TEMPERATURE EFFECTS IN THE STIMULUS-SECRETION PROCESS FROM ISOLATED CHROMAFFIN CELLS

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ABSTRACT Temperature effects on the stimulus-secretion coupling process was studied by inducing release of catecholamines (CA) from isolated chromaffin cells of the bovine adrenal medulla. Use was made of three different secretagogues: acetylcholine (ACH), high potassium concentration, and the calcium ionophore A23187, at various incubation temperatures. The latter two agents induced a monotonic increase in secretion with rise in temperature, suggesting different regions of the dependence of total release on temperature. The ACH-induced secretion was, however, markedly different and exhibited a maximal release at 30°C. Kinetic experiments using ACH stimulus revealed that this maximum is produced by different temperature dependence in the stages of activation and desensitization. A proposed model for the total release process yields temperature-dependent parameters that can be divided into three regions of initial rates of secretory activity corresponding to the above independent findings using high K^+ concentration and the calcium ionophore. The transitions between the various regions indicate possible transitions in the physical properties of the plasma and secretory granule membranes. Elucidation of the interaction between the membranes is of primary importance in the determining mechanism of CA secretion from the isolated adrenal medulla cell.

INTRODUCTION

Secretion via exocytosis is a highly organized and complex process occurring in numerous tissues. Most neurotransmitters, hormones and many enzymes are released into the extracellular domain by this mechanism. The details of this phenomenon are under intense investigation, however, the precise mechanism has yet to be elucidated. Much speculation and controversy has surrounded the mechanism of exocytosis (Viveros [1], Norman [2], Anwyl and Narahashi [3], Zinder and Pollard [4], and many others), and many experimental systems, both animal and microbiological, have been studied, but the molecular mechanism has yet to be fully understood. One of the pioneers and still a leading figure in the field, George Palade, is quoted by Newmark (5) as saying: "Of the complex series of events that culminate in the secretion of products of cells, most can still only be classified as phenomenology." This process, termed by Douglas (6) as stimulus-secretion coupling, is a temporal and sequential event and is considered to involve biochemical and biophysical actions. Many mechanical aspects, which in our opinion play an important role in various stages of secretion (7), were mostly ignored so far in theoretical and experimental reports.

The chromaffin cell of the bovine adrenal medulla has been used as a convenient experimental model for studies of secretory events. This system has been extensively investigated (6, 8–10, others), and is of particular interest since it was observed (6) that when isolated they function as in vivo. An improved method for the preparation of functional isolated cells in high yield has recently been described by Greenberg and Zinder (8). The experimental determination of catecholamines (CA) released from the adrenal medulla cells into the medium is a sensitive and simple procedure, and allows direct monitoring of the kinetics and product concentration.

The immediate physiological stimulus that causes release from chromaffin cells is acetylcholine (ACH) (11). The essential sequence of events leading to CA secretion involves several stages (see e.g., reference 12): (*a*) binding of ACH to its receptors; (*b*) increase of membrane conductance leading to Na^+ influx and membrane depolarization (13) and triggering action potentials (9, 10); (*c*) penetration of Ca^{++} into the cell via voltage-sensitive channels by analogy with action potentials in nerve cells (10); (*d*) approach of the granules to close proximity with the cellular membrane; (*e*) fusion of granular and cell membranes to form an Ω-shaped continuous membrane matrix as is evident also in other systems (see reference 14 concerning endothelium cells); (*f*) diffusion of vesicular content through the stalk to the extracellular environment.

While the first steps involve mainly biochemical and biophysical processes influencing chemical and electrical balances across the membranes, the last three events, usually termed exocytosis, involve mechanical aspects as well. The motion of vesicles toward the cell membrane, the drainage of cytoplasmic fluid from between the membranes, and the failure of membranes undergoing fusion, cannot be ignored in a comprehensive analysis of the process as a whole.

