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Kinetic characteristics of calcium-dependent, cholinergic receptor controlled ATP secretion from adrenal medullary chromaffin cells

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Adrenal chromaffin cells secrete catecholamines (CA) and ATP in response ot acetylcholine (ACh) and high $[K^+]_o$. The release proces is relatively fast making it difficult to measure the early phase of the secretory response. Recently we were able to resolve the time course of the secretory response by measuring the release of ATP using luciferin-luciferase included in the extracellular medium. For the three secretagogues studied, ACh, nicotine and high $[K^+]_o$, the early phase of release followed a complex kinetics. Allowing for an initial delay of the secretory response, the kinetics could be described as the sum of two power exponential processes. Increasing the temperature from 23 to 37 °C induced a marked decrease in the two time constants needed to fit the early time course of the ATP secretion. The activation energies, estimated from Arrhenius plots, were approx. 20 and 16 kcal/mol for both phases of ATP release induced by either cholinergic agonists or high $[K^+]_o$. These results suggest that cholinergic receptor activation and membrane depolarization induce ATP (and CA) secretion through a common pathway. The initial delay in the onset of the secretory response decreased with increasing doses of secretagogue and with temperature. We propose that the delay preceding the actual onset of ATP release represents the time required for generation of intracellular second messengers. The effective concentration attained by these messengers depend apparently on both receptor occupancy by the agonist and the ensuing Ca^{2+} channel activation.

Introduction

Acetylcholine (ACh) stimulation of adrenal medullary chromaffin cells in culture causes the release of catecholamines (CA) and co-factors of either known function such as the enzyme dopamine β-hydroxylase (DBH) [1] or unknown function such as ATP [2–7].

Catecholamine secretion from bovine chromaffin cells in response to cholinergic agonists is a rapid process [8]. This model system has been studied extensively over the past decades as a prototype for exocytosis [9,10]. Nevertheless, the mechanisms by which chromaffin vesicles containing CA eventually fuse with the plasma membrane and release their content outside the cell remain poorly understood [11]. It is known that the concentration of Ca^{2+} in the cytosol increases rapidly after stimulation of the cells [12] and, the increase in Ca^{2+} is dependent on the activation of Ca^{2+}-channels located in the plasma membrane. However, the process by which calcium subsequently induces granule contact and fusion of the vesicles with the plasma membrane is not known with certainty. Once calcium acts, at least two types of membrane fusion processes occur. Simple exocytosis involves fusion of a single vesicle with the plasmalemma. However, chromaffin granules situated more deeply within the cell also fuse with the membrane of the granule which have already secreted by the simple process and are still attached to the plasma membrane. This mode of secretion is named 'compound exocytosis'. An additional type of heterogeneity is found in typical chromaffin cell preparations, which contain cells specific for either epinephrine or norepinephrine. As a result, in face of such complex features, it is anticipated that the kinetics of secretion might be of a complex nature.

Hitherto, the kinetics of secretion in chromaffin cells has been barely studied [13,14]. Moreover, previous kinetic studies of CA secretion from chromaffin cells have suffered from inadequate temporal resolution [13,14], because detection of catecholamine release from

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cells was limited by the bulk diffusion of these compounds for collection and analysis. By contrast, we [15] and others [16] have recently shown that it is possible to measure the time-course of the secretory response in chromaffin cells both quantitatively and virtually on-line using highly purified and stabilized luciferin-luciferase to detect the release of the co-factor ATP [17]. We have also shown that in chromaffin cells ATP and CA release share many properties, including the dependence on ACh receptor activation, the absolute requirement for $[Ca^{2+}]_o$ and the sensitivity to low doses of phenotiazine drugs [18].

In the present paper we have characterized the kinetic properties of ATP secretion, and quantitatively analysed the time-course of the process in a small population of chromaffin cells and we have also attempted to estimate the apparent activation energy of the early phase of the secretory process.

Methods

Luciferin-luciferase enzyme preparation and ATP assay

The luminescent oxidation of luciferin catalyzed by luciferase was used to detect the ATP secreted by the cells. The instrument used to stimulate the cells and to measure the light has been described elsewhere [19]. The contents of a vial of luciferin-luciferase (L-L) kit (Analytical Luminescence Laboratory, San Diego, CA) were dissolved in 5 cm^3 of modified Krebs (KRB) solution (mM: 135, NaCl, 10 $HNaCO_3$, 10 Hepes, 5 KCl, 2.6 $CaCl_2$, 2 $MgCl_2$, 10 D-glucose (pH 7.4)) plus 1 mg/cm^3 of bovine serum albumin (Calbiochem-Boehringer). The assay was carried out in the plastic tubes by mixing 140 μl of the Krebs solution containing freely suspended chromaffin cells plus 50 μl of the L-L solution. In order to maintain ecto-ATPases at low activity, cell density in the reaction mixture was kept below 100 cells/μl. For experiments using high external K^+ as secretagogue, different volumes of KRB (mM: 140 KCl, 10 $NaCO_3$, 10 K^+-Hepes, 2.6 $CaCl_2$, 2 $MgCl_2$, 10 mM D-glucose; 1 mg/ml BSA (pH 7.4)) were added to achieve the desired $[K^+]_o$. Provided the concentration of cells in the reaction mixture was kept below 100/μl, emitted light levels during the experiments with chromaffin cells remained at the steady-state level reached after stimulation. Light output from the reaction mixture containing resting cells was constant, 0.25 to 2.5% of the maximum signal recorded in each experiment. The efficiency of the luciferin-luciferase reaction, i.e. the number of photons emitted divided by the number of molecules of substrate oxidized, is close to 1.0 [20]. During the oxidation of luciferin the energy released (50 to 72 kcal per mole quanta) is utilized to create an electron excited state either directly or by energy transfer to fluorescent molecules formed or present during the reaction [21]. The highest energy emission corresponds to a wavelength maximum of 546 nm. The optimum pH is 7.75 and the optimum temperature is about 25° C [22].

