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Calcium Oscillations in Pancreatic Acinar Cells, Evoked by the Cholecystokinin Analogue JMV-180, Depend on Functional Inositol 1,4,5-Trisphosphate Receptors*

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It has been reported that the synthetic heptapeptide cholecystokinin (CCK) analogue JMV-180 evokes cytosolic Ca^{2+} signals in pancreatic acinar cells via mechanisms that do not include either the generation or action of inositol 1,4,5-trisphosphate ($InsP_3$) (Saluja, A. K., Dawra, R. K., Lerch, M. M., and Steer, M. L. (1992) *J. Biol. Chem.* 267, 11202-11207; Yule, D. I., and Williams, J. A. (1992) *J. Biol. Chem.* 267, 13830-13835). We have now investigated the CCK- and JMV-180-evoked cytosolic Ca^{2+} oscillations by measurement of the Ca^{2+}-sensitive ion currents in internally perfused mouse pancreatic acinar cells. We find that the $InsP_3$ receptor antagonist heparin (500 μg/ml) blocks Ca^{2+} oscillations induced by both CCK (5-20 pM) and JMV 180 (10-40 nM), whereas de-*N*-sulfated heparin (500 μg/ml), which does not affect $InsP_3$ binding to its receptor, fails to inhibit the responses to the two agonists. We conclude that the cytosolic Ca^{2+} oscillations evoked by both CCK and JMV-180 are dependent on functional $InsP_3$ receptors.

Cytosolic Ca^{2+} oscillations evoked by hormones and neurotransmitters are generally mediated by the intracellular messenger inositol 1,4,5-trisphosphate ($InsP_3$)[1] (1). In pancreatic acinar cells, the two main agonists acetylcholine (ACh) and cholecystokinin (CCK) evoke cytosolic Ca^{2+} signals that can be inhibited by intracellular application of the $InsP_3$ receptor antagonist heparin (2, 3) or by the phospholipase C inhibitor U73122 (4). It has been reported that the synthetic heptapeptide CCK analogue JMV-180 releases intracellular Ca^{2+} from an $InsP_3$-independent store in permeabilized pancreatic acinar cells, since this analogue could evoke Ca^{2+} release in the presence of a high heparin concentration (5). In agreement with this, Yule and Williams (4) found that the phospholipase C inhibitor U73122 cannot inhibit cytosolic Ca^{2+} oscillations evoked by JMV-180. These findings suggest that JMV-180 evokes a Ca^{2+} signal via mechanisms that do not include either the generation or action of $InsP_3$ (4, 5). It is, however, difficult to understand the apparent difference in the mechanism of action of CCK and JMV-180, since, at the low agonist concentrations required to evoke cytosolic Ca^{2+} oscillations, both agents are supposed to interact with the same class of high affinity CCK receptors (6).

We have now investigated the CCK- and JMV-180-evoked cytosolic Ca^{2+} signals by measurement of the Ca^{2+}-sensitive ion currents in internally perfused mouse pancreatic acinar cells. We find that the $InsP_3$ receptor antagonist heparin blocks both CCK- and JMV-180-induced Ca^{2+} oscillations, whereas de-*N*-sulfated heparin, which does not affect $InsP_3$ binding to its receptor (7, 8) fails to inhibit the responses to the two agonists. We conclude that the cytosolic Ca^{2+} oscillations evoked by both CCK and JMV-180 are dependent on functional $InsP_3$ receptors.

MATERIALS AND METHODS

Cell Preparation—Isolated single mouse pancreatic acinar cells and small cell clusters were prepared by pure collagenase (Worthington) digestion in the presence of trypsin inhibitor (4 mg/ml; Sigma type 11-S) as described previously (9).

Solutions—The standard extracellular solution contained (mM): 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 11 glucose, 1 mM Ca^{2+}, 10 HEPES-NaOH, pH 7.2. The standard intracellular solution (pipette solution in whole cell experiments) contained (mM): 140 KCl, 1.13 $MgCl_2$, 0.1-0.2 EGTA, 2 ATP, 10 HEPES-KOH, pH 7.2. Bath solution changes at the cell took less than 5 s (10). Two heparin preparations (one of molecular weight < 3000 and one with a mean M_r of 12,000), de-*N*-sulfated heparin, and cholecystokinin were obtained from Sigma. JMV-180 was kindly given to us by Dr. J. Martinez (Montpellier).