The binding of ACH to its receptor has been investigated intensively. Early results were reported by Katz and Thesleff (15) who found that applications of large amounts of ACH caused an initial activation of the receptors followed by a process of desensitization. Under appropriate experimental conditions, washout of the agonist leads to partial recovery of sensitivity. Many researchers have investigated the process of desensitization and resensitization trying to establish theoretical models to describe experimental data studied in various tissues (16–21). In these studies, chemical kinetics were proposed to account for the mechanism of the phenomena.

Paton (16) developed a rate theory on the assumption that excitation by a stimulant is proportional to the rate of drug-receptor interaction. According to this hypothesis, the time scale for the decrease in the rate of release would be similar to the scale associated with the rate of binding of the ACH to its receptor. Such similarity, however, is not supported by experimental evidence (for a review see reference 17).

Katz and Thesleff (15), and later Rang and Ritter (17), proposed the occupation theory in which they suggested that desensitization results from chemical inactivation of a fraction of the receptors pool. Kirpekar and Prat (20) hypothesized that the receptor, after combining with the secretagogue, can exist in at least two stable forms: active for exocytosis to take place (sensitive), and inactive (desensitized). Transition between these two states can be blocked using drugs such as concanavalin A. A recent addition to the possible causes of receptor desensitization is the internalization of the receptor into the cell following its attachment to the secretagogue, resulting in the lack of response of the cell to additional activation by the agonist. This has been postulated for the lipoprotein receptor in liver cells and in the fibroblast (22), the insulin receptor in lymphocytes and adipocytes (23, 24), and other agonist-stimulated receptor systems.

Apart from considerations of chemical kinetics, several investigators suggested a relationship between desensitization and the physical properties of various components of the secretory system. Catterall (25) has suggested the possibility of correlation between the rate of desensitization and morphological maturity in the cultured muscle cell. He noted more rapid desensitization of the ACH receptor in highly differentiated cells as opposed to partially differentiated ones. Saitoh et al. (26) proposed that desensitization results from transition in thermal stability of the ACH-receptor complex (ACH-R). A modified stability was explained either by an extrinsic factor such as the presence of a certain protein in close proximity to the receptor site, or by an intrinsic modification of the receptor molecule.

Physical interpretations were brought forward by El-Fakahany and Richelson (21) who suggested that a certain degree of membrane phospholipid fluidity is required for activation, desensitization, and resensitization to occur. Changes in membrane fluidity may also explain the distribution and stability of ACH-R studied by Axelrod et al. (27), who measured lateral mobility of ACH-R and its aggregation into clusters on the membrane. In the isolated plasma membrane of adrenal medulla cells, Schneeweiss et al. (28) suggest changes in lipid phase behavior induced by cholinergic stimulants and high potassium concentrations. A direct influence on membrane fluidity, granular mobility physical properties of the cytoplasmic fluid, and the rate of biochemical reactions involved in both exocytosis and desensitization can be achieved by controlling the temperature at which these processes occur. The temperature dependence of chromaffin granule fragility has been recently studied and reported (7).

In the present study, we measured secretion of CA from isolated chromaffin cells of the bovine adrenal medulla at various temperatures and under the stimulus of a number of secretagogues. The experimental data is used to present a kinetic model involving activation, secretion, and desensitization. Temperature effects reflected in this model are used to elucidate the role of the physical properties of the membranes involved in the process of secretion.

EXPERIMENTAL PROCEDURES

Preparation of Adrenal Medulla Cells

Chromaffin cells were prepared from fresh bovine adrenal as described by Greenberg and Zinder (8), in a modification of the method of Fenwick et al. (29) and Schneider et al. (30). The procedure involves proteolytic digestion of connecting tissue by collagenase, resulting in the complete disruption of the medulla matrix. All steps were carried out at ambient temperature, as suggested by Greenberg and Zinder (8), except for incubations that were performed as stated for each experiment.