The technique as used here measures the rate at which light is emitted by the ongoing reaction. However, it is easy to infer from the rate of reaction how much ATP is present. To calibrate the light signal, fixed amounts of ATP dissolved in 10 μl of KRB solution were injected into 190 μl of KRB solution containing the L-L enzyme mixture. Fig. 1. shows the time course of the light signal in response to the addition of 10 μl of KRB solution containing 40 pmol of ATP. The rapid ($t_{1/2} < 1$ s) rise of the light signal indicates that mixing was complete in less than 1 s. The initial jump was found to be proportional to the amount of ATP aded in the range from 10 to 200 pmol. For a bimolecular reaction, provided that one of the substrates of the bimolecular reaction is present in excess (in this case luciferin), the rate of the reaction is proportional to the concentration of the other substrate (ATP). Consistent with this interpretation of the L-L reaction, a single rate constant (k) was sufficient to fit the time course of the light emitted following the addition of a given amount of ATP (Fig. 1A). Under these conditions, k was independent of the amount of ATP added. As expected, k depended on the temperature at which the reaction was carried out (Fig. 1A). Lowering the temperature from 37 to 23° C increased the time constant ($\tau = k^{-1}$) of decay from approx. 550 to 1700 s (Fig. 1A). For all the experiments presented here the concentration of luciferin in the L-L mixture was kept constant. Measurements of the extent of ATP release were made approx. 120 s after the addition of the secretagogue. Thus the ATP level measured at 120 s was lower than the actual amount of ATP secreted by the cells.

Similar levels of emitted light were obtained either by single additions of ATP or by consecutive additions. Light levels, although reduced by the addition of small volumes containing the secretagogues due to dilution of the ATP already present in the extracellular medium, were unaffected by ACh (< 100 μM), atropine (< 10 μM), D-tubocuranine (< 10 μM). However, detergents used to release the ATP from cellular pools, i.e. Triton X-100 (1%) and digitonin (20 μM) reduced the light emitted by 30 and 25%, respectively. Light emitted in the presence of high K^+ decreased as $[K^+]_o$ was augmented from 5 to 100 mM. 50% inhibition was observed at 100 mM $[K^+]_o$. In all relevant cases a specific standard curve was prepared. The assays with the cells were performed either at room temperature (approx. 23° C or at 36–37° C).

Preparation of chromaffin cells

Bovine chromaffin cells were prepared and cultured by a modification of the method of Greenberg and Zinder [23]. Briefly, glands were digested in 0.2% collagenase B (Boehringer-Mannheim) at 37° C. After a

purification step in percoll gradient (Pharmacia, Piscataway, NJ), the cells were cultured in a mixture of DMEM/F12 medium (Sigma, St. Louis, MI) in culture flasks ($50 \cdot 10^6$ cells, 30 cm^3 medium) at 37°C in an atmosphere of 5% CO_2. After 48 h in culture the cells were detached mechanically and centrifuged (400 rpm, 10 min). They were then resuspended in 25 cm^3 of a modified Krebs buffer (Na-KRB) of the following composition (mM): 120 NaCl, 5 KCl, 2.5 $CaCl_2$, 10 $NaCHO_3$, 10 glucose, 10 Na-Hepes, bovine serum albumin (BSA, 1 mg/ml) at pH 7.4. Cells were centrifuged and resuspended three times in Na-KRB. Final cell density was 1000 cells/μl.

Data analysis and empirical kinetic model

Records of the secretory response were made from playbacks of the analog tapes. A low pass filter with the corner frequency set at 10 Hz (Frequency Devices, 902LPF) was placed at the input of the analog-to-digital converter used to digitize and to store the records (Digital Storage Oscilloscope, Nicolet-4094B, Nicolet Test Instruments Division, Madison, WI). Each digitized record (16000 points) was transferred to a computer for kinetic analysis. During this process the number of points representing the time course of the signal was reduced to 250 using a skip factor of 64 points. Each set of points representing a record was divided by the mean value of 10 points including the maximum.

The luciferin-luciferase technique presumably allows detection of the ATP secretion at the site of release. However, we consistently found a time lag (now on referred to as dt) between the application of the response in all records in which cholinergic agonists were used. Since the cholinergic secretagogues were added in a small volume of Krebs solution, this time lag (dt) must be due, at least in part, to solution mixing and diffusion of the secretagogue through the unstirred layers around the cells.

In preliminary experiments we observed that the secretory response was made up of two components which could be represented by the sum of two exponential functions of the form

$$\mathrm{atp}(t) = A + B(1-\exp(-t/\tau_f))^n + C(1-\exp(-t/\tau_s))^m \qquad (1)$$

in which the integers n and m were varied from 1 to 4. A represents the basal ATP level at the start of the record, and B (or C) represents the steady-state ATP level reached and, τ_f (or τ_s) the time constant of the corresponding component (f, fast; s, slow).

Each set of points (ATP(i), $i = 1, N$), representing a record, was subjected to a least-squares fit [24] by the empirical function (atp(i), $i = 1, N$). Sixteen pairs of values of m and n were considered. For each pair the time constants τ_f and τ_s were taken as free-parameters.

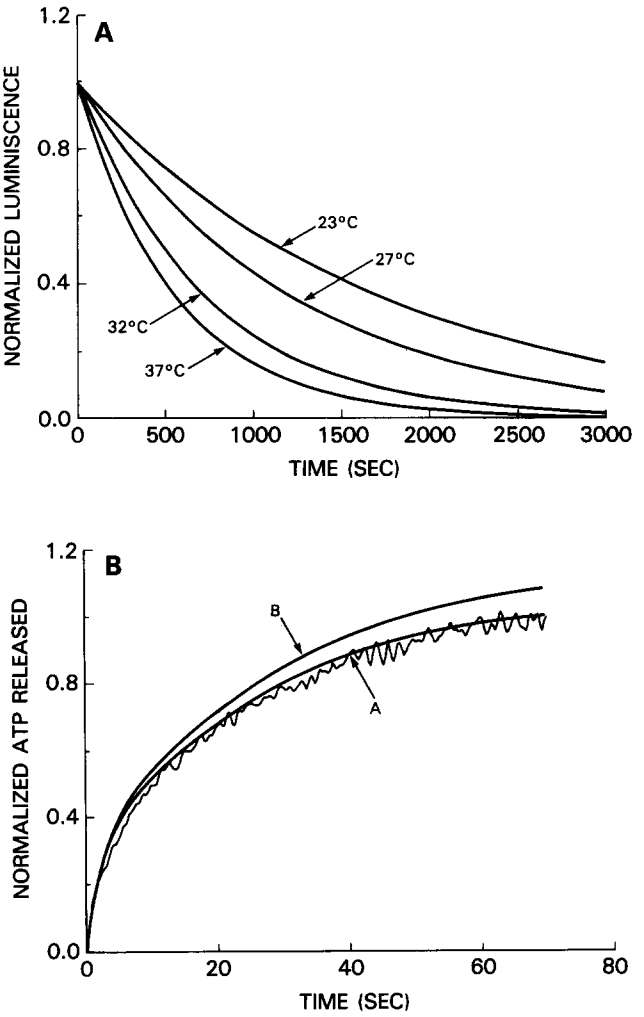

Fig. 1. Time-course of decay of the light signal in the absence of cells. (A) Each curve represents the best fit of a given experimental record and was calculated as

$$\mathrm{ATP}(t) = \mathrm{ATP}_{max} \exp(-t/\tau_D) \qquad (4)$$

where τ_D represents the time constant of decay of the signal. Mean values for the time constant τ_D were used to generate the normalized records. At each temperature four records were made using different amounts of ATP in the range from 10 to 40 pmol. τ_D was found to be independent of the amount of ATP added. Data given as mean ± S.E. with $n = 4$:

