Hormone-induced rise in cytosolic Ca\(^{2+}\) in axolotl hepatocytes: properties of the Ca\(^{2+}\) influx channel

THOMAS LENZ AND JOCHEN W. KLEINEKE
Abteilung Klinische Biochemie, Zentrum Innere Medizin, University of Göttingen, 37075 Göttingen, Germany

Lenz, Thomas, and Jochen W. Kleineke. Hormone-induced rise in cytosolic Ca\(^{2+}\) in axolotl hepatocytes: properties of the Ca\(^{2+}\) influx channel. Am. J. Physiol. 273 (Cell Physiol. 42): C1526–C1532, 1997.—Calcium entry in nonexcitable cells occurs through Ca\(^{2+}\)-selective channels activated secondarily to store depletion and/or through receptor- or second messenger-operated channels. In amphibian liver, hormones that stimulate the production of adenosine 3',5'-cyclic monophosphate (cAMP) also regulate the opening of an ion gate in the plasma membrane, which allows a noncapacitative inflow of Ca\(^{2+}\). To characterize this Ca\(^{2+}\) channel, we studied the effects of inhibitors of voltage-dependent Ca\(^{2+}\) channels and of nonselective cation channels on 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP)-dependent Ca\(^{2+}\) entry in single axolotl hepatocytes. Ca\(^{2+}\) entry provoked by 8-Br-cAMP in the presence of physiological Ca\(^{2+}\)-fomed first-order kinetics (apparent Michaelis constant \(= 43\) μM at the cell surface). Maximal values of cytosolic Ca\(^{2+}\) (increment \(\sim 300\%\)) were reached within 15 s, and the effect was transient (half time of 56 s). We report a strong inhibition of cAMP-dependent Ca\(^{2+}\) entry by nifedipine [half-maximal inhibitory concentration (IC\(_{50}\)) = 0.8 μM], by verapamil (IC\(_{50}\) = 22 μM), and by SK&F-96365 (IC\(_{50}\) = 1.8 μM). Depolarizing concentrations of K\(^{+}\) were without effect. Galdolinium and the anti-inflammatory compound niculflumate, both inhibitors of nonselective cation channels, suppressed Ca\(^{2+}\) influx. This “profile” indicates a novel mechanism of Ca\(^{2+}\) entry in nonexcitable cells.

adenosine 3',5'-cyclic monophosphate; second messenger-operated calcium channel; calcium channel pharmacology; SK&F-96365; fenamates

Very recently, investigations on Drosophila melanogaster have drawn attention to certain proteins (trp, trpl) with an apparent capacity of both channel forming and the sensing of the filling state of the endoplasmic reticulum Ca\(^{2+}\) store (32, 33). Hence, the Drosophila store-operated channel has been put forward as a model for capacitative Ca\(^{2+}\) entry. Analogous proteins, however, have not been detected in liver (43).

In variance, in fish and amphibian liver, the effect of adrenergic agonists and vasotocin is mediated via the generation of cAMP (19, 20, 42), and not via InsP\(_3\). Yet, in parenchymal liver cells from axolotl (Ambystoma mexicanum), hormones that stimulate cAMP formation (the order of efficacy was glucagon \(>\) isoprenaline \(>\) epinephrine \(\geq\) arginine vasotocin) also provoked a pronounced increase in cytosolic Ca\(^{2+}\), which was not due to a mobilization of the cation from internal stores by InsP\(_3\)thapsigargin, but to an increased inflow from the extracellular medium. Thus, in axolotl liver, in contrast to rat liver, hormones that stimulate the production of cAMP also regulate the opening of an ion gate in the plasma membrane, which allows an inflow of Ca\(^{2+}\) (and Mn\(^{2+}\)). The effect is rather specific, since guanosine 3',5'-cyclic monophosphate (cGMP) failed to induce Ca\(^{2+}\) entry (23). We have proposed that this channel could belong to the category of second messenger-operated Ca\(^{2+}\) channels, as defined by Meldolesi and Pozzan (29). In nonexcitable tissues, such channels have so far only been found in blood cells (10, 27, 36).