Chemical Assays

CA were determined by the fluorometric trihydroxyindole method as described by von Euler and Floding (31). Protein determination was carried out by a slight modification of the Bradford method (32) using Coommassie Brilliant Blue Protein Stain.

Procedures

Adrenal medulla cells were preincubated for 10 min at 10 different temperatures in the range of 0–42°C. This time period was sufficient for the cells to reach the desired temperature. A second period of incubation followed the addition of the stimulatory agents, and continued for 20 min. Greenberg and Zinder (8, 33) and Schneider et al. (30) had observed that no further secretion due to ACH occurred after this time period, and this was corroborated in the present experiments. The cells were separated from the medium, at the end of the incubation time, by centrifugation at

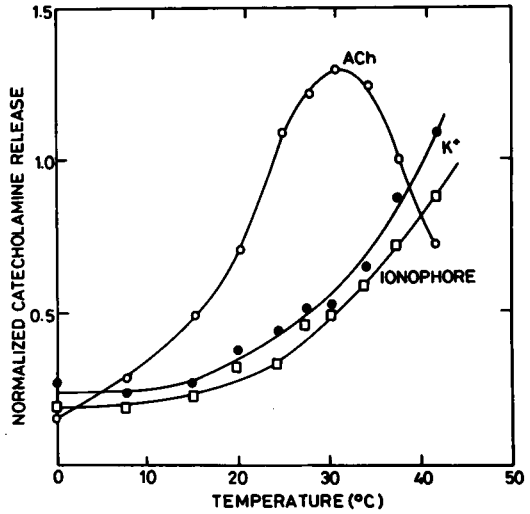

FIGURE 1 Experimental measurements of release due to three different secretagogues after 20 min of incubation. Each data point represents an average of five independent experiments and is normalized with respect to the net amount of ACH-induced secretion at 37°C. (●—●, KCl, 65 mM; □—□, A23187, 10 μg/ml; O—O, ACH, 10^{-4} M).

1,000 *g* for 5 min. The supernatant in each test tube was assayed for released CA, in excess of that released under basal (nonstimulated) conditions. The results are expressed as percent of the net ACH-induced secretion at 37°C. Secretion was initiated by three agents: (*a*) addition of acetylcholine (Sigma Chemical Corp., St. Louis, MO) at a final concentration of 10^{-4}M, (*b*) high potassium concentration, achieved by changing the usual buffer salts to a salt mix containing 65 mM KCl, replacing Na^+ ions, and (*c*) addition of 10 μg of the calcium ionophore A23187 (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA). Each test tube had a total volume of 1 ml and contained ~2×10^5 cells, corresponding to ~200 mg of protein. At each temperature we also measured the basal secretion of an identical chromaffin cell suspension, treated with the same procedure as all other test tubes, but excluding the secretory agents. The amount of CA present at the beginning of the experiment as a consequence of the prior procedures was referred to as the zero-time quantity. All experiments were carried out in triplicate.

Kinetics

Kinetic measurements of ACH-induced release were carried out at various temperatures. 1 ml of chromaffin cell suspension was preincubated at the desired temperatures for 10 min. Stimulation was evoked by addition of 10 μl ACH, resulting in a final concentration of 10^{-4} M. Secretion was terminated at time intervals from 0.5–20 min by placing the appropriate tubes in an ice bath followed by centrifugation for 5 min at 1,000 *g*. The supernatant was then analyzed for CA content. Induced secretion relative to the basal quantity was normalized with respect to the maximum value obtained at 37°C. All experiments were carried out in triplicate.

Resensitization

Our experiments on secretion induced by ACH demonstrated the known feature of desensitization, namely, cessation of release after a short time interval even in the continued presence of the secretagogue. Preliminary work suggested that recovery of secretion could be achieved by precipitating the cells and resuspending them with the same or fresh environment. This recovery mechanism was investigated as follows: secretion with ACH was invoked as explained in the Procedures section at 30 and 37°C.

