 100 μM nicotine in a single chromaffin cell microinjected with aequorin. The cell had not previously been exposed to agonist. Time constants 7 s for resting level, 1 s for transient.

The calcium transient elicited by depolarisation with 55 mM K^+ was essentially the same in magnitude and duration as that due to nicotine. Fig.4 shows results from a cell challenged firstly with 55 mM K^+, followed by 10 μM nicotine. Additional challenges with 55 mM K^+ and 10 μM nicotine give similar responses. Challenge with a combination of 55 mM K^+ and 10 μM nicotine resulted in a slightly larger increase in $[Ca^{2+}]_i$. Expanded plots of the transients elicited by high K^+ (not shown) were similar to those seen with 10 μM nicotine (fig.2) in that the overlying trend of the calcium transient was superimposed on a repetitive series of calcium spikes. These spikes were much larger than could be accounted for by noise and therefore probably reflect a genuine biological phenomenon. Similar spikes were seen at a low frequency in unstimulated cells.

Two major conclusions emerge from these results. First, it is clear that the elevation of $[Ca^{2+}]_i$ in a single chromaffin cell is transient, lasting no more than about 60–90 s and that therefore the data derived from quin 2 experiments on cell populations are misleading. The reason for the difference probably lies in buffering of calcium by quin 2. The presence of up to 1 mM quin 2 in the cytoplasm could prolong the falling phase of the transient by raising the quantity of calcium that has to be removed to restore resting free calcium levels. Buffering by quin 2 reduces the peak free calcium induced by agonists and this reduction in free calcium may slow any calcium-dependent desensitization of receptor or channels thereby prolonging the calcium influx. Experiments using aequorin in quin 2-loaded cells should allow the effects of quin 2 on free calcium to be resolved.

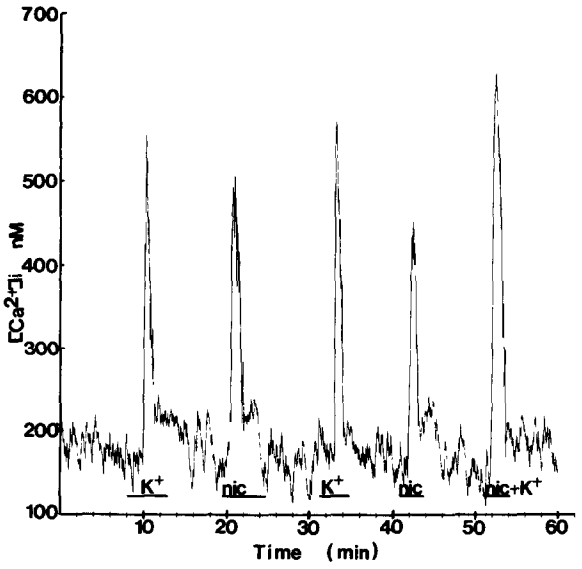

Fig.4. Changes in $[Ca^{2+}]_i$ in response to 55 mM K^+ and 10 μM nicotine in a single chromaffin cell microinjected with aequorin. The computer plot shows effects of challenges with 55 mM K^+ or 10 μM nicotine followed by simultaneous challenge with both agents. Expanded plots of the calcium transients elicited by high K^+ or nicotine were essentially the same as those due to 10 μM nicotine in fig.2. Time constants 25 s for resting level, 3 s for transients.

The second major conclusion is that the shape, magnitude and duration of the calcium transient elicited by high K^+ are essentially no different from those elicited by nicotine. This indicates that the lower level of secretion stimulated by high K^+ in comparison to nicotine [8,9,11,16] cannot be explained on the basis of differences in the calcium transient elicited by these two secretagogues. These results further strengthen the evidence that the full secretory response elicited by nicotine requires the generation of a second messenger in addition to calcium. The identity of this additional second messenger is unknown though we have presented evidence [15] suggesting that the additional second messenger pathway generated by nicotinic agonists acts by disassembling the cortical actin network in chromaffin cells.

The present results also give an indication of the level of $[Ca^{2+}]_i$ likely to be associated with secretion from chromaffin cells. A dose of nicotine (10 μM) which results in a maximal secretory response raised peak Ca^{2+} to little more than 1 μM. Not surprisingly, this is higher than the peak level detected by quin 2 in cell populations [3,4,6–8,10]. However, in one study using quin 2 [17] much higher values have been reported. The authors claimed that cholinergic agonists elevated $[Ca^{2+}]_i$ to 5–10 μM and suggested that the lower levels reported by other workers were a result of calcium buffering by high levels of quin 2. However, the precision of aequorin measurements in the range 1–10 μM free calcium is very good, the rate of consumption rising 250-fold ($10^{-3.25}$ to 10^{-1} s^{-1}) [13] whereas the change in the quin 2 signal (90 to 99% saturation) is unlikely to be measurable with comparable precision. The results

from aequorin, which would also not be expected to buffer calcium significantly, make it unlikely that $[Ca^{2+}]_i$ is raised to the high levels suggested by Kao and Schneider [17] in stimulated chromaffin cells.

The peak level of free calcium detected by aequorin in stimulated cells (~1 μM) is close to the concentration (1–2 μM) eliciting half-maximal secretion but much less than the 10 μM required for maximal in permeabilised chromaffin cells [5]. The aequorin signals have been calibrated assuming that $[Ca^{2+}]_i$ rises homogeneously in the cell and it remains possible that secretion is triggered by local high levels of $[Ca^{2+}]_i$ just beneath the plasmalemma. However, the present results here do suggest that the physiological rise in $[Ca^{2+}]_i$ is not itself sufficient to elicit the full secretory response in chromaffin cells.

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