Temperature (°C)	Time constant τ_D (s)
23	1683.4 ± 62.7
27	1191.3 ± 86.5
32	715.6 ± 88.3
37	549.4 ± 11.6

(B) Noisy record represents the normalized light signal obtained from the reaction chamber containing approx. 20000 chromaffin cells at 23°C immediately after a sudden elevation of the $[K^+]_o$ from 5 to 30 mM. Smooth curve 'A' was calculated using Eqn. 1 and represents the best fit to the experimental curve. The values for the time constants τ_f and τ_s were 6.9 and 32.4 s, respectively. Smooth curve 'B' was calculated using Eqn. 3 as explained in Methods.

The goodness of the fit was evaluated using a computer program designed to minimize the following residual,

$$\text{Res} = \left\{ \left[\sum (\text{ATP}(i) - \text{atp}(i))^2 \right] / N \right\}^{1/2} \quad (2)$$

Correction for ATP degradation

The luciferin-luciferase kit used in the present series of experiments contained luciferin in excess. Therefore, the rate of reaction should just be proportional to the concentration of ATP. To verify this assumption we measured the rate of decay of the light signal as a function of the amount of ATP added to the reaction chamber (10 to 40 pmol). A single time constant was required to fit the decay in the light signal and the time constant of decay was independent of the amount of ATP added (see above; Fig. 1).

Fig. 1B illustrates the effect of the decay of the light signal caused by the ATP hydrolysis on the evaluation of the kinetic parameters of the early phase of the secretory response at 23° C. The best fit of the noisy record gave values for τ_f and τ_s of 6.8 and 32.4 s, respectively (A). To correct for the decay we divided the experimental curve by an exponential function with the appropriate time constant of inactivation,

$$\text{ATP}_{co}(t) = \text{ATP}(t) / \exp(-t/\tau_D) \quad (3)$$

where τ_D represents the time constant of decay. ATP release after correction for ATP hydrolysis was then fitted using Eqn. 1. At 23° C τ_D was approx. 1700 s and the time constants τ_f and τ_s obtained from the fit of the experimental record using Eqn. 1 before and after correction for inactivation decreased from 6.8 and 32.4 s to

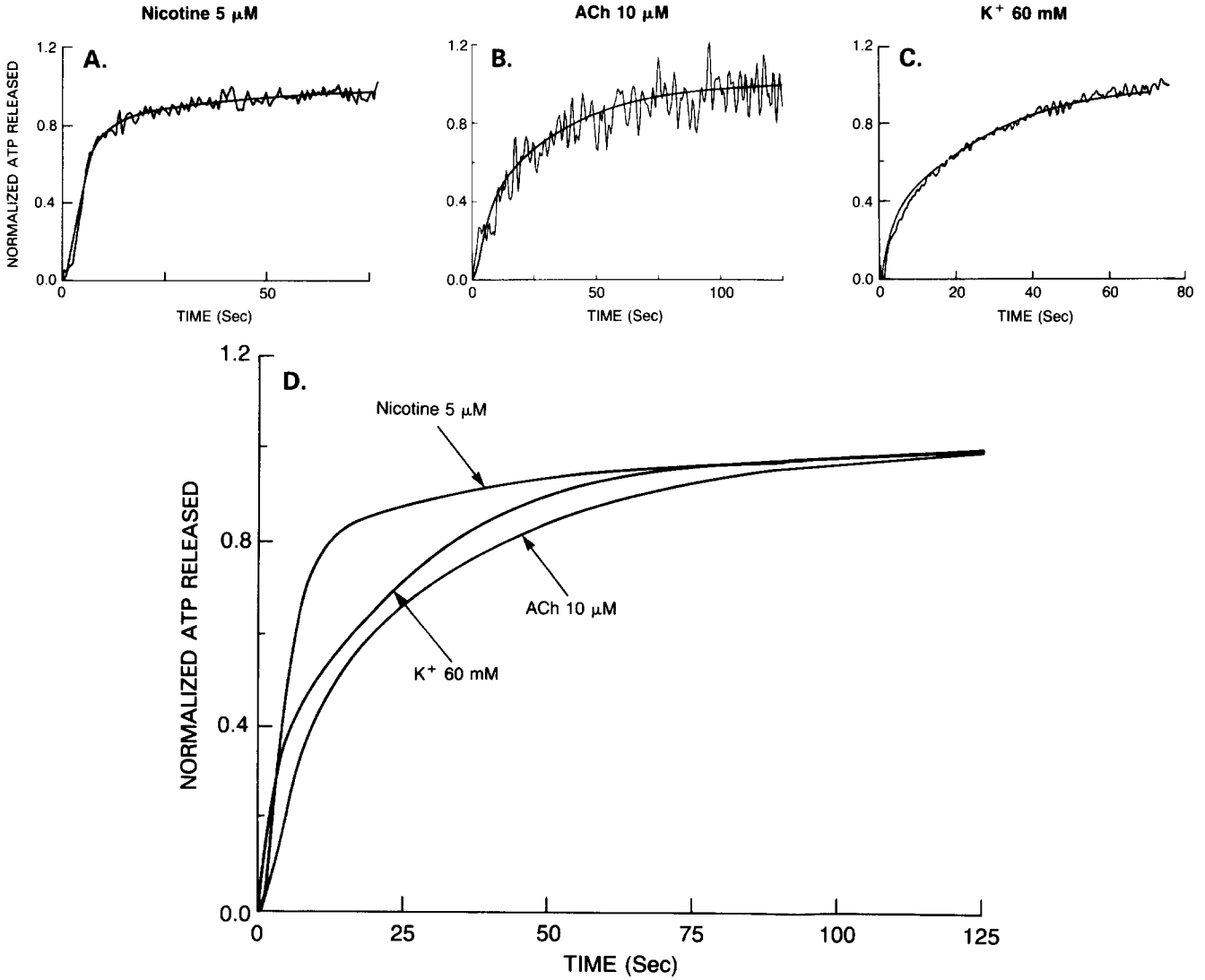

Fig. 2. Comparison between the time course of the ATP release evoked by nicotine (A), ACh (B) and high K^+ (C). Noisy records shown in the upper panels represent typical secretory responses to: (A) nicotine (5 μM), (B) ACh (10 μM) and, (C) high K^+ (60 mM). Records were obtained at 23° C and were normalized as described in Methods. Smooth curves were calculated with Eqns. 1 using n and m as fixed parameters and, A, B, C, τ_f and τ_s as free-parameters. (D) Superimposed fitted records from A, B, and C.