Experimental Protocol—Experiments were all carried out at room temperature (20-25 °C). Standard whole cell recording techniques were used (10, 11). Patch pipettes of between 2 and 4 megohms were pulled from microhematocrit (Assistent) tubes. Seals of >10 gigohms were produced on the cell membrane, and gentle suction or voltage pulses often formed whole cell recordings assessed by an increase in capacitance and noise. Series resistance was measured by the compensation circuitry of the LIST EPC7 amplifier, and whole cell recordings were rejected if the series resistance was greater than 15 megohms. The whole cell current records were obtained by voltage steps from a holding potential of -30 mV to a potential of 0 mV. Each potential was held for 150 ms, and the stepping frequency was 3 Hz. At the resolution shown in the figures, the two current traces obtained at the two holding potentials appear continuous. Using our solutions, the reversal potential of both the Cl^- and nonselective cation currents are at 0 mV. Small deviations in E_{Cl} and E_{cation} and in the holding potential sometimes produce small inward or outward currents at 0 mV. At -30 mV we obtain a measure of both the Ca^{2+}-dependent currents that correlates well with the $[Ca^{2+}]_i$ signal (9, 12).

The pulse protocol and data storage were carried out using a program developed by Smith (13) with a 286 IBM-compatible microcomputer and a Cambridge Electronic Devices analogue to digital converter.

In the figures shown (except Fig. 1), the current traces are taken from the start of the whole cell configuration. Therefore, the left-hand edge of the traces indicates the start of the equilibration of the pipette contents with the cell cytoplasm. In the case of large molecules such as heparin, equilibration would be expected to take a relatively long time (14). In our experiments, effective concentrations of heparin are reached within about 5 min.

Ca^{2+}-dependent Currents—The experiments described here use the Ca^{2+}-dependent ionic currents to monitor changes in $[Ca^{2+}]_i$. These currents have been shown to accurately reflect local and global $[Ca^{2+}]_i$ signals (12). Heparin has no direct effect on these currents as shown in

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[1] The abbreviations used are: $InsP_3$, inositol 1,4,5-trisphosphate; ACh, acetylcholine; CCK, cholecystokinin; JMV-180, synthetic heptapeptide CCK analogue JMV-180; $[Ca^{2+}]_i$, cytosolic free calcium ion concentration.

experiments where currents directly activated by intracellular Ca^{2+} infusion could not be blocked by intracellular heparin application (2).

RESULTS

The Effects of CCK—Fig. 1*A* shows the typical current response to 15 pM CCK previously described (3, 10, 15). Such responses were obtained using 5–20 pM CCK in the present series of experiments. Both the short spikes and the broader transients are due to increases in $[Ca^{2+}]_i$; however, whereas the short spikes are associated with a rise in $[Ca^{2+}]_i$ exclusively in the secretory pole, $[Ca^{2+}]_i$ is elevated throughout the cell during the broad transients (12). When heparin (M_r = 3000) was present in the pipette solution at a concentration of 500 μg/ml, the response to CCK was abolished (n = 3). We also tried a heparin preparation with a higher molecular weight (mean molecular weight about 12,000) and, using a concentration of 500 μg/ml, found that within 10 min after establishment of the whole cell recording configuration the CCK response was abolished in 3 out of 4 experiments.

The Effects of CCK-JMV-180—Fig. 1*B* shows the effect of 10 nM JMV-180. Effects corresponding to those seen with 5–20 pM CCK were obtained using 10–40 nM JMV-180 (n = 14). The quoted concentration ranges of the agonists reflect cell to cell variations in sensitivity. We used concentrations in each experiment required to obtain oscillatory responses. The relative potencies of CCK and JMV-180 found in our experiments are in agreement with those found earlier (5, 16).