The aim of this investigation was to further characterize the nature of this cAMP-activated Ca\(^{2+}\) channel of axolotl liver cells, using a variety of compounds that influence Ca\(^{2+}\) entry in excitable and nonexcitable cells: the phenylalkylamine verapamil, the dihydropyridine nifedipine, both potent inhibitors of Ca\(^{2+}\) entry in heart and skeletal muscle, and the imidazole derivative SK&F-96365, which inhibited receptor-mediated Ca\(^{2+}\) entry (as compared with receptor-mediated Ca\(^{2+}\) release) in nonexcitable cells (human platelets, neutrophils, and endothelial cells) and which has been used as a tool to discriminate between voltage-gated Ca\(^{2+}\) entry and receptor-mediated Ca\(^{2+}\) entry in GH 3 and artery smooth muscle cells (30). Because lanthanides (10^{-7} to 10^{-3} M) block stretch- and receptor-activated nonselective cation channels, but also Ca\(^{2+}\) entry through voltage-dependent channels (15), we investigated the effect of Gd\(^{3+}\) on cAMP-dependent Ca\(^{2+}\) entry. As an additional inhibitor of nonselective cation channels in membranes, we examined the effect of niflumate, a nonsteroidal anti-inflammatory drug (12, 15).

Using single-cell dual-wavelength epifluorescence measurements of cytosolic Ca\(^{2+}\) in amphibian hepatocytes, we report a strong inhibition of cAMP-dependent
Ca\textsuperscript{2+} entry by SK&F-96365 [half-maximal inhibition concentration (IC\textsubscript{50} = 1.8 \times 10^{-6} M], by the dihydropyridine nifedipine (IC\textsubscript{50} = 8 \times 10^{-7} M), and by verapamil. Furthermore, the lanthanide Gd\textsuperscript{3+} and niflumate, both potent inhibitors of nonselective cation channels, suppressed Ca\textsuperscript{2+} influx. It is concluded that in axolotl hepatocytes the rise in intracellular Ca\textsuperscript{2+} after hormonal stimulation is due to a Ca\textsuperscript{2+} inflow via a novel dihydropyridine- and SK&F-96365-sensitive nonselective cation channel.

**MATERIALS AND METHODS**

**Materials.** Fura 2 acetoxyethyl ester (AM) was purchased from Molecular Probes (Eugene, OR). SK&F-96365 1-[(b-3-(4-methoxyphenyl)propoxy)-4-methoxyphenethyl]-1H-imidazole hydrochloride was a kind gift from SmithKline Beecham Pharmaceuticals (Welwyn, UK). BAY K 8644 was from Bayer. Collagenase ("Worthington" type CLS II, 206 U/mg) came from Biochrom (Berlin, Germany). 3-Aminobenzoic acid ethyl ester (MS-222) was from Sigma (Munich, Germany). All other chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Isolation of hepatocytes. Axolotls (A. mexicanum) were maintained in aerated water tanks at 20°C. The animals were fed twice weekly on fish pellets (Fisch-Fit, Interquell Wehringen, Germany) and had a body weight of 60–80 g when used. Results from both males and females are presented together, because there were no differences observed between sexes (19).

The animals were anesthetized by immersion in 0.05% (wt/vol) MS-222. The cannulation and extirpation of the liver were described previously (19, 23).

Hepatocytes were prepared using Ca\textsuperscript{2+}-free amphibian Krebs-Ringer bicarbonate buffer (aKRB) (80 mM NaCl, 3 mM KCl, 0.6 mM KH\textsubscript{2}PO\textsubscript{4}, 0.8 mM MgSO\textsubscript{4}, and 16 mM NaHCO\textsubscript{3}, pH 7.4) as the perfusate. Briefly, the liver was perfused via the portal vein for 15 min with the above medium in an open recirculating system, and then after readdition of Ca\textsubscript{Cl\textsubscript{2}} (1 mM) and collagenase (0.05 g/100 ml), the perfusion was continued for 40–50 min in a recirculating system. After this step, the liver was minced, and the disintegrating tissue fragments as well as single cells were collected and passed through a double layer of cheesecloth. This suspension was washed three times with aKRB by centrifugation (150 g for 1 min). Usually >85% of the cells were viable as judged by trypan blue exclusion (0.2% trypan blue, 1% bovine serum albumin in aKRB).