6.8 and 31.8 s, respectively. This result shows that the uncorrected time constants are very similar to the true time constants. For this reason, in the analysis presented here we considered only the original uncorrected records (Fig. 1B, record A). It should be emphasized that the extent of ATP release measured 80 s after the addition of the secretagogue approx. 90% of the ATP level which would have been reached if the reaction had occurred in the absence of ATP hydrolysis (Fig. 1B, record B).

Results

Early time-course of the ATP secretion evoked by different secretagogues

Cholinergic receptor stimulation of the cells was achieved by addition of a small volume (10 μl) of the Na-KRB solution containing the secretagogue at a concentration 10-fold higher than the desired final concentration in the reaction chamber. Since mixing and diffusion times through the unstirred layers are presumably similar for both cholinergic agonists, differences in the time course of the response to nicotine and ACh should represent genuine differences in the kinetics of activation of the ATP release process.

Shown in the upper part of Fig. 2 are three typical records of the ATP secretion evoked by nicotine (A), ACh (B) and by a sudden elevation in $[K^+]_o$ from 5 to 60 mM (C). To facilitate the comparison between the records, the time course of the response ATP(t) was divided by the average of the last 10 ATP(t) values, usually taken 120 s after the addition of the secretagogue (referred to as 'normalized ATP released'). The smooth curves were calculated using the empirical model and represent the best fit to the points. Fitted curves can be compared in part D. The records illustrated in Fig. 2 represent typical responses obtained with each of the three secretagogues used in this work. All our records with the cholinergic agonists required values of n between 2 and 4, the best fit being obtained by setting n equal to 3 and m equal to 1. These components gave the best fit regardless of the concentration of the cholinergic agonist used to elicit secretion. The values of the time constants τ_f and τ_s were 2.7 and 33.4 s for nicotine (Fig. 2A), and 4.5 and 49.1 sec for ACh (Fig. 2B). In contrast, the best fit of the early time course of the ATP secretion elicited by a sudden elevation in $[K^+]_o$ at the same temperature was obtained if n and m were made equal to 1 and 2, respectively. For the experiment illustrated in Fig. 2C, the time constants τ_f and τ_s which minimized the residual were 3.3 and 20.2 s, respectively.

It was also possible to obtain a good fit of the early part of the release process evoked by either cholinergic agonist or high $[K^+]_o$ by setting n equal to 1 provided we introduced a lag (dt) in the start of the response. This means that from the time of the addition of the secretagogue until the initiation of the response, the computed changes in atp(t) values were assumed to be equal to zero. To test the possibility that the lag (dt) in the secretory response could represent the time required for the activation of the cholinergic receptors and the generation of intracellular messengers [25], we evaluated the effect of temperature on dt.

Effects of temperature on the kinetics of ATP release evoked by cholinergic agonists

Warming the incubation solution from 23 to 37 °C had a marked effect on the early time course of ATP secretion induced by the two cholinergic agonists used

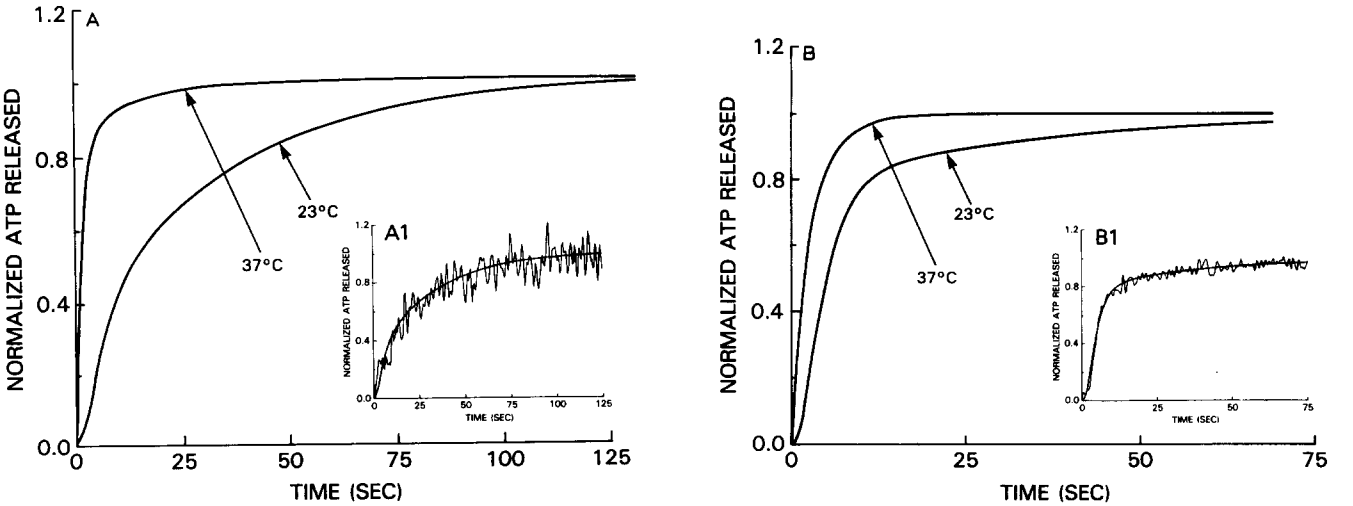

Fig. 3. Effect of temperature on the time course of ATP secretion induced by ACh (A) and nicotine (B). (A) Time course of the ATP release evoked by ACh (10 μM) at two different temperatures. Smooth curve represents the best fit to the original record using Eqn. 1 as described in Methods. At 23 °C: $\tau_f = 4.5$ s and $\tau_s = 49.1$ s. At 37 °C: 1.6 and 12.6 s, respectively. (A1) Normalized original record and the best fit at 23 °C. (B) Time course of nicotine (5 μM) evoked ATP secretion at two different temperatures. At 23 °C values for τ_f and τ_s were 2.7 and 33.5 s, respectively; at 37 °C the corresponding values were 0.8 and 7 s, respectively. (B1) Normalized original record and best fit at 23 °C.

here. It may be seen that the rate of ACh-evoked ATP release was dramatically increased by warming (Fig. 3). Figs. 3A1 and 3B1 depict the actual secretory responses to the application of the cholinergic agonists at 23°C. In the case of the stimulation with ACh, a temperature increase from 23 to 37°C induced a decrease in the value of dt from 4.5 to 1.6 s; τ_f decreased from 2.7 to 0.8 s and τ_s from 33.5 to 7.0 s, respectively (Fig. 3B). The extent of release was rather insensitive to temperature changes in the range from 23 to 37°C (data not shown).

