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CALCIUM-INDEPENDENT DESENSITIZATION OF RISES IN INTRACELLULAR FREE Ca^{2+} CONCENTRATION AND CATECHOLAMINE RELEASE IN CULTURED ADRENAL CHROMAFFIN CELLS

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Summary: Exposure of adrenal chromaffin cells to carbamylcholine (CCh) in the absence of extracellular Ca^{2+} suppressed rises in intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) induced by subsequent addition of Ca^{2+} into the incubation medium. The extent of the suppression was dependent on the concentration of CCh and the duration of exposure. A similar inhibitory effect of CCh was also observed in the case of catecholamine release. In contrast, pretreatment with 56 mM K^+ did not affect these two responses induced by Ca^{2+}. Recovery from the desensitized state was rapid, since the responses became normal within 3 min following washout of the maximum concentration of CCh. These results show that, in Ca^{2+}-free medium, exposure of the cells to CCh induces desensitization as indicated by diminished rise in $[Ca^{2+}]i$ and reduced release of catecholamines. These phenomena were not due to direct inhibition of voltage-dependent Ca^{2+} channels by CCh, but seem to be due to an uncoupling of signal transduction between the nicotinic receptor and Ca^{2+} channel.

Stimulation of adrenal medulla with acetylcholine causes a rapid increase in the rate of catecholamine (CA) release; however, the response declines in spite of the continued presence of the agonist (1, 2). This decrement in CA release, "desensitization", has been partially attributed to depletion of the readily releasable store of CA (3). However, it remains unclear whether other factors, such as affinity change of acetylcholine receptor, inactivation of Ca^{2+} channel, etc., also contribute to this phenomenon (4, 5, 6, 7). Furthermore, the modes of desensitization induced by several kinds of stimulants

The abbreviations used are: CCh, carbamylcholine; $[Ca^{2+}]i$, intracellular free Ca^{2+} concentration; CA, catecholamine.

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seem somewhat controversial (6, 7, 8, 9). Thus, the mechanisms involved in this desensitization are poorly understood.

In the present study, we attempted to elucidate the mechanism of desensitization caused by nicotinic receptor stimulation in adrenal chromaffin cells in relation to rises in $[Ca^{2+}]i$.

Materials and Methods

Primary culture of adrenal chromaffin cells: Chromaffin cells were isolated from fresh bovine adrenal glands by retrograde perfusion with 0.025% collagenase as described previously (10, 11). The cells were plated on 270 ml culture flask and 16 mm diameter well of 4-well plastic multidish at a concentration of $5-7x10^5$ cells/ml in minimum essential medium, supplemented with 10% fetal calf serum, according to the previous method (11, 12). The cells were used for experiments between 4 and 7 days of culture.

Measurement of intracellular free Ca^{2+} concentration: The cultured chromaffin cells were collected by centrifugation (200xg, 6 min) and suspended in a Locke's solution of the following composition; 154 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5.6 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 0.1% bovine serum albumin (pH 7.4). The cells were incubated at a density of $3-4x10^6$ cells/ml with 20 μM Quin-2 acetoxymethyl ester for 60 min at 37°C. After the incubation, cells were washed and resuspended in the solution without Quin-2 acetoxymethyl ester and incubated for a further 30 min. Immediately before use, 1.5 ml cell suspension ($2x10^6$ cells/ml) was centrifuged (5000xg, 10 sec) and the cells were washed with Ca^{2+}-free Locke's solution, and resuspended in the solution. The cell suspension was transferred to the thermostatted quartz cuvette (37 °C) settled in a fluorescence spectrophotometer (HITACHI, model 650-60). The cells were stirred continuously by a syringe pump which operated in a push-pull manner. Fluorescence intensity was measured at an excitation wavelength of 339 nm (5 nm slit) and an emission wavelength of 492 (5 nm slit). Test agents were added with a microsyringe directly into the cuvette without interrupting continuous recording. The procedure for calculating the $[Ca^{2+}]i$ from the fluorescent signal was essentially the same as that described elsewhere (13, 14).

