

# Muscarinic receptor-mediated intracellular $\text{Ca}^{2+}$ mobilization in embryonic chick heart cells

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Activation of muscarinic receptors of heart cells elevates the intracellular  $\text{Ca}^{2+}$  concentration. The increase is considered to be due to influx of extracellular  $\text{Ca}^{2+}$ . We show that intracellular  $\text{Ca}^{2+}$  mobilization is involved. Cell suspensions prepared from hearts of 6-day-old chick embryos were loaded with the fluorescent  $\text{Ca}^{2+}$  chelator chlortetracycline. Muscarinic stimulation induces a dose-dependent fluorescence decrease ( $\text{ED}_{50} = 2.6 \times 10^{-6}$  M) indicating intracellular  $\text{Ca}^{2+}$  mobilization.

$\text{Ca}^{2+}$  mobilization; Muscarinic receptor; Chlortetracycline; (Chick heart)

## 1. INTRODUCTION

Activation of cardiac muscarinic receptors results in increased  $\text{K}^+$  permeability [1,2], decreased adenylate cyclase activity [3,4], increased cyclic GMP formation [5] and stimulation of phosphoinositide turnover [6–8].

In most tissues and cells, phosphoinositides are hydrolysed to give diacylglycerol and phosphatidylinositol phosphates [9] from which inositol trisphosphate triggers intracellular  $\text{Ca}^{2+}$  mobilization [10,11].

In the heart, stimulation of phosphoinositide turnover [6–8] and synthesis of  $\text{IP}_3$  [12] by muscarinic agonists have been demonstrated. However, there is still controversy about the effect of  $\text{IP}_3$ . Hirata et al. [13] were able to demonstrate  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum of canine ventricular muscle after addition of  $\text{IP}_3$ ,

whereas Movsesian et al. [14] did not find  $\text{Ca}^{2+}$  release on permeabilized cardiac myocytes and sarcoplasmic reticulum of the rat.

High concentrations of muscarinic agonists have a positive inotropic effect which might be due to increased intracellular  $\text{Ca}^{2+}$  concentration [15–17]. An increased intracellular  $\text{Ca}^{2+}$  level after stimulation of muscarinic receptors has recently been demonstrated in rat ventricular myocytes with quin2 [18]. The increase in intracellular  $\text{Ca}^{2+}$  concentration was accompanied by an increase in intracellular  $\text{Na}^+$  and was dependent on extracellular  $\text{Na}^+$  and  $\text{Ca}^{2+}$ . In addition, the increase was independent of intracellular  $\text{Ca}^{2+}$  mobilization because inhibiting intracellular  $\text{Ca}^{2+}$  mobilization with ryanodine and discharging  $\text{Ca}^{2+}$  stores with caffeine were without effect on the quin2-measured  $\text{Ca}^{2+}$  increase. The authors therefore assume that the increase in intracellular  $\text{Ca}^{2+}$  is caused by influx of extracellular  $\text{Ca}^{2+}$  in exchange for intracellular  $\text{Na}^+$  which increases after muscarinic stimulation [15,16,18].

In contrast to the above-cited experiments we show with the fluorescent  $\text{Ca}^{2+}$  chelator chlortetracycline that in chick embryonic heart cells activation of muscarinic receptors leads to intracellular  $\text{Ca}^{2+}$  mobilization.

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*Abbreviations:*  $\text{IP}_3$ , myo-inositol 1,4,5-trisphosphate; CTC, chlortetracycline

## 2. MATERIALS AND METHODS

Suspensions of 6-day-old chick embryonic heart cells (stage 29) were prepared by collagenase/hyaluronidase (Boehringer) treatment as in [19] for the isolation of chick limb bud cells. Isolation was performed at room temperature. The isolated cells were kept in Hanks solution at 25°C until use.

Intracellular  $\text{Ca}^{2+}$  mobilization after muscarinic stimulation was measured by CTC fluorescence. The procedure is described in detail by Oettling et al. [20]. A 2 ml aliquot of cell suspension ( $4 \times 10^6$  cells/ml) was preincubated with 20  $\mu\text{M}$  CTC for 25 min in modified Hanks (10 mM Hepes, 2.1 mM  $\text{NaHCO}_3$ ). The cell suspension was supplemented with  $1 \times 10^{-5}$  M eserine in order to inhibit cholinesterase.

Acetylcholine, added in 2- $\mu\text{l}$  units to the stirred cell suspension, triggered a fluorescence decrease. The reaction had reached completion 4 min after addition of acetylcholine. The fluorescence change was determined graphically and corrected for dilution produced by the volume added as in [20]. The relative fluorescence change was calculated as the percentage of the maximal inducible fluorescence decrease. The parameters of the dose-response curves were fitted to the function

$$E = E_{\max}[A]/ED_{50} + [A]$$

where  $E$  denotes the effect,  $E_{\max}$  the maximal effect,  $ED_{50}$  the concentration of drug giving half-maximal effect and  $[A]$  the concentration of drug. Parameters were fitted with the non-linear least-squares regression programme BMDPAR (BMDP statistical software).