Measurement of cytosolic Ca\textsuperscript{2+}. The cells were washed once (100 g for 1 min) and resuspended in a medium containing 80 mM NaCl, 3.2 mM KCl, 0.8 mM MgSO\textsubscript{4}, 1 mM Ca\textsubscript{Cl\textsubscript{2}}, 10 mM d-glucose, and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4 (medium A) to give a concentration of 20 nM fura 2-AM (5 µM) for 30 min at 25°C in a shaking water bath (100 cycles/min). After this, the cells were spun down (1 min, 100 g), the supernatant was discarded, and the pellet was resuspended in the same volume of medium A and further incubated for up to 30 min at 4°C. Thereafter, the cells were washed twice with medium A (100 g, 1 min) and resuspended in medium A at a concentration of 80–100 mg wet wt/ml. This suspension was kept for up to 30 min at room temperature to allow deesterification of fura 2-AM. The latter was controlled during this period by monitoring the epifluorescence of single hepatocytes at 360-nm excitation. Ca\textsuperscript{2+} concentration was calculated from the fluorescence ratio 360/380 nm (31). Hepatocytes (1–2 mg wet wt) were suspended in 2 ml medium A (plus additions as specified) in a petri dish (Falcon 3001) with a central quartz window (diameter = 15 mm). Water-insoluble compounds were prepared as concentrated stock solutions in dimethyl sulfoxide (DMSO). The concentration of DMSO in the petri dish never exceeded 1% (vol/vol). The same amount of DMSO was added to control incubations.

Ca\textsuperscript{2+} measurements were performed on single hepatocytes using a fura 2 data acquisition system (Luigs and Neumann, Ratingen, Germany) mounted to an inverted microscope (Zeiss IM 35) equipped with epifluorescence, a xenon lamp (Osmar, XBO 75 W/2), a rotating filter wheel (357/380- to 390-nm excitation, 480- to 540-nm emission), and a photomultiplier (Hamamatsu 928 SF). The sampling rate was 2/s. For a more detailed description and evaluation of the equipment, see Neher (31). Calibration of the system was done using fluorescent beads.

Application of agonists. Application of agonist [8-bromoadenosine 3',5'-cylic monophosphate (8-BrcAMP)] was done using a micropipette to direct a flow of solution of agonist under constant pressure (1,000 hPa) from a distance of 30 µm for 5–10 s onto the equatorial surface of the single cell under investigation. The capillary (2–3 µm diameter) was positioned using an Eppendorf ECET 5170 micromanipulator, and an ECET micromanipulation system (Eppendorf 5242) coupled to the capillary was activated for the time and pressure specified to generate the flow of agonist. All other compounds were dissolved in medium A and were present in the "bath" (petri dish) at concentrations given in Figs. 1–5.

The rate of Ca\textsuperscript{2+} increase (nM/s) and the maximum level in cytosolic Ca\textsuperscript{2+} (ΔCa\textsuperscript{2+}) were calculated from fura 2 recordings of individual hepatocytes. The initial rate of Ca\textsuperscript{2+} increase reflects the rate of Ca\textsuperscript{2+} entry into single hepatocytes and is related to the proportion of active "open" channels in the membrane. The initial rate is largely independent of signal distortion by compensating mechanisms (desensitization). At high concentrations of inhibitor(s), the determination of this value was more reliable.

If not otherwise stated, values given are means ± SE from 7–10 single cells exposed and stimulated individually under identical conditions per dish. The experiments were repeated at least three times with independent cell preparations.

**RESULTS**

Kinetics of cAMP-dependent Ca\textsuperscript{2+} uptake. 8-BrCAMP (1 mM) when applied from the outside using a microinjection glass capillary for 5 s from a distance of 30 µm onto the surface of single axolotl hepatocyte led after a short delay to an increased influx of Ca\textsuperscript{2+} as shown for six of seven individual hepatocytes in the same petri dish (Fig. 1). In most cells, the increase of cytosolic Ca\textsuperscript{2+} was transient with a half-life of decay of 57 ± 4 s (n = 6). Some cells however exhibited longer-lasting responses, some also with superimposed oscillations (not shown). Maximum levels of cytosolic Ca\textsuperscript{2+} (ΔCa\textsuperscript{2+}: 211 ± 20 nM; n = 5) were obtained within 15 s (rate: 13 ± 1.5 nM/s; n = 6).