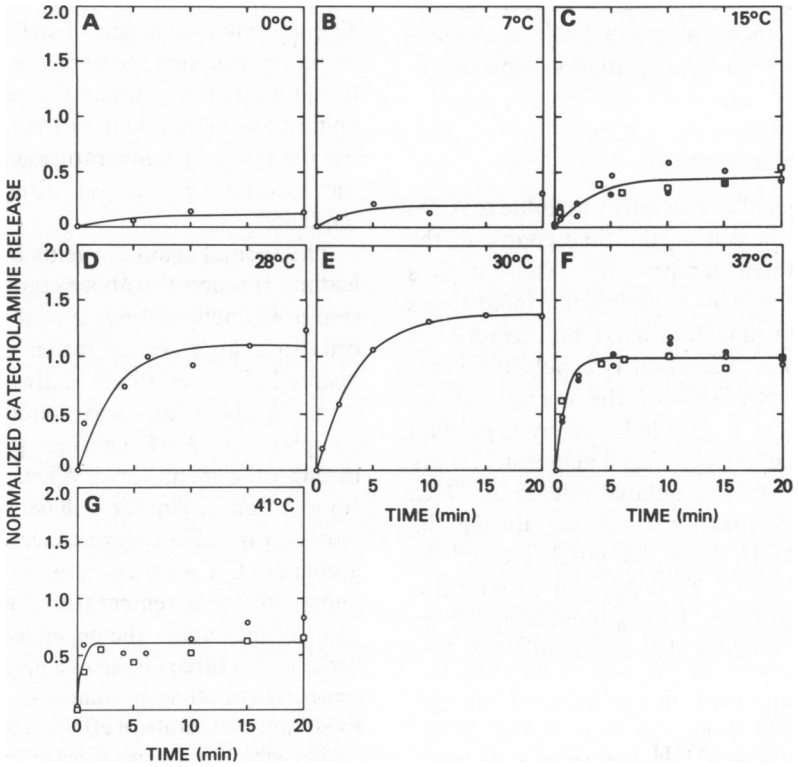

FIGURE 2 The dynamic change of net CA secretion by ACH stimulus at different temperatures. The continuous curve represents the least-squares fit of Eq. 5. The various labels denote independent runs. The data is normalized as in Fig. 1.

Next, cells were sedimented by centrifugation. The supernatants were collected separately for each tube and were reintroduced into their original tubes after various time periods ranging from 5 s to 10 min. The cells and the supernatants were kept at the incubation temperature during the entire procedure. The tubes were then further incubated for 20 min, and the supernatant separation with CA content determination followed. In one set of control tubes the supernatant was removed after the first incubation period and CA levels were determined without reintroduction. In the other sets of control tubes the supernatant was not removed but rather incubation was continued for the entire duration of each lag period and the second incubation. The increment in secretion due to this resensitization was evaluated taking into account the control values. Total CA level was normalized with respect to net secretion at 37°C.

RESULTS

Release at Various Temperatures

Determination of the effect of temperature on the different stages of CA secretion, was achieved by incubating the cells with three agents: ACH, high K^+ concentration, and Ca^{++} ionophore. CA secreted at the various temperatures was measured and normalized with respect to the net amount of ACH-induced secretion at 37°C. Fig. 1 shows that a similar behavior was detected at high K^+ concentration and by addition of Ca^{++} ionophore, namely, an exponential increase in secretion with temperature rise. The ACH-induced secretion is, however, markedly different and exhibits a maximum ~30°C. Note that ACH-stimulated releases at various temperatures are taken from steady state levels, while those seen in Fig. 1 for the other secretagogues are derived from a nonsteady state situation. These data indicate that the mechanism involving the binding of ACH to its receptor and possible conformational changes of the receptor are highly sensitive to temperature.

