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INACTIVATION OF THE EARLY CALCIUM UPTAKE AND NORADRENALINE RELEASE EVOKED BY POTASSIUM IN CULTURED CHROMAFFIN CELLS

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SUMMARY : Upon stimulation with a 59 mM K solution (59K), ^{45}Ca uptake into cultured bovine adrenal chromaffin cells quickly enhanced to reach a plateau within 60 sec. ^{45}Ca transients could be clearly measured with a time resolution (10 sec) and a net Ca uptake (75 times the basal uptake) that considerably improve data reported in other recent papers; this experimental design allows the direct comparison of ^{45}Ca transient data with electrophysiological measurements of chromaffin cell Ca currents. In addition, it is shown that upon sustained depolarization with 59K both, the rates of ^{45}Ca uptake and ^{3}H-noradrenaline release decline in a parallel manner, suggesting that the voltage-dependent Ca channel activity modulates the kinetics of the early secretory response.

The correlation between the activity of voltage-dependent Ca channels and the kinetics of activation and inactivation of adrenomedullary catecholamine release has been restrained because of the poor time resolution (several minutes) of ^{45}Ca transient studies and the scarce net uptake of ^{45}Ca (2-3 times the basal) by cultured chromaffin cells depolarized with high K concentrations. In this paper, we present experimental designs that allow the evaluation of ^{45}Ca transients during high-K depolarization with a time resolution (10 sec) and a net ^{45}Ca uptake (75 times the basal) that considerably improve those reported in other recent papers on cultured bovine chromaffin cells (1,2). This approach facilitates the direct comparison of ^{45}Ca transient studies with electrophysiological measurements of chromaffin cell Ca currents (3). In addition, by using these protocols it was possible to demonstrate that the early Ca influx through voltage-sensitive Ca channels precedes noradrenaline (NA) release, indicating that the activity of such channels tightly modulates the kinetics of the early secretory response during depolarization of the chromaffin cell.

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METHODS

Chromaffin cells were prepared from bovine adrenal medulla (4,5), and plated on uncoated plastic culture wells containing 1 ml of Dulbecco's modified Eagle's medium ($5x10^5$ cells per well).

Loading of the cells with DL-[7-^{3}H]-noradrenaline (NA; 27 Ci/mmol; Amersham) was performed as described by Almazan et al. (5). Spontaneous or high K-evoked NA release was studied at 37ºC with two procedures : the first, by incubating every individual well during periods of time between 10 sec and 10 min either in the presence of Krebs-Hepes (KH) solution (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4.7H_2O$, 11 mM glucose 0.01 M mM EDTA, 0.56 mM ascorbic acid and 15 mM Hepes, at pH 7.4) or in the presence of KH containing 3x (17.7K) or 10x (59K) the normal concentration of K (NaCl reduced isoosmotically). In a second procedure, NA release was "continuously" monitored by replacing the bathing fluids (KH, 17.7K or 59K) at 10 sec intervals. To terminate the secretory process, test solutions were removed and 0.5 ml of 10% trichloroacetic acid (TCA) was added to the cells.

^{45}Ca uptake into chromaffin cells was studied by incubating the cells at 37ºC with $^{45}CaCl_2$ (10-40 mCi/mg Ca, New England Nuclear) at a final concentration of 8 uCi/0.5 ml of medium, in the presence of KH (basal uptake) or high K solutions (evoked uptake). Two experimental protocols were used : first, by adding simultaneously ^{45}Ca and high K for different time periods, and second by exposing the cells first to the high K solution and then giving a 10-sec ^{45}Ca pulse at several time periods after the initiation of depolarization. Unless indicated otherwise, ^{45}Ca uptake studies were performed in the presence of 2.5 mM $CaCl_2$. At the end of each incubation period the test medium was rapidly removed and the uptake reaction was ended by adding a Ca-free KH containing 2 mM $LaCl_3$ and lacking $MgSO_4$ and K_2HPO_4, at 37ºC. Several washes with this solution at 30 sec intervals were then given during a 10-min period before removing the cells with 0.5 ml of TCA to count the radioactivity retained.

RESULTS AND DISCUSSION

Time course of ^{3}H-noradrenaline release and ^{45}Ca uptake evoked by high K

^{3}H-NA release evoked by 59K exposure (fig. 1) was 4 times the basal output after only 10 sec of depolarization. The release (usually 10 times the basal) was linear during the first 2 min of depolarization, and reached a plateau around the fith min (18.7 $\pm$ 0.13 %; n=9). ^{45}Ca uptake could be clearly measured after only 10 sec of depolarization; by using repetitive washes with a Ca-free solution containing 1 mM $LaCl_3$, the net ^{45}Ca uptake evoked by 59K amounted to as much as 75 times the basal uptake. Earlier papers (1,2) reported a net ^{45}Ca uptake of only 3 times the basal. Fig. 1 also shows that the net accumulation of Ca was very fast, preceded in 10 sec NA release and terminated earlier than the secretory response.