The rate of Ca\textsuperscript{2+} influx and the maximum increase of cytosolic Ca\textsuperscript{2+} of cells treated as defined above were dependent on the concentration of 8-BrCAMP (Fig. 2, A and B). The influx of Ca\textsuperscript{2+} followed first-order kinetics with an apparent affinity constant of 8.6 \times 10^{-4} M. Maximal levels of cytosolic Ca\textsuperscript{2+} were observed at a 8-BrCAMP concentration of 1–2 mM. These data compare favorably with earlier measurements of hepatocytes in suspension (23). Because of the experimental
The availability of extracellular Ca\(^{2+}\), as has been reported earlier (23).

Effect of SK&F-96365 on cAMP-dependent Ca\(^{2+}\) entry. The imidazole derivative SK&F-96365 has been introduced as a tool to discriminate between voltage-gated Ca\(^{2+}\) entry and receptor-mediated Ca\(^{2+}\) entry (30). SK&F-96365 inhibited CAMP-dependent Ca\(^{2+}\) inflow in axolotl hepatocytes in a dose-dependent manner. The dose-response curves for the rate of Ca\(^{2+}\) entry and for the maximal increase are shown in Fig. 3. The \(IC_{50}\) values for SK&F-96365 were 1.4 and 1.7 \(\times 10^{-6}\) M for the rate of Ca\(^{2+}\) entry and maximal increase (\(\Delta C a^{2+}\)), respectively.

Effect of dihydropyridines and verapamil on cAMP-dependent Ca\(^{2+}\) entry. The dihydropyridine (8, 9) nifedipine, when tested under comparable conditions, inhibited markedly the cAMP-dependent Ca\(^{2+}\) influx (IC\(_{50}\) = 8 \(\times 10^{-7}\) M). This sensitivity is 20–50 times more pronounced than that reported for liver by others (18, 26). BAY K 8644, an agonistic dihydropyridine, which binds during the open state of L-type Ca\(^{2+}\) channel and prolong their open time (24), when present in equimolar concentration had no additional effect (Fig. 4A, open square). BAY K 8644 at 2.5 \(\mu\)M on its own, however, increased the basal Ca\(^{2+}\) by 17\% and cAMP (1 M)-dependent \(\Delta C a^{2+}\) by 47\%.

The potency of the phenylalkylamine verapamil to block Ca\(^{2+}\) entry was about one order of magnitude lower (IC\(_{50}\) = 2.2 \(\times 10^{-5}\) M) than that of nifedipine.

Dihydropyridines are the “classical” inhibitors of L-type Ca\(^{2+}\) channels that are abundant in electrically excitable tissues, like muscle and brain cells. These cells are depolarized in the presence of K\(^{+}\). In experiments in which KCl was applied at a concentration of 100 mM onto single axolotl hepatocytes, we could not detect any effect of such depolarizing concentrations of KCl on intracellular Ca\(^{2+}\) (data not shown).

Effect of Gd\(^{3+}\) on cAMP-dependent Ca\(^{2+}\) entry. The lanthanide Gd\(^{3+}\) inhibited CAMP-dependent Ca\(^{2+}\) entry very efficiently. A 50\% inhibition of the rate of Ca\(^{2+}\) entry and of the maximal increase of cytosolic Ca\(^{2+}\) was observed at a concentrations of 2.5 \(\times 10^{-6}\) M (Fig. 5).
Effect of niflumate on cAMP-dependent Ca\textsuperscript{2+} entry. Niflumate inhibited cAMP-dependent Ca\textsuperscript{2+} entry by \(~90\%\) (rate: 9.6 and 7.2\%; \(\Delta\text{Ca}^{2+}\): 13.6 and 13.2\% of control at 1 or 5 \(\times 10^{-4}\) M niflumate, respectively).

DISCUSSION

Using dual-wavelength excitation epifluorescence measurements of Ca\textsuperscript{2+} on single hepatocytes, we demonstrate here unique properties of this Ca\textsuperscript{2+}-conducting channel in axolotl hepatocytes (a nonexcitable splanchnic cell).