Dynamic Measurements

A dynamic determination of the secretory rate due to ACH stimulus was made by measuring the time course of the amount released at different temperatures. Fig. 2 depicts curves obtained in independent experiments. Incubations carried out up to 40 min show that maximum secretion is obtained after 10–15 min. Secretion is a pulsatile event being initially rapid, with 85% of the overall release occurring during the first 5 min, followed by a gradual decay to zero in the rate of release. Fig. 3 demonstrates the pulsatile nature of the ACH-stimulated release at 37°C. Thus a process of desensitization is evident during the continued exposure to ACH. Since the initial rate and the desensitization process seem to be dependent on temperature in a different manner (Fig. 2), an optimal temperature for maximum secretion is achieved as depicted in Fig. 1.

Resensitization

Upon prolonged exposure to ACH a process occurrs in which secretion ceases. A recovery or resensitization of the cell, in which further secretion is detected, can be achieved.

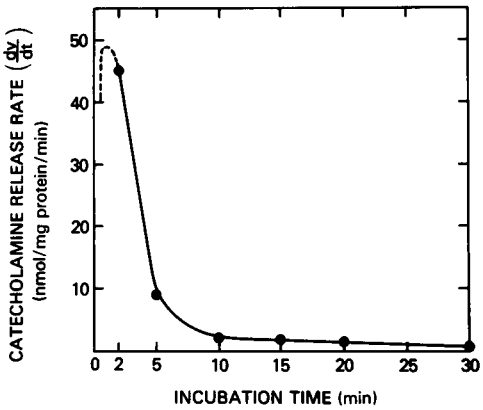

FIGURE 3 Rate of CA secretion over time upon stimulation by ACH at 10^{-4} M.

It is of particular interest that this induction can be achieved by physical, or more precisely mechanical means, and can be effected by centrifugation and sedimentation of the cells, followed by reincubation in ACH-containing medium. In these preliminary observations on resensitization, the qualitative estimates suggest that the level of additional secretion is ~40% of that measured in the original stimulation of the cells, and was practically uninfluenced by the duration of the mechanical agitation (Y. Hiram, A. Nir, A. Greenberg, and O. Zinder, unpublished results).

DISCUSSION

The accepted sequence of major events in the stimulus-secretion coupling process are (*a*) binding of ACH to receptors, (*b*) membrane depolarization, (*c*) Ca^{++} entry, and, (*d*) secretion. Our experiments were aimed at isolating the effect of temperature on these stages by initiating the secretory process at different levels of the above sequence.

The normal chain of events begins by ACH stimulation leading through the above steps to secretion. A bypass of step *a* was achieved by depolarization via exposure of the cells to a high concentration of K^+. Steps *b* to *d* then resulted. Further direct activation at a more advanced stage in the chain was induced by use of the Ca^{++} ionophore A23187 causing penetration of Ca^{++} ions through the membrane. When the process is initiated at steps *b* and *c*, similar behavior and an almost identical temperature effect were observed. Fig. 1 shows that the amount of CA secreted by use of K^+ and Ca^{++} ionophore is augmented with temperature increase. By taking the tangents of the curves, the dependence on temperature can be divided into three distinct regions (*a*) a region in which the temperature effect is small ($T \lesssim 15$°C) (*b*) a region with moderate temperature effect (15°C $\lesssim T \lesssim 30$°C), and (*c*) a region where a steep increase in temperature influence is seen. In these partial chain of events (steps *b* and *d*) the change in temperature should have a profound influence on

the physical properties of the granule and cell membranes, the intracellular fluid, and on the biological activity of the cells (such as enzyme action). It would be expected that as the temperature increases, the fluidity of the membranes will become higher and the viscosity of the cytoplasm lowered. These changes favor a facilitation of fusion and secretion (21, 34).