CA release: Cultured cells were washed two times with Ca^{2+}-free Locke's solution and were incubated in a 0.5 ml of the same solution. To determine the effects of carbamylcholine (CCh) or K^+, the cells were preincubated at 37°C for several time periods with different concentrations of each of them. After preincubation, Ca^{2+} (final concentration: 2 mM) was added into the medium and the incubation was continued for another 3 min to measure CA release. Cellular CA and released CA in the medium were extracted with 0.4 N perchloric acid and analyzed by a high performance liquid chromatography unit (Waters Assoc., Milford. MA) equipped with an electrochemical detector (Bioanalytical Systems Inc., W. Lafayette, IN) (15).

Results and Discussion

Since there is no reported investigation which demonstrates the desensitization of $[Ca^{2+}]i$, we investigated the effects of pretreatment with various concentration of CCh or K^+ in the absence of extracellular Ca^{2+} on $[Ca^{2+}]i$ induced by subsequent addition of Ca^{2+}.

As shown in Fig. 1-a, simultaneous addition of 0.3 mM CCh and 2 mM Ca^{2+} into the medium caused $[Ca^{2+}]i$ to rise from about 120 nM to 540 nM within 20 sec. When Ca^{2+} (2 mM) was added subsequent to CCh (0.3 mM) pretreatment for 1 min in the absence of extracellular Ca^{2+}, the rise in $[Ca^{2+}]i$ was markedly decreased; the maximal level of 360 nM was attained about 40 sec after the Ca^{2+} addition (Fig. 1-b). When the cells were pretreated with CCh for 3 min, a further decrease was observed in both the rate of rise and maximal level of $[Ca^{2+}]i$ in response to Ca^{2+} addition (Fig. 1-C). Similar results were obtained in the presence of 1 μM atropine, which was sufficient to block cyclic GMP response to muscarinic cholinergic stimulation by CCh (data

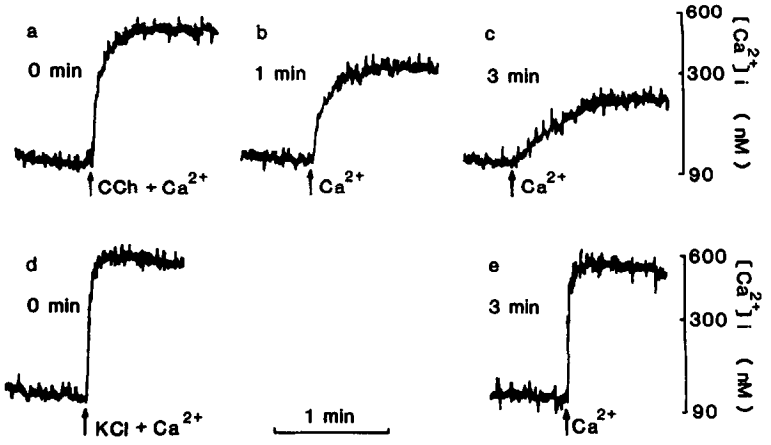

Fig. 1 Suppression by pretreatment with CCh of rise in $[Ca^{2+}]i$ induced by Ca^{2+} addition in a Ca^{2+}-free medium. Chromaffin cells were pretreated with 0.3 mM CCh (a), (b) and (c) or 56 mM K^+ (d) and (e) for indicated time periods. Ca^{2+} (2 mM) was added at the points indicated by the arrow. Typical tracings obtained from more than three different cell preparations are presented.

not shown). Simultaneous stimulation by 56 mM K^+ and Ca^{2+} induced the rise in $[Ca^{2+}]i$ to about 600 nM within 15 sec (Fig. 1-d). Pretreatment with 56 mM K^+ in the absence of external Ca^{2+} for 3 min (Fig. 1-e) or 10 min (data not shown), did not prevent the rise in $[Ca^{2+}]i$ induced by addition of Ca^{2+}. Although data obtained are not presented, pretreatment of the cells with CCh for 3 min did not affect the rise in $[Ca^{2+}]i$ induced by addition of 56 mM K^+ plus Ca^{2+}. These results indicate that the suppression by pretreatment with CCh of the rise in $[Ca^{2+}]i$ in response to Ca^{2+} addition was mediated by the nicotinic cholinergic receptor system and that this phenomenon could develop independent of extracellular Ca^{2+}. Moreover, the difference between the effect of CCh and high K^+ suggests that the desensitization by CCh is not caused by a direct inhibition of voltage-dependent Ca^{2+} channel. The affinity change of nicotinic cholinergic receptors and/or uncoupling of signal transduction between the receptors and Ca^{2+} channels may be responsible for the decreased rise in $[Ca^{2+}]i$ after CCh pretreatment.