The rate constant of ATP release evoked by cholinergic receptor activation is not affected by concentration of the agonist

The extent of ATP release augmented with [ACh] in a steep sigmoidal manner, the [ACh] to elicit 50% of the maximum secretion being approx. 10 μM (Fig. 4A). The relative contribution of both fast and slow components as a function of the dose of secretagogue is shown for both ACh and nicotine (Figs. 4B, 4C). Only at very high doses of secretagogue there is an increase in the contribution of the fast component over the small component. As the rate constants of high K^+ induced ATP release are profoundly dependent on $[K^+]_o$ (see Fig. 7), we expected that the kinetics of ACh-induced secretion might also depend on the concentration of the cholinergic agonist. However, the rate constants of ACh-evoked release are quite insensitive to the dose of ACh (Fig. 5A). As expected, following this result obtained with ACh, both rate constants for the faster and slow component of secretion are also insensitive to $[\text{nicotine}]_o$ (Fig. 5B).

Effects of membrane potential on the kinetics of ATP release evoked by membrane depolarization

The membrane potential of the chromaffin cell is determined by $[K^+]_o$ [26]. To study the effects of membrane potential on the time course of ATP secretion we applied sudden changes in $[K^+]_o$. Increasing $[K^+]_o$ from the physiological to a higher concentration always evoked prompt release of ATP as illustrated in Fig. 6B. The extent of release increased with $[K^+]_o$ in the range from 5 to 100 mM reaching levels close to 12% of the total ATP in the cells (data not shown). To compare the ATP secretion in response to different $[K^+]_o$ step changes, we have superimposed the normalized (fitted) responses to three different potassium concentrations in Fig. 6A.

The time constants τ_f and τ_s decreased with the size of the $[K^+]_o$ change. For the records of the responses to changes in $[K^+]_o$ from 5 to 30 mM and from 5 to 100 mM shown in Fig. 6A, the time constants decreased from 6.9 and 32.4 s to 0.5 and 6.1 s, respectively. Semilogarithmic plots of the rate constants ($1/\tau$) as a function of the membrane potential (Fig. 7) show that,

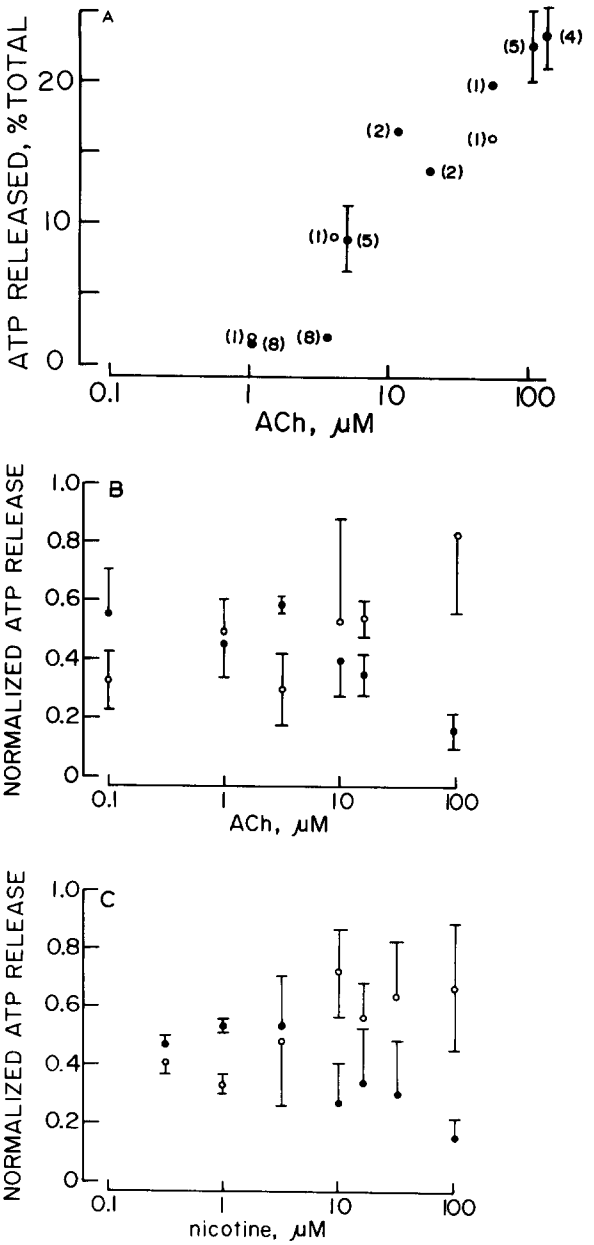

Fig. 4. Extent of ATP release as a function of ACh concentration. (A) Extent of ATP release as a function of ACh concentration. The % of total ATP released from chromaffin cells is plotted as a function of ACh concentration in both absence (filled circles, ●) or in the presence (open circles, ○) of tetrodotoxin (TTX, 3 μM). Data represent the mean values from several experiments (number given in parenthesis) done in triplicate. Vertical bars represent ± S.D. Temperature 37°C. (B) Relative contribution of the two components: fast (○) and slow (●) to the extent of the ACh-evoked ATP release at 23°C. Similar results were obtained at 37°C. Data represent mean ± S.D. of 3–5 experiments. (C) Relative contribution of the two components: fast (○) and slow (●) to the extent of nicotine-induced ATP release at 23°C. Data represent mean ± S.D. of 2–4 experiments.

while 24 mV caused an e-fold change in the rate during the fast component (open circles), 39 mV induced an e-fold change in the rate of ATP secretion during the slow phase (filled circles).