Decline of the rates of ^{45}Ca uptake after pre-depolarization

Because the chromaffin cell voltage-dependent Ca channel inactivates very slowly (3) it was likely that exposure of chromaffin cells to

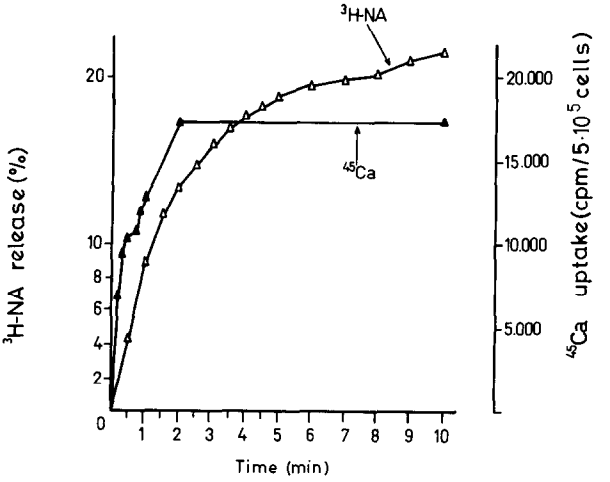

FIG. 1. Time course of ^{3}H-noradrenaline (^{3}H-NA) release and ^{45}Ca uptake during exposure of cultured bovine adrenal chromaffin cells to a Krebs-Hepes (KH) solution containing 59 mM K (59K). Cells of individual wells preloaded with ^{3}H-NA, were incubated in 59K for the time periods shown in the abscissa. The net release was calculated by subtracting evoked output from the basal release and expressed as % of the total ^{3}H-NA retained by the cells (fractional release). ^{45}Ca uptake by cells that were incubated also in 59K for time periods between 10 sec and 5 min was 75 times the basal uptake. Data from two experiments made in triplicate wells.

^{45}Ca pulses of only 10-sec-duration after different periods of previous sustained depolarization with 59K, would constitute an adequate index of the rates of Ca uptake and the relative number of open channels at a given time after depolarization.

Fig. 2 shows that upon sustained exposure of the cells to 59K, the rate of ^{45}Ca uptake declined very fast during the first minute of depolarization to 20% of the initial uptake. However, the decline of Ca uptake during maintained depolarization may be due either to termination of Ca influx through Ca channels or to a combination of decreased influx and increased efflux of ^{45}Ca previously taken up. The fact that after 2 min of previous depolarization Ca uptake was insignificant, yet in the presence of Sr or Ba was still very high (fig. 3), is consistent with the expected properties of Ca channels that are known to inactivate in several neuronal systems in the presence of Ca, but not when the permeant cation was Sr or Ba (6-10). These results, and the lack of inactivation of Ca uptake in the presence of the Ca channel activator Bay-K-8644 (see later), strongly suggest that during depolarization with 59K, ^{45}Ca entered the chromaffin cell through voltage-sensitive Ca channels; the decline of the rate of uptake upon sustained depolarization seems to be a direct consequence of the inactivation of such channels.