Heparin Effects on JMV-180-evoked Responses—We used two protocols to determine the effect of heparin on Ca^{2+} mobilization by JMV-180. In one type of experiment, heparin was included in the patch pipette solution at the concentrations shown. After forming a whole cell, the contents of the patch pipette are in continuity with the cell interior. In previous experiments (2), it has been shown in our cells that effects of heparin applied from the pipette solution can be observed after about 3–4 min. The main limit to diffusion is the access resistance of the patch, which was always monitored, and we rejected cells where this resistance increased (see "Materials and Methods"). To monitor the entry of heparin into the cell, brief applications of ACh were applied, as in the example of Fig. 2*A*, and the reduction in the response was observed. It is clear that, at the point where the calcium-dependent currents in response to ACh are abolished, a subsequent application of JMV-180 fails to elicit a response (n = 3). In the second protocol (Fig. 2*B*), we simply observed the effect of repeated applications of JMV-180 (20 nM) during the period immediately after establishment of the whole cell configuration. The experiments demonstrate a rapid abolition of the response (n = 3).

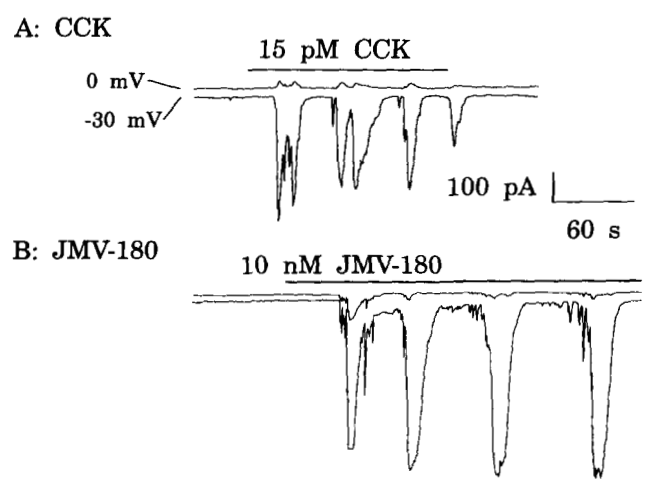

FIG. 1. **Whole cell patch-clamp current oscillations obtained in response to cholecystokinin (15 pM, *A*) and JMV-180 (10 nM, *B*).** The *upper line* of each record was obtained at 0 mV holding potential, the reversal potential of the Cl^- and cation currents. The *lower line* was obtained at a holding potential of −30 mV and represents the combination of the calcium-dependent Cl^- and nonselective cation currents. Small deviations in E_{Cl} and E_{cation} give rise to small currents at 0 mV.

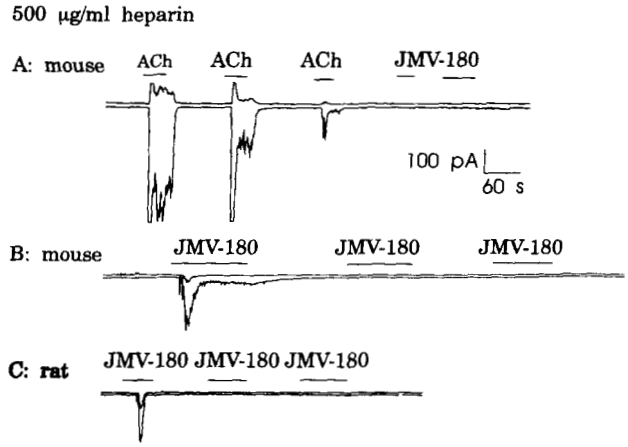

FIG. 2. **Current records obtained in the presence of 500 μg/ml heparin in the patch pipette.** *A*, repeated applications of ACh were used to monitor the heparin-induced antagonism of the $InsP_3$ receptor. Subsequent application of JMV-180 failed to induce current activation. *B*, repeated application of JMV-180 demonstrates block of Ca^{2+}-dependent currents. *C*, the same type of experiment as in *B* but carried out using acutely isolated rat rather than mouse pancreatic acinar cells.

The previous studies concluding that the effect of JMV-180 is not mediated by $InsP_3$ (4, 5) were carried out on pancreatic acinar cells from rats rather than mice; therefore, we also performed a few experiments using rat cells. Fig. 2*C* shows an example of an experiment in which heparin blocked the JMV-180 (40 nM) induced current oscillation (n = 3).