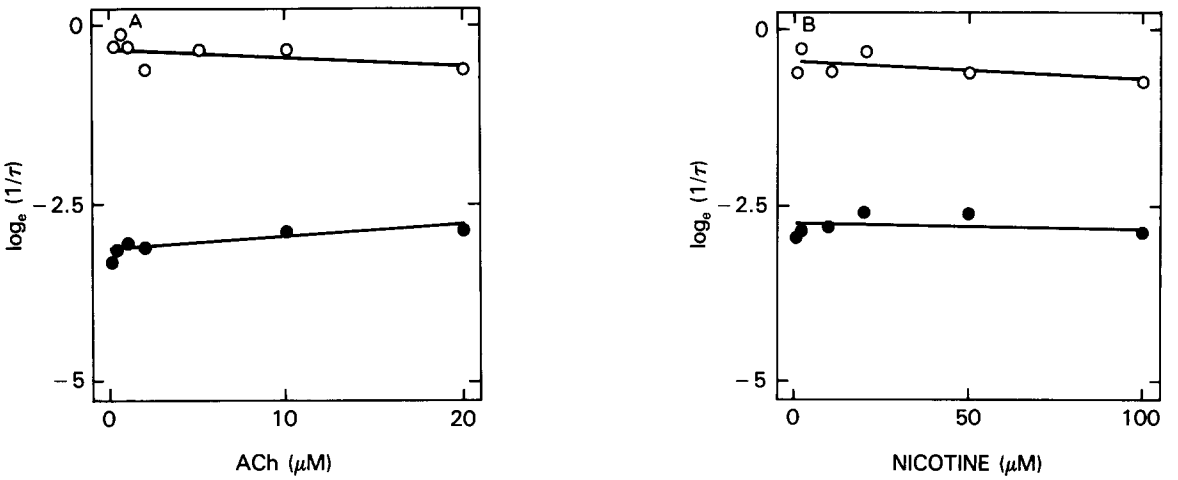

Fig. 5. Effect of agonist concentration on the rate of ATP release induced by ACh and nicotine. (A) Semilogarithmic plot of the rate constant as a function of [ACh]. (B) Semilogarithmic plot of the rate constant as a function of [nicotine]. Rate constant for both the fast (○) and the slow (●) components of secretion are plotted.

The relationship between membrane potential and the extent of release (corrected both for decay and for $[K^+]_o$ inhibition of the signal) is depicted in Fig. 8A. The curve was calculated to fit the experimental points and represents the Boltzmann function often used to describe charge movement and channel gating across excitable membranes [27]. Membrane potentials were calculated assuming that $[Na^+]_i$ was 38 mM, that $[K^+]_o$ plus $[Na^+]_o$ equal 150 mM, and taking P_{Na}/P_K as 0.025 [26]. At the mid-point potential E_o of −6.8 mV, a change of 9 mV in membrane potential evoked an e-fold change in the steady-state value of the ATP released. Open symbols represent data points obtained in the presence of Cd^{2+} (1 mM) a potent blocker of voltage-gated Ca^{2+} channels. The relative contribution

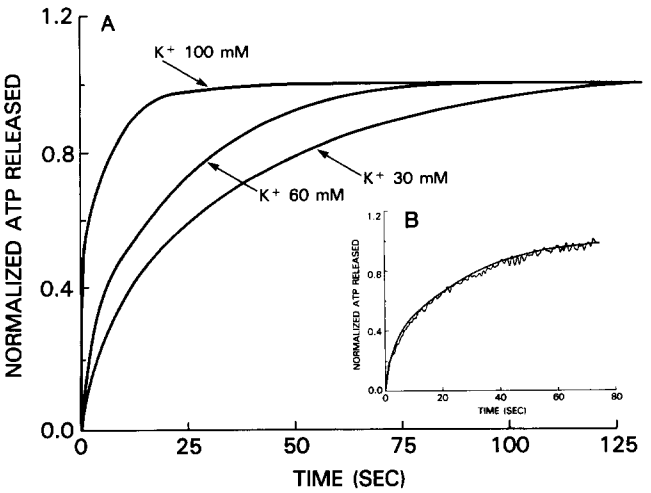

Fig. 6. Effects of membrane potential on the early time course of ATP release. (A) Superimposed fitted records representing the experimental time course of the ATP release evoked by a sudden elevation of $[K^+]_o$ from 5 to 30, 60 and 100 mM (approx. 20000 chromaffin cells at 23° C). (B) Noisy record represents the time course of the ATP release evoked by a sudden elevation of $[K^+]_o$ from 5 to 30 mM. Smooth curve was calculated with Eqn. 1 and represents the best fit of the experimental record. The time constants determined from these records had the following values:

$[K^+]_o$ (mM)	τ_f (s)	τ_s (s)
30	6.9	32.4
60	3.2	20.2
100	0.6	6.1

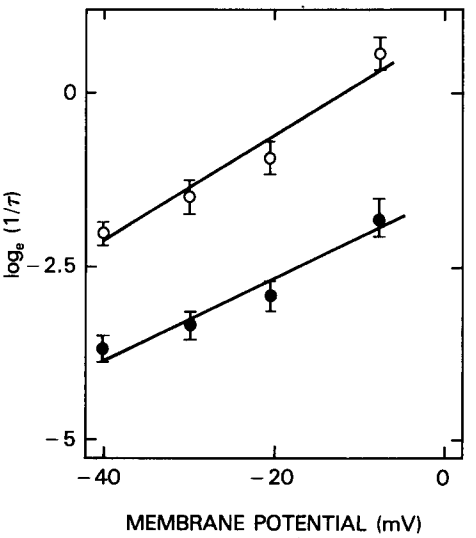

Fig. 7. Voltage dependence of the apparent rates of ATP release. Symbols represent the natural logarithm of the 'apparent' rate constant for the fast (○) and for the slow component (●) of high K^+-evoked ATP release. Membrane potential was estimated as previously described in Results. The apparent rate constants (±S.E.) were calculated from the following mean values of the time constants:

$[K^+]_o$ (mM)	n	τ_f (s)	τ_s (s)
30	8	10.5±3.3	39.9±9.4
45	6	5.7±1.7	28.8±5.1
60	6	2.1±0.8	22.4±6.6
100	3	0.5±0.1	5.8±1.4