The entry of Ca\textsuperscript{2+} evoked by cAMP was strongly inhibited by the imidazole derivative SK&F-96365 (IC\textsubscript{50} \(\sim 2 \times 10^{-6}\) M), whereas that after microinjection of InsP\textsubscript{3} was inhibited only at concentrations \(>10^{-4}\) M (data not shown). This inhibition is about one order of magnitude more effective than that described for a variety of different cells including rat hepatocytes (0.8–3 \(\times 10^{-5}\) M, see Refs. 10, 11, 25, 30, 40). SK&F-96365, which belongs to a group of imidazole antimycotics that have been originally used to block cytochrome P-450 but also Ca\textsuperscript{2+} and Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels (3), was introduced as a novel inhibitor of receptor-mediated Ca\textsuperscript{2+} entry into cells (30). In addition, inhibition of voltage-gated Ca\textsuperscript{2+} entry in GH\textsubscript{3} and rabbit ear artery smooth muscle cells by SK&F-96365 has been observed (30). The mechanism of this inhibition is still elusive. The proposal, however, that cytochrome P-450 may link intracellular Ca\textsuperscript{2+} stores with plasma membrane influx (2) has been questioned by others (36). In axolotl liver, we could exclude a participation of intracellular, capacitative stores in cAMP-dependent Ca\textsuperscript{2+} influx, which is in support of a cytochrome P-450-independent interaction (23).

Opposing effects of SK&F-96365 on HL-60 cells have been recently reported by Leung et al. (25). At low concentrations (<16 \(\mu\)M), SK&F-96365 inhibited Ca\textsuperscript{2+} entry, whereas at higher concentrations (16–100 \(\mu\)M), it provoked release of intracellular Ca\textsuperscript{2+}, and by this promoted even Ca\textsuperscript{2+} entry (30–100 \(\mu\)M). The latter could be inhibited by La\textsuperscript{3+}, but not by nifedipine.

The comparably sensitive inhibition of cAMP-dependent Ca\textsuperscript{2+} entry observed in the presence of the dihydropyridine derivative nifedipine was not expected. Dihydropyridines are known to block rather selectively L-type voltage-dependent Ca\textsuperscript{2+} channels of excitable tissues (9), a channel type which is absent in hepatocytes, as judged by electrophysiological measurements (37) or Northern analysis (17). This is confirmed by our failure to demonstrate Ca\textsuperscript{2+} entry after membrane depolarization in the presence of K\textsuperscript{+} (100 mM), which reveals that the channel described here although sensi-
Ca\textsuperscript{2+} influx channels of nonexcitable cells sharing these properties have been recently found in B lymphocytes from rat, which showed dihydropyridine but no voltage sensitivity (1), and in an erythroleukemia cell line from mouse, where a truncated \( \alpha_1 \)-subunit lacking the first four transmembrane segments was expressed (27). Furthermore, the trp/trpl gene product from Drosophila that forms a nonselective cation channel presumably involved in capacitative Ca\textsuperscript{2+} entry in invertebrates and vertebrates shows some sequence homology to the voltage-operated Ca\textsuperscript{2+} channel \( \alpha_1 \)-subunit, but lacks arginine residues of the S4 region (33, 41).

The phenylalkylamine verapamil inhibited Ca\textsuperscript{2+} entry in axolotl hepatocytes (50% effective concentration = 22 \mu M) but in comparison with nifedipine with lower sensitivity. In contrast to dihydropyridines, phenylalkylamines enter the cell to interact with a high-affinity binding protein on the endoplasmic reticulum, which has been identified in guinea pig and human liver (14). As for nifedipine, the effects of verapamil reported so far for liver (and hepatocytes) are rather controversial. Studying capacitative Ca\textsuperscript{2+} entry, Llopis et al. (26) failed to see effects of verapamil or nifedipine (up to 50 \mu M), whereas Striggow and Bohnensack (36) observed an incomplete inhibition of this Ca\textsuperscript{2+} entry mechanism at verapamil or diltiazem concentrations between 200 and 400 \mu M. Others have reported complete inhibition of \( ^{45}\text{Ca}^{2+} \) exchange across the liver cell plasma membrane in the presence of 50–100 \mu M nifedipine or verapamil (18). A stretch-activated nonselective cation channel found in rat hepatocytes and rat hepatoma cells was not affected by nifedipine, verapamil, or La\textsuperscript{3+} (4).