A transition in chromaffin granule membrane physical properties may exist at T = 16–19°C. Perlman (35) observed a sharp change in the permeability of the granule membrane to high molecular weight nonelectrolytes. Such a transition though, was not evident in granule membrane tensile strength as found by us in a previous publication (7). If both granular and cellular membranes exhibit a high crystalline state below this temperature, insensitivity of the secretion process to this temperature range would be expected. The absence of such sensitivity at this range is evident in our experimental results of release using high K^+ concentration and Ca^{++} ionophore as secretagogues (Fig. 1). Above this temperature (16°C), the granule membrane exhibits a change in physical properties most probably indicating a morphological phase change (35, 36). Zinder et al. (37) reported a higher content of proteins as well as lower cholesterol to phospholipid ratio for the cellular plasma membrane than for the granular membrane. These suggest that the transition point for the plasma membrane and the granular membrane may well be different. Indeed, our data suggest a second transition point at ~30°C where the increase of secretion with temperature rise became most sensitive. These results correlate extremely well with the findings of Schneeweiss et al. (28) who reported a transition in fluorescence polarization in isolated adrenal medulla plasma membranes at ~30°C, which they attributed to changes in the phase behavior of the membrane lipids. It is remarkable that this transition is strongly evident even in our complex system that involves whole cells rather than isolated membranes. Above this temperature both membranes and the cytoplasm are in a state of high fluidity, which most probably facilitates membrane conductance to ions, more rapid depolarization, fusion, and release. Below 30°C, yet above 19°C, a moderate temperature influence is evident probably due to the fact that at this temperature range only the granular membrane has gone through a phase transition. Temperature dependence of membrane fluidity and transition states was studied by Papahadjopoulos (36) using artificial phospholipid membranes. He found that different proteins have specific effects on the transition temperature, which may shift its value by ~10°C.

When secretion was induced by adding ACH, maximum release was realized at ~30°C (see Fig. 1). When this mechanism is activated two additional processes are involved: (*a*) binding of ACH to its receptor and (*b*) deactivation or desensitization in the activity of the ACH-bound receptors. The results of the kinetic experiment shown in Fig. 2 indicate that the initial rate of release, associated with binding of ACH to its receptor, increases monotonically with temperature rise, while the finite level of secretion, after complete desensitization, reaches peak levels at 30°C and declines at higher temperatures. This suggests that the process of desensitization is also influenced by temperature, though at a different rate than the initial release activity. Desensitization does not appear to be a pure chemical process, but is probably associated with the physical state of the ACH receptor (ACH-R) complex in the membrane relative to the solute distribution in the immediate external vicinity. Hence desensitization should be strongly affected by the membrane morphology and fluidity as was also suggested earlier (21, 26, 27). It would then be expected that desensitization will be accelerated when the cellular membrane is beyond the transition temperature (30°C) and should compete strongly with the initial fast processes leading to secretion. Also, the possibility of receptor internalization, as shown for the low density lipoprotein (LDL) receptor (22) and the insulin receptor (23, 24), could also be an explanation for the time-dependent down regulation of the ACH receptor.

The proposed model involves three rate constants, k_1, k_2, and k_3. The rate coefficients do not necessarily represent chemical processes but rather a total measure for each stage as outlined in Fig. 4. Using excess ACH, the rate at which the cellular receptor reacts is given by

$$\frac{d(R)}{dt} = -k_1(R), \tag{1}$$

where R is the measure for the free receptor concentration; ACHR*, the active complex of ACH and the receptor, is produced by the binding process and consumed by desensitization, hence

$$\frac{d(\text{ACH-R}^*)}{dt} = k_1(\text{R}) - k_2(\text{ACH-R}^*). \tag{2}$$

The change of ACHR* with time t, is then given by

$$\frac{(\text{ACH-R}^*)}{(R_0)} = \frac{k_1}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}), \tag{3}$$

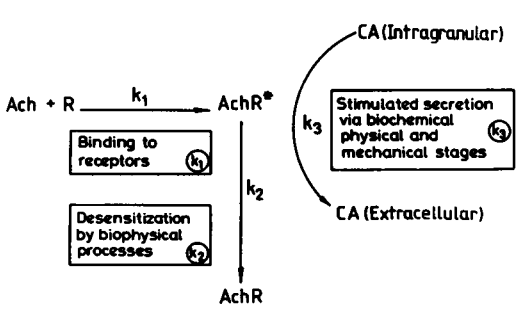

FIGURE 4 Scheme of a kinetic model for CA secretion following stimulation by ACH.