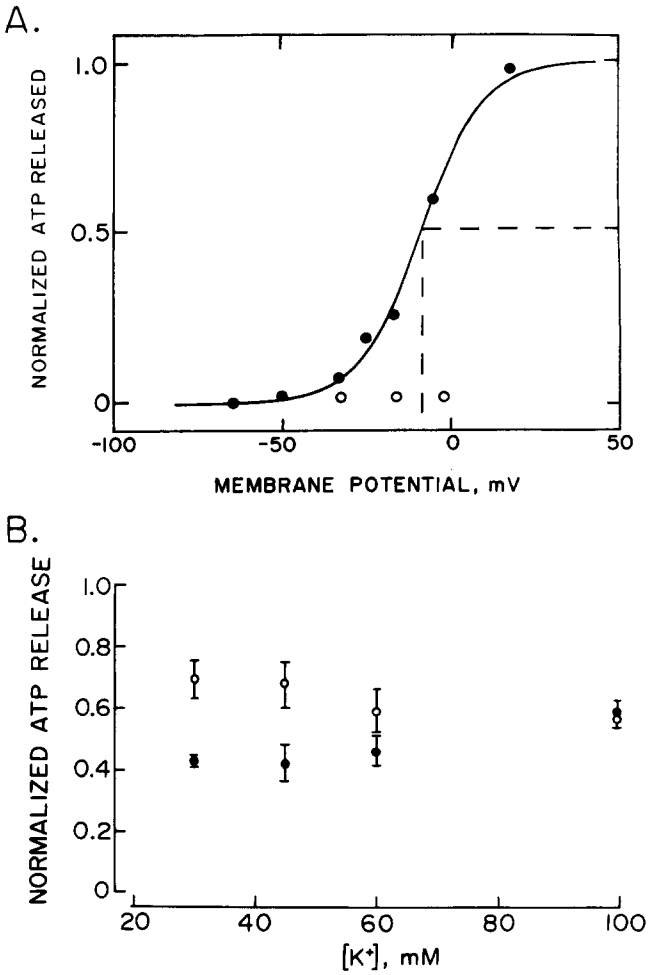

Fig. 8. Dependence on membrane potential of high K^+-evoked ATP secretion. (A) Each point represents the mean value of three measurements. Data points were corrected to take into account the inhibition of the L-L system by high $[K^+]$. The curve represents a least-squares regression fit of the following function shown to the data points:

$$\frac{ATP}{ATP_{max}} = \frac{1}{1+\exp\left\{\frac{a(E-E_o)}{kT}\right\}} \quad (5)$$

In this equation, E represents the membrane potential calculated as described in Results. kT is the Boltzmann factor (= 25.6 meV at 20 °C). From the fit, the following values were obtained: Midpoint potential (E_o) = −6.7 mV. a = −2.7 electronic charges. Empty circles represent single measurements carried out after incubation of the cells in the presence of Cd^{2+} (1 mM). (B) Relative contribution of each component of secretion: fast (○) and slow (●) to the extent of ATP secretion evoked by high K^+ at 23 °C.

of each component to the extent of release was found to be independent of $[K^+]_o$ as illustrated in Fig. 8B). Taken together these results suggest that high K^+-stimulated ATP release is mediated by the activation of voltage-gated Ca^{2+} channels.

Effects of temperature on kinetics of ATP release evoked by high K^+

The marked voltage sensitivity of the kinetics (Fig. 6) together with the absolute dependence on extracellular calcium of the extent of the ATP release [18] suggest that calcium entry through voltage-gated calcium channels are involved in both components of ATP secretion [28,29]. Since membrane channels exhibit large temperature coefficients [27,30,31], we examined the effects of temperature on the kinetics of ATP release induced by membrane depolarization.

Consistent with the idea that high $[K^+]_o$ induces Ca^{2+} entry by activation of voltage-gated Ca^{2+}-channels [29], we found that warming decreased the time constants for both components of ATP secretion (Fig. 9). For example, the kinetics of ATP release induced by a constant elevation of $[K^+]_o$ from 5 to 60 mM at 23, 27 and 37 °C, could be accounted for using the empirical model described in Eqn. 1 with τ_f values of 3.2, 1.4 and 0.8 s, respectively. Similarly, τ_s decreased from 20.2 to 12.0 and 4.17 s. Arrhenius plots of the rate constants for the fast and slow phase of ATP release (Fig. 10A, circles) gave activation energy values of 20 and 16 kcal/mol, respectively. Similar values were obtained for nicotine-induced ATP secretion (Fig. 10A, squares). As observed in the case of ATP release evoked by cholinergic agonists, the extent of the ATP secretion induced by high K^+ was not affected by temperature in the range from 23 to 37 °C (data not shown).

Effect of membrane potential on activation energies

Another important observation is the marked dependence of the activation energy on membrane potential

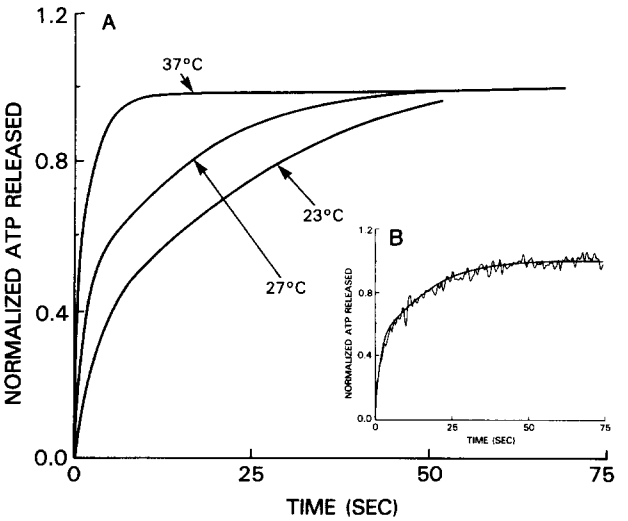

Fig. 9. Effect of temperature on the early time course of ATP release evoked by high $[K^+]_o$. (A) Time-course of high $[K^+]_o$ (60 mM) induced ATP secretion measured at three different temperatures. The time constant for both the fast and the slow component of secretion decreased markedly when temperature was increased. The mean values for the time constant were, respectively, for the fast and the slow component: 2.2 ± 0.6 s and 22.4 ± 6.6 s (n = 6) at 23 °C; 1.7 ± 0.2 and 15.7 ± 2.2 s (n = 5) at 27 °C; 0.9 ± 0.1 and 8.5 ± 2.9 s (n = 6) at 32 °C; 0.7 ± 0.1 and 4.5 ± 0.9 s (n = 6) at 37 °C. (B) Original record and best fit obtained at 27 °C.