Fig. 2-a shows the effects of duration of pretreatment with CCh on rises in $[Ca^{2+}]i$ induced by Ca^{2+} addition. CCh inhibition of $[Ca^{2+}]i$ response was clearly observed as early as 30 sec of 0.3 mM CCh pretreatment. When the cells were pretreated with CCh for 3 min or longer, the rise in $[Ca^{2+}]i$ was maximally depressed by about 60%. As shown in Fig. 2-b, the rise in $[Ca^{2+}]i$ induced by addition of Ca^{2+} was prevented by CCh in a concentration-dependent manner. About 60% inhibition was observed in the rise in $[Ca^{2+}]i$ following pretreatment of the cells with CCh at the concentration of 0.3 mM or above.

To examine the significance of above data, we measured CA release from the cells in the same experimental conditions

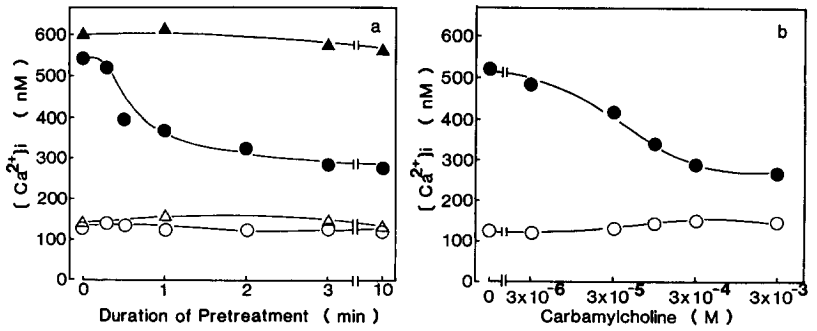

Fig. 2 Effects of the length (a) and concentration (b) of pretreatment with CCh or 56 mM K^+ on the suppression of rise in $[Ca^{2+}]i$ induced by Ca^{2+} addition. (a): Cells were pretreated with 0.3 mM CCh or 56 mM K^+ for indicated time periods. The maximum rises in $[Ca^{2+}]i$ induced by the addition of 2 mM Ca^{2+} were plotted. (b): Cells were pretreated with various concentration of CCh for 3 min. In the case of CCh concentrations below 0.3 mM, CCh needed to obtain 0.3 mM was further added with Ca^{2+}. The maximum rises in $[Ca^{2+}]i$ were plotted. Each of these data represents the mean of duplicate or triplicate determinations. Typical data obtained from two different cell preparations are presented. CCh pretreatment, ● (Ca^{2+} +) and ○ (Ca^{2+} -); K^+ pretreatment, ▲ (Ca^{2+} +) and △ (Ca^{2+} -).

adopted in the study on $[Ca^{2+}]i$. Time course and concentration-dependency on CCh of decrease in CA release induced by Ca^{2+} addition were similar to those of rises in $[Ca^{2+}]i$ (Fig. 3-a,b). As in the case of $[Ca^{2+}]i$, pretreatment with 56 mM K^+ in the