where R_0 is the initial value for R. Using Eq. 3 we can evaluate the progress of the release process

$$\frac{(CA)}{(R_0)} = \int_0^t k_3 \frac{(ACH\text{-}R^*)}{(R_0)}\,dt$$

$$= \frac{k_3}{k_2 - k_1}(1 - e^{-k_1 t}) - \frac{k_1 k_3}{k_2(k_2 - k_1)}(1 - e^{-k_2 t}). \quad (4)$$

k_1, k_2 and k_3 were evaluated at different temperatures by fitting Eq. 4 to the dynamic experimental results by the least-squares technique. In Eq. 4 it is implicitly assumed that the internal stores of CA do not become depleted during the experiment as is demonstrated elsewhere (8). The model confirms that at all temperatures examined, the binding of ACH to its receptors is relatively very fast, i.e., $k_1/k_2 \rightarrow \infty$ and $k_1/k_3 \rightarrow \infty$. Since the amount of ACH-R* reaches its maximal value after the time

$$t_m = \frac{\ln \frac{k_1}{k_2}}{k_1 - k_2},$$

it is evident that in this special case $t_m \rightarrow 0$, and Eq. 4 can be reduced to the simple form

$$\frac{(CA)}{(R_0)} \simeq \frac{k_3}{k_2}(1 - e^{-k_2 t}). \quad (5)$$

This implies that our experimental determination of release and desensitization started with the full potential of ACH-R*, which is achieved almost instantaneously following addition of the stimulant. The integrated results for each temperature are shown in Fig. 2 (*a-g*) as the continuous curves.

A combination of the theoretical curves at various temperatures is shown in Fig. 5. It is evident that at short time intervals, when desensitization is not fully active, the initial rate of release increases monotonically with temperature rise. Asymptotic consideration of Eq. 5 shows that when $t \rightarrow 0$ the rate of secretion is simply k_3. Fig. 6 depicts the dependence of k_3 on the temperature. The existence of a transition at ~30°C is striking. Since k_3 is a measure of the rate of the biochemical, physical, and mechanical processes involved in exocytosis, the sharp change may be a reflection of a change in the internal properties of the cellular membrane. A possible transition at ~15°C is also indicated by the results in Fig. 6. These are in agreement with the results obtained by use of excess K^+ or Ca^{++} ionophore as secretion stimulants (see Fig. 1), where the possible transitions at 30°C and ~15°C are shown. The second critical change at ~15°C seen in Fig. 1 was also noticed by El-Fakahany and Richelson (21), where no observable changes in activation and desensitization were seen below 15–20°C. The possible relation to the granular membrane fluidity was discussed earlier in this section.

The resemblance in the temperature dependence of k_3 and total release induced by the different secretagogues, K^+ or Ca^{++} ionophore, is evident. It implies that indeed the use of K^+ or Ca^{++} ionophore isolates the effect of temperature on the fourth stage in the sequence of stimulus-secretion coupling.

At high temperatures desensitization dominates the secretion process at a relatively early stage resulting in low total level of CA released. The dependence of the rate of desensitization on temperature predicted by our model is shown in Fig. 7. The transition at ~30°C is again clear. However, there appears to be only minute dependence on temperature below this point indicating that the desensitization is associated with the properties of the cellular membrane (21).