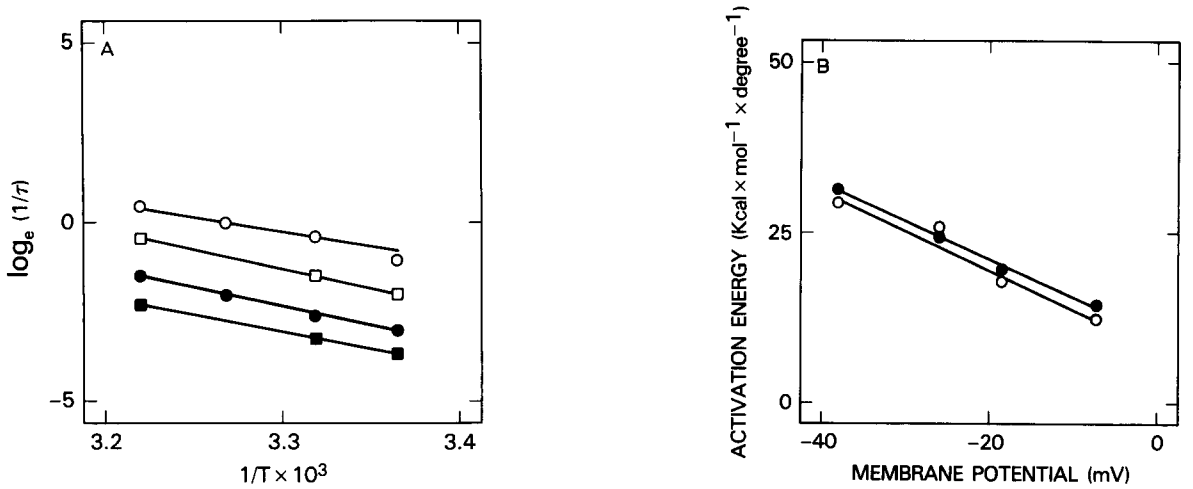

Fig. 10. Estimation of the eneryg of activation for ACh- and high K^+-induced ATP secretion. (A) The rate constant for the fast (open symbols, ○,) and slow (closed symbols, ●,) components of ATP secretion induced by high [K^+] (60 mM) and by ACh are plotted against the inverse of the absolute temperature. The activation energy of the underlying mechanism is approx. 17 kcal/mol for the fast component and 20 kcal/mol for the slow component for both secretagogues. (B) Activation energy as a function of membrane potential for high K^+ evoked ATP release: (○) fast and (●) slow component. The four membrane potential studied corresponded to $[K^+]_o$ of 30, 45, 60 and 100 mM. The activation energy values obtained were (in kcal/mol), respectively, for the fast and the slow component: 29.4 and 31.4 at 30 mM; 26.5 and 25.6 at 45 mM; 17.6 and 20.3 at 60 mM. 12.5 and 15.1 at 100 mM.

(Fig. 10B). For the fast component of release, a 14 mV change in membrane potential determined a 3-fold change in activation energy. Similar change in the rate of ATP release during the slow component of secretion was induced by 18.8 mV. These results are consistent with the idea that high $[K^+]_o$ induces Ca^{2+} entry by direct activation of voltage-gated Ca^{2+} channels.

Discussion

Cholinergic receptor activated ATP secretion is biphasic

The most important feature of the luciferin-luciferase method is the temporal resolution with which one can monitor the secretory process. We exploited this feature to analyze the early (< 2 min) time course of the release process and, as a result, we have established for the first time the existence of two kinetically distinct components in the release process. The simplest interpretation of this result is to consider that secreted ATP originates in two different pools. The fastest release process (time-constant τ_f approx. 2–5 s at 23° C), may correspond to nucleotide released from a vesicular pool in close proximity to the cell membrane. This value of τ_f is similar to the time constant of release estimated from light microscopy, which showed images resembling exocytotic event just a few seconds after the stimulation of chromaffin cells [32]. The slow secretory process (time constant τ_s approx. 20–50 s at 23° C), may correspond to ATP release from another pool, further away from the membrane.

Another explanation for the two components of release could be the concurrence of both direct and compound exocytosis [9]. However, regardless of the origin of the secreted ATP, our results clearly establish the biphasic nature of ATP secretion induced by cholinergic agonists and by membrane depolarization.

High activation energy values suggest a common coupling mechanism for the two components of ATP secretion

The effects of increasing temperature on secretion indicate that there is an increase in the rate of ATP secretion for both cholinerigc agents and high $[K^+]_o$. This confirms previous data obtained on CA secretion using carbachol and K^+ [13]. We propose that warming increases the rate of Ca^{2+} entry through voltage-gated Ca^{2+} channels [28,29] which in turn accelerates the elevation in $[Ca^{2+}]_i$ required to initiate ATP secretion. It should be noted that the voltage-dependence of the activation energy for ATP secretion is rather similar to that reported for the activation and gating of sodium channels [31].

Other possible explanations for the effect of temperature may involve the action of temperature on the fusion event itself, on the plasma membrane proteins (ACh receptors) and lipids, with the general effect of increasing the efficiency of the secretory response. In line with the idea that the primary effect of tempearature is on the fusion event itself, Hiram, Nir, Greenberg and Zinder [32] reported recently on the effects of temperature on activation and inactivation of the ACh receptor-channel and on the transition point of the lipids (at about 30° C) known to be present in the chromaffin cell membrane.

Finally, it is interesting to notice that the activation

energy reported here for K^+-evoked secretion (approx. 20 kcal/mol) is similar to the value previously reported for a single component of CA release [13].

Differential effect of concentration of the secretagogue on ATP release kinetics

The kinetics of ATP release induced by ACh and nicotine was found to be independent of the dose used to stimulat the chromaffin cells (Fig. 3). In contrast, the kinetics of ATP secretion was markedly dependent on $[K^+]_o$ (Fig. 6). It is generally accepted that many of the steps in the sequence of events leading to exocytosis may be common to both modes of activation of the secretory response. However, some profound differences must exist between the two modes of activation for, otherwise, the rates of ATP secretion should be similarly affected by the concentration of both types of secretagogues.

Several observations support this idea. In bovine medullary chromaffin cells ACh induces a dose-related membrane depolarization which in turn is due to the activation of nicotinic receptor-channels [34]. However, we [26] and others [34] have shown that the extent of the membrane depolarization evoked by a maximal dose of ACh (100–200 μM) is comparable to that obtained with a minimal dose of K^+ (12.5 mM). Thus, K^+ at all concentrations tested (30–100 mM) was more effective than ACh (or nicotine) at maximal dose in depolarizing the chromaffin cell membrane. Therefore, we may expect that a larger number of non-inactivating Ca^{2+} channels [29] might be activated with K^+ than with ACh (or nicotine). Consistent with this idea are the results by Heldman et al. [8] showing that the increase in $[Ca^{2+}]_i$ evoked by high $[K^+]_o$ remains elevated whereas the $[Ca^{2+}]_i$ increase evoked by nicotine is transient. Thus, we propose that the rate of ATP secretion is markedly dependent on $[K^+]_o$ because the rate of Ca^{2+} entry through voltage-gated Ca^{2+} channels is greater in high $[K^+]_o$ than in high $[ACh]_o$.

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