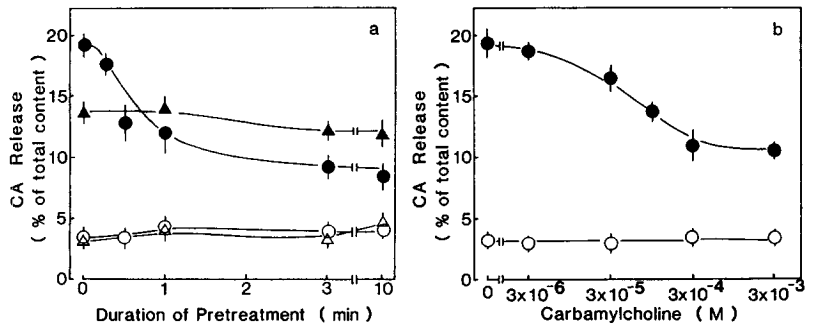

Fig. 3 Effects of the length (a) and concentration (b) of pretreatment with CCh or 56 mM K^+ on the suppression of CA release induced by Ca^{2+} addition. (a): Cells were pretreated with 0.3 mM CCh or 56 mM K^+ for indicated time periods. The CA release induced by the addition of 2 mM Ca^{2+} within 3 min was plotted. (b): Cells were pretreated with various concentrations of CCh for 3 min. In the case of CCh concentration below 0.3 mM, CCh needed to obtain 0.3 mM was further added with Ca^{2+}. The CA released within 3 min was plotted. Each of these data represents the mean ± S.E. of triplicate determinations. Typical data obtained from two different cell preparations are presented. CCh pretreatment, ● (Ca^{2+} +) and ○ (Ca^{2+} -); K^+ pretreatment, ▲ (Ca^{2+} +) and △ (Ca^{2+} -).

absence of Ca^{2+} did not affect Ca^{2+}-induced CA release (Fig. 3-a). These results suggest that the decrease in CA release by pretreatment with CCh in the absence of external Ca^{2+} is due to decrease in the rise of $[Ca^{2+}]i$.

Schiavone and Kirpeker (6), Boksa and Livett (7) and Holtz et al. (9) have reported that pretreatment with nicotinic agonists results in desensitization of CA release, at least in part, through mechanisms independent of extracellular Ca^{2+}. The present data are in agreement with their results. On the other hand, as to the effect of pretreatment with high K^+, whether desensitization can be induced or not remains somewhat controversial. Although Holtz et al. (9) demonstrated reduced CA release by high K^+ plus Ca^{2+} following exposure to high K^+ in a Ca^{2+}-free medium, Boksa and Livett (7) showed that pretreatment with high K^+ in a Ca^{2+}-free medium did not cause any decrement in CA response to subsequent addition of high K^+ plus Ca^{2+} following washout of the pretreatment medium. Our results for both $[Ca^{2+}]i$ and CA release are in accordance with the findings of these latter two authors.

Fig. 4 shows the time course of recovery from desensitization of the rise in $[Ca^{2+}]i$ produced by pretreatment with 0.3 mM CCh for 3 min in the absence of Ca^{2+}. Within a 3 min washout period, responses to CCh plus Ca^{2+} of the rise in $[Ca^{2+}]i$ returned to about 90% of those of the control group, indicating rapid recovery from desensitization. Similar resuls were obtained concerning the CA release. These results suggest that the decreased responsiveness to Ca^{2+} is not the result of cellular toxicity of CCh and/or Ca^{2+}-free condition.

In conclusion, exposure of cultured adrenal chromaffin cells to CCh in the Ca^{2+}-free medium was shown to decrease responses to Ca^{2+} of rise in $[Ca^{2+}]i$ and release of CA. The desensitization

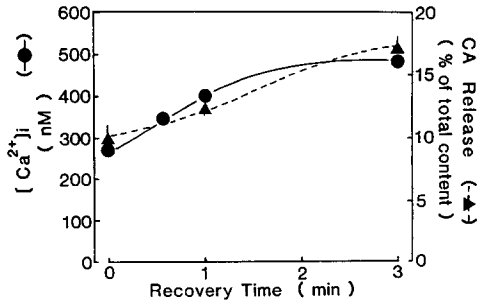

Fig. 4 Time course of recovery from suppression by pretreatment with CCh of rise in $[Ca^{2+}]i$ and CA release. Cells were pretreated with 0.3 mM CCh for 3 min in a Ca^{2+}-free medium. The cells were washed 2 times with a Ca^{2+}-free medium, immediately after the indicated time period, and stimulated by 0.3 mM CCh plus 2 mM Ca^{2+}. The maximum rises in $[Ca^{2+}]i$ and released CA within 3 min were plotted. Values of rise in $[Ca^{2+}]i$ and CA release obtained from the cells without the pretreatment were 540 nM and 18.8% of total cellular content, respectively.

caused by CCh was not due to inhibition of voltage-dependent Ca^{2+} channel, since $[Ca^{2+}]i$ response to K^+ plus Ca^{2+} after CCh was normal. Uncoupling of the linkage between receptor stimulation and Ca^{2+} channel activation might be the cause of the desensitization.

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