Asymptotic examination of Eq. 4 at $t \rightarrow \infty$ shows that the total amount of CA released approaches the ratio

$$\frac{(CA)}{(R_0)} \rightarrow \frac{k_3}{k_2}. \quad (6)$$

Following the experimental results, the kinetic model predicts that this asymptotic level does not change monotonically with temperature, as is shown in Fig. 5, where the

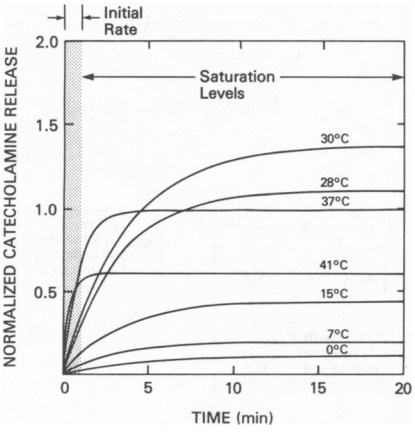

FIGURE 5 Superposition of the theoretical predictions of dynamic ACH-induced secretion at various temperatures.

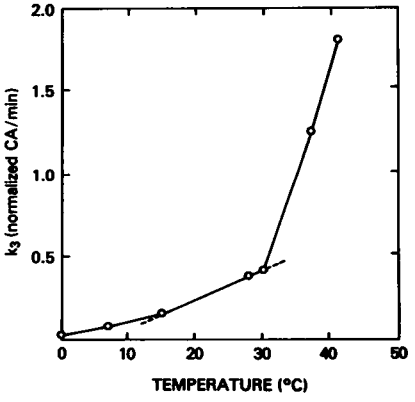

FIGURE 6 The dependence of k_3, the uninhibited secretion rate, on temperature.

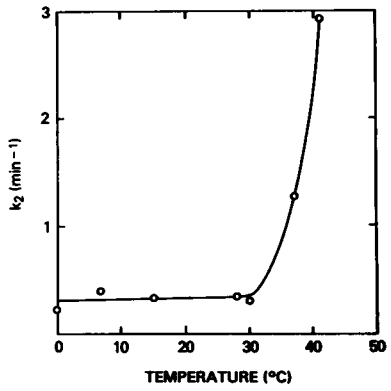

FIGURE 7 The dependence of k_2, the rate of desensitization, on temperature.

curves at various temperatures intersect with each other. A comparison of the dependence on temperature of k_3/k_2 determined from the kinetic data of Fig. 2, with independent experimental results obtained after 20 min of incubation (Fig. 1) is depicted in Fig. 8. The fitted curve closely follows the experimental measurements over the wide spectrum of temperature and agrees well with the position of the maximum value for total CA release.

SUMMARY AND CONCLUSIONS

The kinetic model presented for secretion and desensitization agrees closely with the experimental measurements. The temperature dependence transitions observed experimentally suggest that physical and mechanical properties of the plasma and granular membranes can play an important role in CA release. It is of course very difficult to isolate the effect of temperature on these properties from other temperature dependent parameters in intact cells. Nevertheless, our preliminary experiments in resensitization of CA secretion, induced by physical procedures such as centrifugation or mechanical agitation and almost unaffected by their duration, serve to support the conjecture of an important involvement of physical and mechanical properties in the process. The use of various secretagogues and the observed different responses to temperature change facilitate the discrimination between the various stages leading to secretion. These results suggest that membrane composition and properties are significant in the control of secretion by exocytosis.

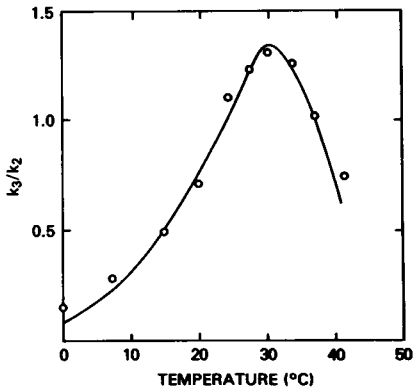

FIGURE 8 A comparison of the predicted total amount of CA release to the measured quantity at various temperatures. k_3/k_2 is predicted using a fit of the theoretical model to the dynamic experiments (continuous curve). The data points represent the independent runs depicted in Fig. 1.

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