The effect of nifedipine (or verapamil) shown here on axolotl hepatocytes appears to be more specific, since the effective concentration of nifedipine (1–5 \mu M) is the order of magnitude used to block voltage-dependent L-type Ca\textsuperscript{2+} channels of excitable cells in vitro, i.e., 1–10 \mu M.

The inhibition of cAMP-dependent Ca\textsuperscript{2+} entry observed in axolotl hepatocytes in the presence of niflu- mate or the lanthanide Gd\textsuperscript{3+} was not surprising. Both compounds are potent inhibitors of Ca\textsuperscript{2+} entry through nonselective cation channels (Ref. 15 and references therein). Because of an ionic radius close to that of Na\textsuperscript{+} and Ca\textsuperscript{2+}, Gd\textsuperscript{3+} (0.2–100 \mu M) can block efficiently stretch- or receptor-activated nonselective cation channels (4, 11, 40) but, like La\textsuperscript{3+}, also voltage-dependent channels (39).

The nonsteroidal anti-inflammatory fenamates have been applied to block Ca\textsuperscript{2+} entry via nonselective cation channels in cells from rat exocrine pancreas (12), in human polynuclear leucocytes (21), and in mucosa-type mast cells (35). Apart from nonselective cation channels, Cl\textsuperscript{−} channels are blocked by fenamates (13, 35). In mucosa-type mast cells, the Cl\textsuperscript{−} channel blocker 4,4′-dioctyloxyanisobenzene-2,2′-disulfonic acid, however, fully obstructed Cl\textsuperscript{−} currents without affecting Ca\textsuperscript{2+} influx, thus indicating that the effect of niflumate on Ca\textsuperscript{2+} influx may be dissociated from that on Cl\textsuperscript{−} channels (35).

As discussed above, all compounds used here, except for nifedipine and verapamil, are to a variable degree inhibitory on capacitative Ca\textsuperscript{2+} entry (11, 16, 21, 25, 26, 40).

The agonist-induced Ca\textsuperscript{2+} influx in axolotl hepatocytes may be characterized as follows. The influx depends totally on the generation of CAMP, which in turn acts indirectly via protein phosphorylation catalyzed by protein kinase A (23). The influx of Ca\textsuperscript{2+} measured in the presence of 8-BrcAMP as a surrogate follows first-order kinetics, with a maximal rate of \( \sim 60 \) nM/s and an apparent Michaelis constant of \( \sim 5 \times 10^{-6} \) M 8-BrcAMP, as calculated for the concentration present on the cell surface. Protein phosphorylation(s) could be coupled to and/or modulate the open state of an ion-gating channel in the membrane, as demonstrated for voltage-gated ion channels (8, 9). The pharmacological profile of the Ca\textsuperscript{2+} influx channel in amphibian hepatocytes reveals certain relationships to these channels, as well as to nonselective cation channels. The Ca\textsuperscript{2+} entry shows a remarkable dihydropyridine sensitivity but lacks the ability of voltage sensing, indicating certain homologies to the dihydropyridine binding site of the \( \alpha_1 \)-subunit, but apparently differences in the S4 segment. Examples of other nonexcitable cells sharing these properties have been discussed above. This relationship is reinforced by the distinct effects of verapamil and SK&F-96365 or Gd\textsuperscript{3+}, because the selectivity of the latter compounds for voltage-gated Ca\textsuperscript{2+} entry and nonselective cation channels appears to be low (30, 39). The sensitivity to SK&F-96365, Gd\textsuperscript{3+}, and niflumate, all of which act by different mechanisms (15), discloses the properties of a receptor-activated nonselective cation channel.

This novel dihydropyridine-sensitive channel, which to our knowledge is absent in rodent liver, could serve...
as an example for a diversity of types and subtypes of channels in various tissues and species.

The generous gift of axolotls by Prof. Dr. W. Hanke, Karlsruhe, Germany, is gratefully acknowledged.


Received 4 April 1997; accepted in final form 7 July 1997.

REFERENCES