Effects of De-N-sulfated Heparin—Although the evidence showing that heparin is an $InsP_3$ receptor antagonist is very extensive and convincing (17), this agent may also inhibit $InsP_3$ formation (1). A G-protein-uncoupling action of heparin has been described for muscarinic receptors in cardiac myocytes (18) and α-adrenoceptors in hepatocytes (19). To control for possible nonspecific actions of heparin, we used de-*N*-sulfated heparin, which has been shown not to bind to the $InsP_3$ receptor (8). Using a concentration of 500 μg/ml de-*N*-sulfated heparin, the effects of 5–20 pM CCK were not reduced, even more than 10 min after establishment of the whole cell recording configuration (n = 3). Similar results were obtained with 10–40 nM JMV-180, and Fig. 3 shows an example of a recording where JMV-180 still evoked a response more than 15 min after de-*N*-sulfated heparin gained access to the cell interior (n = 3).

DISCUSSION

Our experiments show that the cytosolic Ca^{2+} oscillations evoked by CCK and the synthetic CCK analogue JMV-180 can be blocked by intracellular application of the $InsP_3$ receptor antagonist heparin, whereas de-*N*-sulfated heparin, which has been shown not to bind to $InsP_3$ receptors (7, 8), failed to inhibit responses to both these agonists. The most straightforward conclusion to be drawn from these experiments is that both the CCK- and JMV-180-evoked cytosolic Ca^{2+} oscillations are dependent on functional $InsP_3$ receptors. It does not necessarily follow from this that the actions of CCK and JMV-180 are initiated by an increase in the intracellular $InsP_3$ concentration.

The sulfhydryl reagent thimerosal induces cytosolic Ca^{2+} oscillations in both pancreatic acinar cells (20) and HeLa cells (21) without elevating $InsP_3$ levels (21), but the effects of thimerosal are abolished by heparin (20, 22). This has led to the

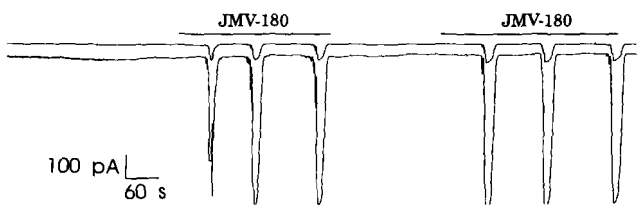

FIG. 3. **Whole cell current record obtained in response to 40 nM JMV-180.** In this experiment the patch pipette contained 500 μg/ml de-*N*-sulfated heparin, which does not act as a competitive antagonist at the $InsP_3$ receptor and fails to block the currents.

proposal that $InsP_3$ receptor sensitivity to $InsP_3$ can be regulated and that thimerosal and oxidized glutathione act by increasing the sensitivity of the $InsP_3$ receptor to such an extent that the resting $InsP_3$ level becomes effective in releasing Ca^{2+} (1).

There is much evidence indicating that agonist-evoked cytosolic Ca^{2+} oscillations are mediated by a Ca^{2+}-induced Ca^{2+} release mechanism (2, 9, 17, 23), which could in principle depend on either $InsP_3$ or ryanodine receptors (1). With regard to the actions of CCK and JMV-180, our data indicate a role for the $InsP_3$ receptors. Since it has not been possible to demonstrate $InsP_3$ formation in response to CCK concentrations lower than 100 pM or to concentrations of JMV-180 below 1 μM (16), it is possible that the cytosolic Ca^{2+} oscillations are initiated by a Ca^{2+} release process not dependent on $InsP_3$ generation. An action mediated entirely by sensitization of $InsP_3$ receptors to the resting $InsP_3$ level, similar to that described for thimerosal, is one possibility. Another $InsP_3$ receptor-independent Ca^{2+} release pathway could also be involved, since experiments on permeabilized pancreatic acinar cells show that high concentrations of JMV-180 (10–100 μM), but not CCK, can evoke Ca^{2+} release from an $InsP_3$-insensitive Ca^{2+} store (5).

Further work on the mechanism by which CCK receptor interaction initiates cytosolic Ca^{2+} signals is clearly needed, but our results show that functional $InsP_3$ receptors are involved in mediating the cytosolic Ca^{2+} oscillations.

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