## 3. RESULTS AND DISCUSSION

CTC is known to form fluorescent complexes with  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ . Cellular CTC fluorescence is mainly caused by CTC molecules complexed to membrane-associated  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  [21]. The fluorescence intensity depends on the concentration of free  $\text{Ca}^{2+}$  in the storage vesicles [22,23]. Thus, intracellular  $\text{Ca}^{2+}$  mobilization is accompanied by a decrease in CTC fluorescence.

Fig.1 shows that acetylcholine triggers a concentration-dependent decrease in fluorescence in cell suspensions of embryonic heart cells.  $3 \times 10^{-6}$  M atropine prevents the effect of acetylcholine.

We recorded the excitation spectra of CTC-labeled cells before and after stimulation with acetylcholine (fig.2). As shown in [19], changes in CTC- $\text{Ca}^{2+}$  fluorescence should be reflected by a peak at 397 nm in the difference spectrum and in CTC- $\text{Mg}^{2+}$  fluorescence by a peak at 383 nm. The difference spectrum of chick heart cells (fig.2) peaks at 397 nm indicating that changes in CTC- $\text{Ca}^{2+}$  fluorescence are responsible for the

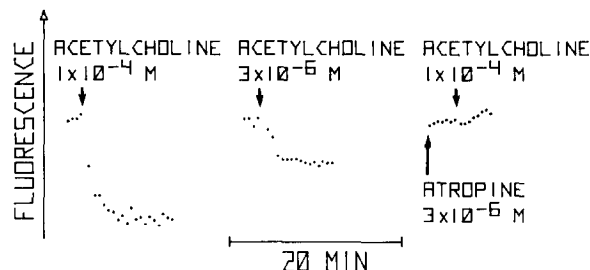


Fig.1. Effect of acetylcholine on CTC fluorescence of chick embryonic heart cells. On-line plots of fluorescence intensity of representative experiments. Cells were preincubated with 20  $\mu\text{M}$  CTC for 25 min at 37°C in the dark and then transferred to a thermostatted fluorometer cuvette (37°C). Drugs were added (arrow) to the stirred cell suspension in a volume of 2  $\mu\text{l}$ .

fluorescence decrease after addition of acetylcholine.

Fig.3 shows the dose-response curve of acetylcholine for  $\text{Ca}^{2+}$  mobilization. The  $ED_{50}$  was calculated to be  $2.6 \times 10^{-6}$  M ( $2.4$ – $2.8 \times 10^{-6}$  M; asymptotic SD; BMDP manual).

In embryonic chick heart cells muscarinic agonists are 100-fold more potent at inhibiting cyclic AMP formation than at stimulating phosphoinositide turnover [24]. The concentrations of muscarinic agonists for inducing the positive inotropic effect and the  $\text{Ca}^{2+}$  increase measured by quin2 fall within the range where phosphoinositide turnover is stimulated ( $>10^{-6}$  M) [15–18]. The concentrations of acetylcholine

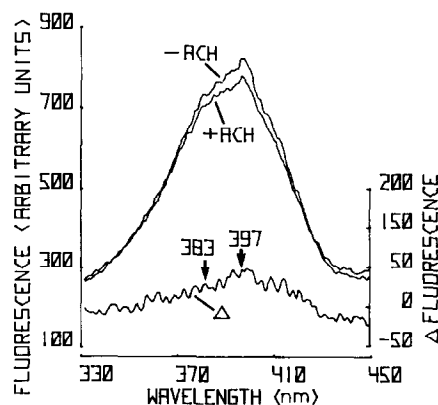


Fig.2. Excitation spectra. A cell suspension was scanned before (–ACH) and after (+ACH) stimulation with  $1 \times 10^{-4}$  M acetylcholine. Emission wavelength 529 nm. The difference spectrum ( $\Delta$ ) was calculated from the data of these spectra.

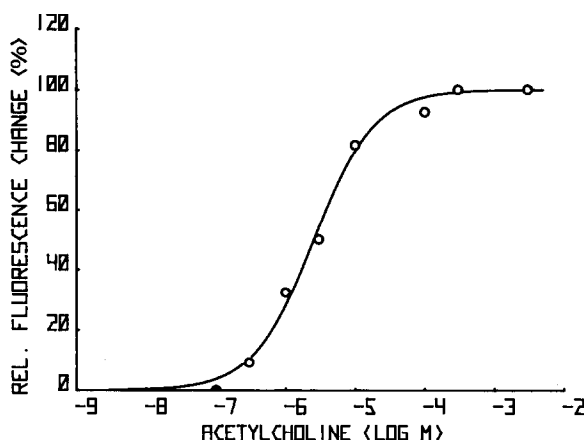


Fig.3. Concentration dependence of the acetylcholine effect. The results are taken from a single experiment. The experiment was repeated 3 times with essentially identical results.

necessary for triggering intracellular  $\text{Ca}^{2+}$  mobilization are in the same range. We therefore assume that the intracellular  $\text{Ca}^{2+}$  mobilization is triggered by  $\text{IP}_3$ .

The CTC method is not appropriate for investigations on the influx of  $\text{Ca}^{2+}$  into cells. Therefore, our experiments do not exclude the possibility of concomitant influx of  $\text{Ca}^{2+}$  into the cells. Both phenomena, influx of extracellular  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$  mobilization out of intracellular vesicles, may contribute to the increased intracellular  $\text{Ca}^{2+}$  concentration after muscarinic stimulation of heart cells.

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