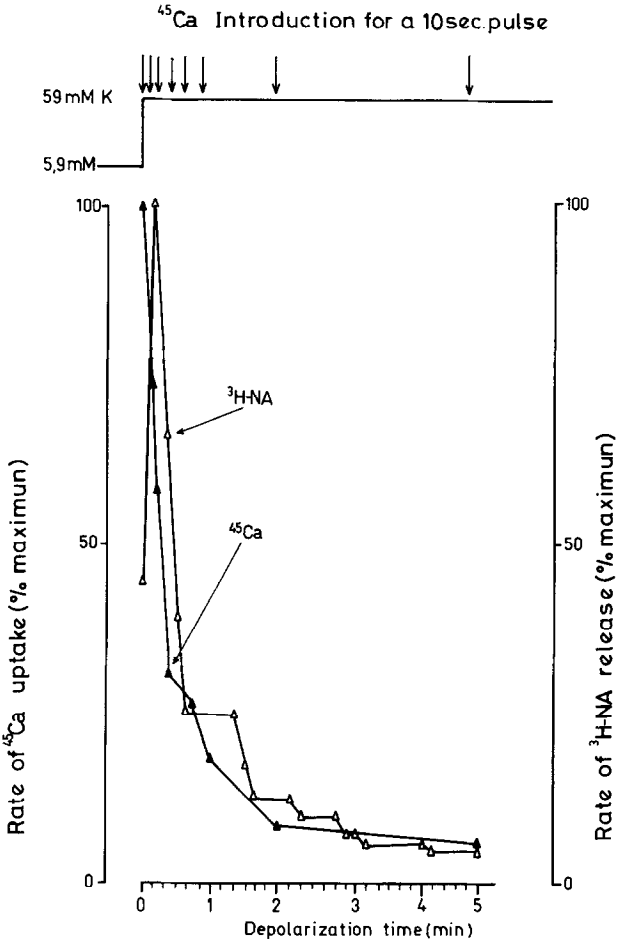

FIG. 2. Rates of release of ^{3}H-noradrenaline (^{3}H-NA) from and ^{45}Ca uptake into 3-days-old cultured cells exposed to a 59 mM K solution. The experiment to monitor ^{3}H-NA release was performed in an individual single well, replacing the solution at 10 sec intervals. The rates of ^{45}Ca uptake after pre-depolarization of the cells was studied by giving 10-sec ^{45}Ca pulses (top arrows) after different periods of pre-depolarization. Data are expressed as % of maximum peak of ^{3}H-NA release or ^{45}Ca uptake. Net ^{45}Ca uptake during the first initial 10 sec of depolarization amounted to 13.688 $\pm$ 1.152 cpm/10 sec. 5×10^{5} cells (4.02 $\pm$ 0.08 fmol/cell; n=9). Data from two experiments made in triplicate wells.

The rates of noradrenaline release parallel the rates of calcium uptake during high potossium-depolarization

Fig. 2 shows that the decline of the rates of ^{3}H-NA release closely follows the rates of inactivation of ^{45}Ca uptake. If Ca channels couple K depolarization to the secretory mechanism, the manipulation of the activity of such channels should lead to parallel changes in ^{3}H-NA release. This assumption was tested by using Sr and Ba and the Ca channel agonist Bay-K-8644.

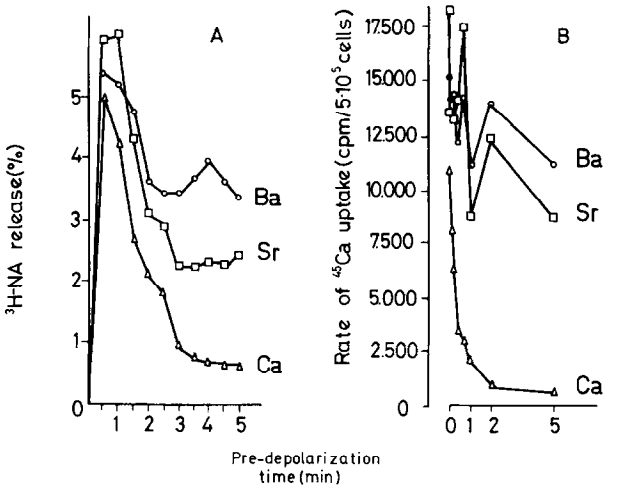

FIG. 3. Rates of inactivation of ^{3}H-noradrenaline (^{3}H-NA) release (A) and ^{45}Ca uptake (B) after pre-depolarization with 59K, when using 2.5 mM Ca, Sr, or Ba as divalent permeant cations. Experimental protocols similar to those of fig. 2. Data from two experiments made in triplicate.

Sr and Ba are known to replace Ca as a cation necessary to trigger the adrenomedullary secretory process (11) yet they permeate through the Ca channel at different rates and they inactivate such channel to different extent (6-10). If it is true that Ca plays a role in controlling the rate of inactivation of the secretory response, these cations might exhibit different rates of inactivation. This assumption was supported by the experiments shown on fig. 3, where both parameters (^{45}Ca uptake and ^{3}H-NA release) inactivated at the same rates in the presence of 2.5 mM Ca (fast inactivation), Sr (moderate inactivation) or Ba (little inactivation).

A second procedure to manipulate the activity of Ca channels was the use of the Ca channel activator Bay-K-8644, that is known to potentiate adrenomedullary catecholamine release or ^{45}Ca uptake evoked by high K (12). In cultured chromaffin cells (data not shown), Bay-K-8644 (10^{-6}M) potentiated NA release (7 times) and Ca uptake (14 times).

It is well established that upon prolonged stimulation of the cat (13-15) and bovine (16) adrenal glands with high K, the amounts of catecholamine release into the perfusate decline exponentially during the first few minutes and fall to about 10% of the initial rate by the 10th min. Two hypothesis are presently handled to explain the cause for this reduction: one suggests that the rate limiting step might be the inactivation of voltage-dependent Ca channels (16,17); the second, that an intracellular site (refractoriness of the exocytotic mechanism, Ca

buffering or Ca extruding systems) is involved (15,18,19). All these experiments were performed in a time scale of several tens of minutes. Our present results show that al least at earlier times (tens of seconds), the kinetics of the secretory response closely parallels the activity of Ca channels.

In conclusion, experimental designs are reported that allow the study of ^{45}Ca transients during depolarization with a time resolution (10 sec) and a net Ca uptake (75 times the basal) that considerably improve those reported in previous recent papers on cultured bovine chromaffin cells (1,2). This will allow, for the first time, the direct comparison of ^{45}Ca transient studies with electrophysiological measurements of chromaffin cell Ca currents (3). In addition, this paper shows that Ca influx through voltage-sensitive Ca channels precedes NA release and indicate that the activity of such channels tightly modulates the kinetics of the early secretory response during depolarization of the chromaffin cell.

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